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Tti2 in PIKK Biosynthesis and Its Use in Identifying Missense Suppressor tRNAs

Kyle S. Hoffman
The University of Western Ontario

Supervisor
Dr. Chris Brandl
The University of Western Ontario

Graduate Program in Biochemistry

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Abstract

Protein biosynthesis is an essential process for all cells. It involves the translation of genetically encoded information into peptides, folding and assembly of peptides into three-dimensional molecules and complexes, and post-translational modification. Molecular chaperones facilitate protein folding so that a native state is achieved. Misfolded proteins and aggregates are toxic within the cell and accumulate due to stress conditions, mutations, and cell aging. Moreover, essential proteins rely on chaperones and co-chaperones for their regulation and activity.

The TTT complex, consisting of Tel2, Tti1, and Tti2, is considered an Hsp90 co-chaperone with specificity for phosphatidylinositol 3-kinase-related kinases (PIKKs). I show that yeast expressing low levels of Tti2 are viable under optimal growth conditions, but are sensitive to a number of stress conditions that involve PIKK pathways. In agreement with this, depleting Tti2 levels decreased expression of Tra1, Mec1, and Tor1, and affected their localization. I also find that overexpressing Hsp90 or its co-chaperones is synthetic lethal when Tti2 is depleted, an effect possibly due to imbalanced stoichiometry of a complex required for PIKK assembly. These results indicate that Tti2 does not act as a general chaperone, but may have a specialized function in PIKK folding and/or complex assembly.

Using a stress sensitive tti2 allele with a Leu to Pro mutation at residue 187, I performed a genetic selection of spontaneous second site mutations. The selection identified a single nucleotide mutation at the same position (C70U) in four tRNA\textsuperscript{Pro\_UGG} genes. Since the mutation incorporates the identity element (G3:U70) for alanyl-tRNA synthetase into tRNA\textsuperscript{Pro}, I hypothesized that suppression results from mistranslation of Pro187 in Tti2\textsubscript{L187P} as Ala. In vitro, tRNA\textsuperscript{Pro\_UGG\_C70U} was mis-aminoacylated with alanine by alanyl-tRNA synthetase. Mass spectrometry from protein expressed \textit{in vivo} and a novel GFP reporter for mistranslation confirmed substitution of alanine for proline at a rate of ~6%. Mistranslating cells expressing \textit{SUF9\textsubscript{G3\_U70}} induce a partial heat shock response but grow nearly identically to wild-type.
Keywords

Protein biosynthesis, chaperones, PIKK proteins, TTT complex, genetic code, mistranslation, suppressor genetics.
Co-Authorship Statement

In regard to Chapter 2:


K.S. Hoffman contributed to experimental design and performed experiments for figures 2-2c, growth curves (table 2-1), 2-3c-d, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9b,c,d, 2-10a,b, 2-11a-c, 2-12, S 2-2, S 2-3, S 2-6, S 2-7, S 2-8, and wrote the manuscript with C.J. Brandl. M.L Duennwald contributed to experimental design and provided plasmids, strains, and antibodies indicated in the methods. J. Karagiannis helped with fluorescence microscopy for figure 2-7. J. Genereaux performed experiments for figures 2-2, 2-9a, and generated tti2 mutant alleles and yeast strains. A.S McCarton contributed to the cloning of HSP82, HSC82, CDC37, and AHA1, and performed experiments for figure S 2-4. C.J Brandl performed experiments for figure 2-1 and S 2-5, and created the tti2 deletion strain.

In regard to Chapter 3:


K.S. Hoffman performed experiments and data analysis for figures 3-1a,c, 3-2b, 3-3, 3-4, 3-7, 3-8a, S3-2, S3-3, S3-4, S3-7, tables 3-1 and 3-2, and wrote the manuscript with C.J Brandl and P. O’Donoghue. M.D. Berg performed experiment for figure S3-8, generated R boxplot for figure S3-5, calculated GFP intensities, and created strains for fluorescence microscopy. B.H. Shilton contributed to designing the GFP$_{D129P}$ molecule to use as a fluorescent reporter and preparation of the manuscript. C.J. Brandl performed experiments for figures 3-1b, 3-5b, 3-6, 3-8b, isolated suppressor strains, dissected tetrads for linkage analysis and to generate strains, and prepared genomic DNA for next-generation sequencing.
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<th>Full Form</th>
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<tbody>
<tr>
<td>A site</td>
<td>Aminoacyl site</td>
</tr>
<tr>
<td>AAA+</td>
<td>ATPase associated with various cellular activities</td>
</tr>
<tr>
<td>aaRS</td>
<td>aminoacyl-tRNA synthetase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3-related</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie brilliant blue</td>
</tr>
<tr>
<td>CDC</td>
<td>cell division cycle</td>
</tr>
<tr>
<td>CEN</td>
<td>Centromeric</td>
</tr>
<tr>
<td>CHIP</td>
<td>carboxy terminus of Hsp70-interacting protein</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase 2</td>
</tr>
<tr>
<td>DED1</td>
<td>Defines essential domain 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase, catalytic subunit</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>Elongation factor thermo unstable</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic reticulum associated degradation</td>
</tr>
<tr>
<td>E site</td>
<td>Exit site</td>
</tr>
<tr>
<td>FAT</td>
<td>FRAP-ATM-TRRAP</td>
</tr>
<tr>
<td>FATC</td>
<td>FAT C-terminal</td>
</tr>
<tr>
<td>GAL</td>
<td>Galactose</td>
</tr>
<tr>
<td>GPD</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEAT</td>
<td>Huntington, Elongation Factor 3, PR65/A, TOR</td>
</tr>
<tr>
<td>HSE</td>
<td>Heat shock element</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>Htt</td>
<td>Huntingtin</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>IPOD</td>
<td>Insoluble protein deposit</td>
</tr>
<tr>
<td>JUNQ</td>
<td>Juxtanuclear quality-control</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>Mec1</td>
<td>Mitosis entry checkpoint 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NAC</td>
<td>Nascent-chain-associated complex</td>
</tr>
<tr>
<td>NEF</td>
<td>Nucleotide exchange factor</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>P site</td>
<td>Peptidyl site</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGK</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIKK</td>
<td>Phosphatidylinositol 3-kinase-related kinase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RAC</td>
<td>Ribosome-associated complex</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase P</td>
<td>Ribonuclease P</td>
</tr>
<tr>
<td>Rpn4</td>
<td>Regulatory particle non-ATPase 4</td>
</tr>
<tr>
<td>SAGA</td>
<td>Spt-Ada-Gcn5 Acetyltransferase</td>
</tr>
<tr>
<td>SBD</td>
<td>Substrate binding domain</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SMG-1</td>
<td>Suppressor with morphological effect on genitalia family member 1</td>
</tr>
<tr>
<td>STRE</td>
<td>Stress response element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TF</td>
<td>Trigger factor</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TOR</td>
<td>Target of rapamycin</td>
</tr>
<tr>
<td>TORC</td>
<td>TOR complex</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide repeats</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>tRNase Z</td>
<td>Transfer ribonuclease Z</td>
</tr>
<tr>
<td>TRRAP</td>
<td>Transformation/transcription domain-associated protein</td>
</tr>
<tr>
<td>Tel2</td>
<td>Telomere maintenance protein-2</td>
</tr>
<tr>
<td>Tti</td>
<td>Tel2-interacting protein</td>
</tr>
<tr>
<td>TTT</td>
<td>Tel2, Tti1, and Tti2 complex</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract, peptone, dextrose</td>
</tr>
</tbody>
</table>
Chapter 1

Scope of Thesis

There are two focuses of this thesis: the role of Tti2 in stress response and PIKK regulation, and defining the mechanism and consequences of mistranslation caused by a missense suppressor tRNA.

This thesis begins with an overview of peptide synthesis and protein folding. It will provide detail on tRNA maturation, aminoacylation, and mRNA decoding, which will be required for later discussions on mistranslation. This will be followed by a review on protein folding in relation to the Hsp90 and Hsp70 chaperone systems. Next, the importance of molecular chaperones and protein quality control pathways in managing translation infidelities and toxic aggregates will be introduced.

Chapter 2 will characterize the role of Tti2, a proposed Hsp90 co-chaperone protein part of the TTT complex, in stress response and regulation of a family of proteins, known as the PIKKs. Following the investigation on the function of Tti2, Chapter 3 will describe how a defective tti2 allele was used to identify a missense suppressor tRNA. This study investigates the mechanism of the suppressor tRNA and the protein quality control pathways that permit mistranslation.

Chapter 4 will briefly review the findings presented in each data chapter and provide future directions of the research. The main discussion topics will include the emerging theme of mistranslation as an adaptive response and the pathways that maintain the stability of a mistranslated proteome. The thesis will conclude with examples of how engineered translation systems are being used for designing therapeutic proteins and describe the potential for suppressor tRNAs in gene therapy.
1 Protein Biosynthesis

Protein biosynthesis is an essential process for cell viability, growth, and proliferation. It is a critical aspect of the central dogma, which describes the flow of information from DNA to protein molecules. This process starts with transcription of the coding regions of DNA and processing of the transcript into a messenger RNA (mRNA). The subsequent decoding of mRNA by the cell’s translation machinery determines the amino acid sequence of the polypeptide being synthesized.

While translation is considered a high fidelity aspect of protein biosynthesis, translational errors are surprisingly frequent (1). Amino acid mis-incorporation, or mistranslation, can exploit proteome diversity as an adaptive response (2-6). Molecular chaperones and protein quality control pathways maintain the stability of the proteome in the midst of mistranslation (7-11).

As a polypeptide is synthesized it becomes available for folding and assembly of a functional molecule. Protein folding, modification, and complex assembly achieves a three-dimensional stable structure. Molecular chaperones and co-chaperones ensure that folding and assembly occurs in a controlled manner. Furthermore, chaperones are required for managing aggregations and misfolded proteins within the cell (12,13). Thus, protein folding, managing toxic protein species, and introducing proteome diversity relies on the work of chaperones.

Co-chaperones are involved in recruiting proteins to chaperone systems, chaperone remodeling and catalytic regulation, and assembly of protein complexes (14-20). The TTT complex is a proposed co-chaperone of Hsp90, and is required for the stability of a family of kinases, known as phosphatidylinositol 3-kinase-related kinases (PIKKs). In addition to discussing the relationship between protein quality control pathways and mistranslation, this chapter will introduce Tti2, a member of the TTT complex, and describe its role in PIKK protein function and stress response.
1.1 Translation of the Genetic Code

The standard genetic code is considered universal across all organisms and consists of 61 codons that specify translation of 20 amino acids, and three additional codons designating termination. Translation involves the covalent attachment of amino acids, sequentially defined by the genetic code. As a prerequisite for peptide synthesis, a diverse pool of aminoacyl-tRNA molecules is found in the cytoplasm, ready for recruitment to the ribosome by translation factors. The fidelity of translation relies on two processes: tRNA aminoacylation and codon reading at the ribosome. Aminoacyl-tRNA synthetases (aaRSs) are responsible for charging the correct tRNA molecule with an amino acid. With a few exceptions, each tRNA has its own aaRS that recognizes both structural and chemical features of a tRNA molecule and in some cases has an editing domain to hydrolyze mis-activated amino acids or mischarged tRNAs (21,22). If correct pairing between a codon and an aminoacyl-tRNA anticodon is met in the ribosome A site, a peptide bond will attach the amino acid to the growing peptide. While the genetic code was considered a “frozen accident” that all forms of life universally translate (23), deviations from the standard genetic code are evident and suggest that the code is evolving (24-28).

The concept of tRNA molecules as carriers of amino acids for translation of an RNA template was first postulated by Francis Crick. He hypothesized that, “the amino acid is carried to the template by an adaptor…” and that each adaptor molecule may contain a di- or tri-nucleotide sequence to base pair with an RNA template (29). While some of Crick’s predictions on peptide synthesis have been refuted, such as a unique aaRS being required for the synthesis of each aminoacyl-tRNA (30,31), many were surprisingly accurate considering the experimental evidence available at that time.

The tRNA molecule is represented as a cloverleaf in secondary structure. Nucleotide pairing results in stem-loops that form the three arms of the cloverleaf. From 5’ to 3’ tRNAs contain an acceptor stem, dihydrouridine (D) arm, anticodon stem-loop, variable loop, and the ribothymidine (T) arm (32). In three dimensions, tRNAs take on a similar L-shaped structure (33-36). To adopt an L-shape, the acceptor stem stacks on top
of the T-arm to form the shorter branch, and the long branch consists of an anticodon stem interaction with the D-arm.

Transfer RNA maturation involves a series of events that regulate tRNA levels, availability, and decoding properties (37-39). Once a tRNA gene is transcribed, the pre-tRNA will undergo processing that includes nucleotide excision of 5’ and 3’-ends, addition of the 3’-end CCA sequence, intron splicing, and modification of nucleotides. Pre-tRNAs contain 5’ and 3’ nucleotide extensions that require processing for tRNA maturation and function. The 5’ leader sequence is cleaved by RNase P (40), an endonuclease that contains an RNA catalytic subunit and is conserved in all three domains of life (41-43). Processing of the 3’ trailer sequence requires both exonucleases and tRNase Z, which cuts the phosphodiester bond at the 3’ end of the discriminator base (the first unpaired base of the acceptor stem just prior to the CCA sequence) (44-48). Once end processing is complete, tRNA nucleotidyltransferase catalyzes the addition of a CCA tail to the 3’end of the discriminator base, and likely plays a role in tRNA end repair (49,50).

Introns are present in tRNAs of bacteria, archaea, and eukaryotes, and their removal is essential for tRNA function (51,52). Intron splicing is accomplished by a tRNA splicing endonuclease, tRNA ligase, and 2’-phosphotransferase (53-55). While end processing and modification typically occur in the nucleus, evidence from yeast suggests that premature tRNAs are exported to the cytoplasm and tRNA splicing endonuclease localizes to the mitochondrial surface (56,57). In some cases, a spliced tRNA structure is a prerequisite for recognition of tRNA modification enzymes (58-60) and retrograde nuclear import of processed tRNAs can be a way of separating nucleoside modification from tRNA splicing (61).

Nucleotides throughout the tRNA molecule undergo extensive modifications (illustrated in Figure 1-1) (62; for schematics of tRNA processing and modifications see Phizicky and Hopper (63)). As indicated in the RNA Modification Database, there are currently 108 different tRNA modifications found across eukaryotes, bacteria, and archaea (http://mods.rna.albany.edu/mods/). Although not all are essential for viability, a
Figure 1-1. Structure and modifications of tRNAs in *Saccharomyces cerevisiae*.

Each nucleotide is represented as a circle and base pairing is indicated with horizontal or vertical lines. Blue circles are unmodified nucleotides, red circles are bases that are modified in some but not all tRNAs, white circles are nucleotides found in only certain tRNAs and may or may not be modified, and the green circles represent the CCA acceptor sequence. Abbreviations used are according to the Modomics database (http://modomics.genesilico.pl). (Ψ) Pseudouridine; (Am) 2’-O-methyladenosine; (Cm) 2’-O-methylcytidine; (m1G) 1-methylguanosine; (m2G) 2-methylguanosine; (ac4C) 4-
acetylcytidine; (D) dihydrouridine; (Gm) 2’-O-methylguanosine; (m2,2G) N2, N2-di"ystemylguanosine; (m3C) 3-methylcytidine; (I) inosine; (m5C) 5-methylcytidine; (m5U) 5-methoxycarbonylmethyluridine; (m5s2U) 5-methoxycarbonylmethyl-2-thiouridine; (nmc5U) 5-carbamoylmethyluridine; (ncm5Um) 5-carbamoylmethyl-2′-O-methyluridine; (m1I) 1-methylinosine; (i6A) N6-isopentenyl adenosine; (yW) wybutosine; (t6A) N6-threonylcarbamoyladenosine; (Um) 2′-O-methyluridine; (m7G) 7-methylguanosine; (rT) ribothymidine; (Ar(p)) 2′-O-ribosyladenosine (phosphate). The subscripted numbers associated with modification abbreviations indicate the nucleotide number relative to -1 position at the 5’ end. Anticodon bases are numbered 34, 35, 36, and the discriminator base is numbered 73. This figure was adapted from (63).
number of modifications are conserved in all forms of life (64). Modification of nucleotides in regions distal to the anticodon play a role in tRNA stability and prevent degradation by the rapid tRNA decay quality control pathway (65,66). Modifying the wobble position in the anticodon, and of other nucleotides in the anticodon loop, are important for tRNA function, decoding, and response to stress conditions (67-70). Loss of tRNA modification is also linked to human disease and causes temperature sensitivity in yeast (68,71,72).

Despite the structural similarity across all tRNAs, an aaRS recognizes its cognate tRNA with a high degree of specificity (73). Aminoacyl-tRNA synthetases are divided into two classes based on structural differences. The class I aaRS catalytic domain contains a Rossmann fold and a five-stranded parallel β-sheet (74,75). Class I catalytic domains bind the minor groove side of the tRNA acceptor stem, and the 2’-OH group of A76 is ligated with an amino acid (21). Class II aaRSs are defined by a seven-stranded parallel β-sheet in the active site (76,77). Binding occurs on the major groove side of the tRNA acceptor stem (with the exception of alanyl-tRNA synthetase), and amino acids are attached to the 3’-OH group of A76 (with the exception of phenylalanyl-tRNA synthetase) (21). For both aaRS classes, aminoacylation occurs as a two-step reaction (78,79). First, an amino acid and ATP are bound and an aminoacyl adenylate is formed. Secondly, transfer of the amino acid and esterification of the 2’ or 3’-OH of nucleotide A76 forms an aminoacyl-tRNA and releases AMP. The rate-limiting step for class I aaRSs is the release of the tightly bound aminoacyl-tRNA product, which is mediated by the formation of a ternary structure with elongation factor EF-Tu (80). Conversely, aminoacylation by class II aaRSs is limited by the activation of amino acids in the presence of tRNAs (79,81,82).

Aminoacyl-tRNAs are recruited to the ribosome by translation elongation factors for the incorporation of amino acids into a growing peptide chain. There are three tRNA binding sites in the ribosome, which include the aminoacyl (A), peptidyl (P), and exit (E) sites. To initiate peptide synthesis, eukaryotic initiation factor 2 (eIF2) bound to GTP recruits the initiator methionyl-tRNA to the P site of the small ribosome subunit. This leads to the assembly of the translation initiation complex (83) and enables mRNA
binding and scanning for a start codon (84). Next, the larger 60S ribosomal subunit is recruited to the initiation complex by the GTPase, eIF5B (IF2 in bacteria and aIF5B in archaea)(85). Upon binding, GTP hydrolysis and release of eIF5B promotes translation elongation (85,86).

During elongation, EF-Tu coupled with GTP forms a ternary complex with an aminoacyl-tRNA and pairs the tRNA anticodon with the mRNA codon in the ribosome A site. GTP hydrolysis releases EF-Tu when complementary base pairing and the required tRNA-ribosomal interactions are met (87). Upon release of EF-Tu, the aminoacyl-tRNA is properly positioned for the peptidyltransferase reaction. The peptide bound to the tRNA in the P site is transferred to the amino acid of the tRNA in the A site. The translocase, EF-G (eEF2 in eukaryotes), bound to GTP stimulates the translocation of peptidyl-tRNAs from the A site to the P site after peptidyltransferase activity (87). The peptidyl-tRNA is moved to the P site, the deacylated tRNA to the E site, and the free A site can accommodate an aminoacyl-tRNA to continue elongation of the peptide (87). During termination of translation, the binding of a release factor in the ribosome A site when a stop codon is encountered signals the hydrolysis of the ester bond that links the P site bound tRNA to the full-length polypeptide (88,89).

1.2 Protein Folding and Molecular Chaperones

Peptide folding into a stable three-dimensional structure is needed for protein function. While some proteins fold spontaneously in an unassisted manner, others require molecular chaperones to overcome kinetic barriers and reach their native state (90). A chaperone is any molecule that interacts with a protein only to assist in folding, unfolding, or complex assembly (91). Without the help of chaperones, proteins can adopt misfolded conformations with propensity to form toxic aggregates within the cell (92). Chaperones are also involved in targeting irreversible misfolded proteins for degradation by ATP-dependent proteases, to eliminate their toxic affects within the cell. For these reasons, cells invest a great deal of energy in protein folding and express a high number of chaperone molecules for managing the proteome.
Protein folding is driven by hydrogen bonds, ionic and covalent interactions, dipole interactions, van der Waals forces, and hydrophobic interactions (93). The sequence and composition of amino acids within a peptide determines the structure that will be formed (94). The folding of an unfolded peptide to its native state is not often a linear process (95). Figure 1-2 depicts the folding landscape that a typical protein may have to traverse to form a stable native structure. Most peptides adopt partially folded intermediates or transition states containing non-native interactions and have higher free energy states compared to their native structure (95,96). This is due to the vast number of weak interactions that must be met to achieve the native state but cannot all be satisfied simultaneously during folding. Characterizing the folding landscape and the diversity of folded intermediates has been improved in studies using innovative techniques involving nuclear magnetic resonance (NMR), Förster resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FCS) (97-100).
Figure 1-2. The folding energy landscape.

Unfolded peptides with high potential energy progress down the folding landscape and adopt various folding intermediates before reaching a stable native state. Chaperones assist partially folded or misfolded peptides in overcoming kinetic barriers to reach native state. The green (left) side represents folded states driven by intramolecular interactions that are stabilized during folding. Aggregates, oligomers, and fibrils can form due to intermolecular interactions between partially folded proteins (red). This figure was adapted from (90).
As a peptide is synthesized in eukaryotes, folding into a tertiary structure can commence as a co-translational process. This is true for multi-domain containing proteins and is a way to avoid the formation of unproductive intermediates and accelerate protein folding (12,101,102). Ribosome-associated chaperones bind protein domains, specifically hydrophobic residues, as they exit the ribosome. These chaperones include Trigger Factor (TF) in prokaryotes, ribosome-associated complex (RAC) in eukaryotes, and nascent-chain-associated complex (NAC) in archaea and eukaryotes (103). TF binds hydrophobic residues of newly synthesized peptides in an ATP-independent manner and prevents the misfolding of highly aggregation prone motifs (104,105). RAC consists of Hsp70 chaperones Ssb1/2p, Ssz1p, and the Hsp40 chaperone Zuotin (Zuo), all of which cooperate in binding of nascent peptide chains (106). NAC is present at 1:1 stoichiometry with ribosomes and is a general co-translational chaperone that binds nascent peptides and prevents their misfolding. Moreover, NAC interacts with the signal recognition particle (SRP), thereby mediating protein sorting (107,108).

The Hsp70 chaperones are present in bacteria and eukaryotes and function in the cytosolic network of chaperones, along with the co-chaperone Hsp40 and nucleotide exchange factors (NEFs) (109,110). Together the system binds newly synthesized peptides, both co- and post-translationally, and acts early in protein folding. Structurally, Hsp70 contains an N-terminal nucleotide-binding domain (NBD), a C-terminal substrate-binding domain (SBD), and a linker domain that loosely connects the SBD and NBD (111-113). When ATP is bound in the NBD, Hsp70 takes on an open conformation and the SBD can interact with hydrophobic residues of peptides recruited by Hsp40. Hydrolysis of ATP is stimulated by the binding of Hsp40 and causes the alpha-helical lid of the SBD to close over top of the bound peptide. An open conformation and release of the substrate is triggered by the binding of NEFs to the NBD and exchange of ADP for ATP (111-114). Thus, the Hsp70-Hsp40 chaperone system functions in cycles of substrate binding and release in an ATP-regulated manner.

To complete maturation of client proteins, Hsp70 transfers folded intermediates to Hsp90. Hsp90 forms a highly flexible homodimeric structure in both bacteria and eukaryotes (115-117). The N-terminal domain contains an ATPase and its activity
regulates folding reaction cycles, similar to Hsp70. Substrate binding is accomplished by the middle domain, which also regulates ATP hydrolysis. The C-terminal domain functions as the dimer interface and in co-chaperone binding. It contains a fold that forms a four-helix bundle upon dimerization and interacts with co-chaperones via an MEEVD motif at the extreme C-terminus (118,119). When bound to ATP, the N-terminal domains of each Hsp90 dimer interact to form a closed conformation (116). ATP hydrolysis can cause the N-terminal domains to dissociate for release of the client protein (115; for a schematic of the ATPase cycle of Hsp90 see Young et al. (119)).

Hsp90 interacts with a range of co-chaperones that recruit client proteins, regulate ATP hydrolysis, and remodel Hsp90 (for a list and descriptions of Hsp90 co-chaperones see Table 4-1). Sti1 physically interacts with both Hsp70 and Hsp90, mediating Hsp70 client protein transfer. Sti1 contains three TPR (tetratricopeptide repeat) domains, one of which interacts with the C-terminus of Hsp90, and the other two interacting with the C-terminal domain of Hsp70 (120). In addition to linking Hsp70 and Hsp90, Sti1 interacts with the middle and N-terminal domain of Hsp90 to stabilize the open conformation (15,121). Similarly, Cdc37 interacts with the N-terminal and middle domains of Hsp90, stabilizes the open conformation and inhibits ATP hydrolysis (122). The co-chaperone Aha1 displaces Sti1, promotes the N-termally dimerized closed conformation, and activates ATP hydrolysis. Aha1 is subsequently displaced by p23, which inhibits ATP hydrolysis by interacting at the interface of the Hsp90 N-terminal domains. This interaction induces a tightly closed conformation of Hsp90 and may be a way to prolong the closed state for protein activation (17,116).

Having a variety of Hsp90 co-chaperones not only regulates the folding cycle but also provides specificity in client protein recruitment. The tethering between Hsp70 and Hsp90 via Sti1 ensures that Hsp70 specific client proteins are delivered to Hsp90 to complete folding. Cdc37 specifically targets protein kinases to Hsp90 and is an important mechanism for regulating protein kinase activity (122,123). In plants, the function of nucleotide binding leucine-rich repeat receptors (NLRs) depends on recruitment to Hsp90, which is mediated by assembly of an Hsp90 complex containing the co-chaperones Sgt1 and Rar1 (124).
The R2TP complex associates with Hsp90 and is involved in the assembly of RNA polymerase II (18), stabilizing PIKK protein complexes (125,126), and the biogenesis of box C/D small nucleolar ribonucleoproteins (snoRNPs) (127-129). In yeast, the R2TP complex was identified by screening for physical and genetic interactions with Hsp90 (130). The subunits of the complex are conserved from yeast to mammals and consist of Tah1/RPAP3 (yeast/mammals), Rvb1/RUVBL1, Rvb2/RUVBL2, and Pih1/PIH1D1 (130). The Tah1 TPR domain interacts with the C-terminal MEEVD motif of Hsp90, physically linking the two complexes. The Rvb1 and Rvb2 are a part of the AAA+ ATPase family (ATPases associated with a variety of cellular activities), and when incubated together form hexameric ring structures in yeast and humans (131,132). The Rvb1/2 ring, while also playing an important role in chromatin remodeling complexes (133,134), associates with the N-terminal region of Pih1 as part of the R2TP complex (135). The Tah1 C-terminal domain binds to an intrinsically disordered region in the C-terminus of Pih1. This interaction prevents Pih1 degradation and increases its stability (129,136,137), and is therefore a critical aspect of R2TP complex assembly.

1.3 Regulation of PIKK proteins by the Hsp90-R2TP-TTT complex

Pih1 tethers the TTT complex to the R2TP, and indirectly to Hsp90 (136). The PIH domain of Pih1 contains a phosphopeptide binding site, which interacts with a motif of Tel2 containing CK2-phosphorylated serine residues. Tel2 is a member of the TTT complex, and its interaction with Pih1 is required for the stability of PIKK proteins (125,136). Based on crystal structures and biochemical data, Pal et al. (136) created a model for the assembly of an Hsp90-R2TP-TTT complex, functioning in the recruitment of PIKK proteins (depicted in Figure 1-3).

The TTT complex consists of Tel2, Tti1, and Tti2, which are all found in yeast and mammals. Tel2 (telomere maintenance 2) was initially identified in a screen for mutant alleles that affect the maintenance of telomere length (138). It was subsequently annotated with functions in circadian rhythm, lifespan, and DNA damage and S-phase checkpoints (139-142). In a screen for interactors with Tor1 and Tor2, Hayashi et al.
Figure 1-3. Associations of the Hsp90, R2TP, and TTT complexes in yeast.
The R2TP complex associates with Hsp90 through an interaction between the TPR
domain of Tah1 and the C-terminal MEEVD motif of Hsp90. Tah1 interacts with Hsp90
as a dimer, however, it is unknown whether the R2TP and TTT complexes can associate
in duplicate. The PIH domain of Pih1 binds to Tel2 phosphoserines, linking the TTT
complex and PIKKs to the R2TP complex and Hsp90. Whether the PIKKs make direct
contact with either the Rvb proteins or Hsp90 remains to be determined. This figure was
adapted from (136).
detected Tel2 and subsequently identified uncharacterized proteins which they termed Tel two-interacting proteins 1 and 2 (Tti1 and Tti2) (143). In a different study, to define a proteome interaction network involved in chromatin regulation, all three TTT components were found in association with Tra1, Asa1, Rvb1, and Rvb2, in a complex termed ASTRA (ASsembly of Tel, Rvb and Atm-like kinase) (144). Moreover, Tti1, Tti2 and Tel2 are all required in the DNA damage response, for PIKK stability and complex assembly, and for chromosome stability (126,145-147).

The PIKK proteins are a family of serine-threonine kinases that includes TOR (target of rapamycin), TRRAP (transformation/transcription domain associated protein), SMG1 (suppressor with morphogenetic effect on genitalia-1), ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit). Functions for each PIKK member include nutrient and growth signaling (TOR), transcriptional regulation (TRRAP), regulating nonsense mediated mRNA decay (SMG1), and the DNA damage response and repair (ATM, ATR, and DNA-PKcs) (148-152). The PIKKs all have similar domain architectures. Starting from the N-terminus there are alpha-helical HEAT (Huntington, EF3A, ATM, TOR) repeats that make up the FAT (FRAP-ATM- and TRRAP) domain, followed by a kinase domain that resembles that of the PI3K (phosphatidylinositol-3-kinase) family, and a FATC (FRAP-ATM-TRRAP C-terminal) domain at the extreme C-terminus (152-154).

It is likely that the TTT complex functions as an Hsp90 co-chaperone in the recruitment of PIKK proteins for their maturation. The TTT members have been implicated in pathways related to PIKK functions, and their depletion results in decreased expression and stability of the PIKKs, and affects PIKK complex assembly (145,155-157). Rather than having individual functions, the TTT members seem to regulate the PIKKs as a complex since impairing the Tel2 interaction with Tti1-Tti2 disrupts PIKK stability and function (126). In support of a co-chaperone role for the TTT complex, Tel2 binds newly synthesized PIKK peptides and is found in complex with Tti1, Tti2 and Hsp90 (126). It is still unclear how Hsp90 interacts with the PIKKs, however, inhibition of Hsp90 ATPase activity reduces PIKK stability (126). The recruitment of the PIKKs to Hsp90 also involves the R2TP complex. Phosphorylation of Tel2 serine residues 487 and
491 by CK2 is required for interaction of Tel2 with Pih1, and regulates PIKK stability
and activity (125,136,158,159). Taken together, the evidence to date supports a model
where the PIKK proteins are recruited to the Hsp90 system by a series of protein-protein
interactions involving the TTT and R2TP complexes.

1.4 Proteome Perturbations

Maintaining the integrity of the proteome extends beyond protein folding. The stability
and function of proteins can be affected by errors during translation, stress conditions, or
defects in protein biosynthesis, and can cause toxicity within the cell. Cells have evolved
to deal with misfolded protein and invest a great deal of energy to ensure proteins are
synthesized correctly. These include upregulation of chaperones, targeting proteins for
degradation, storage of toxic aggregates, and editing functions of the translation
machinery. The pathways that control these processes are all linked to one another by a
network of interactions that maintain proteostasis (13).

Translation is considered a high fidelity process that ensures faithful peptide
synthesis corresponding to the codons of an mRNA. However, incorporation of an amino
acid not specified by a codon, referred to as mistranslation, is more prevalent than
expected and occurs at an approximate frequency between $10^{-3}$ and $10^{-4}$ (160). This
frequency can be significantly increased for rare codons and when a tRNA is in low
abundance (1). Mutations in an aaRS or within tRNA molecules can also increase
mistranslation, but surprisingly, sequence variations are commonly found within
eukaryotic species (10,161). Translational errors include amino acid substitutions,
translational frameshifts, and read-through of stop codons (162-165). While
mistranslation occurs more frequently than initially predicted, it is kept at tolerable levels
by aaRS editing mechanisms that ensure the formation of cognate aminoacyl-tRNA
species (21).

A distinct aaRS is expressed for each canonical amino acid, with the exception of
asparaginyl- and glutaminyl-tRNA synthetases in most bacteria and archaea. In these
organisms, via an indirect pathway, glutamine and asparagine are biosynthesized on their
cognate tRNAs from seemingly mis-aminoacyl-tRNA precursors charged with aspartic or
glutamic acid (reviewed in (166,167)). Misacylation, or mischarging, occurs when aminoacylation involves a non-cognate amino acid and/or tRNA substrate. Such errors are more frequent with near-cognate substrates, as demonstrated by the increased rate of misactivation of valine by *E. coli* isoleucyl-tRNA synthetase (168). In some cases, errors can be circumvented prior to translation by pre- and post-transfer hydrolytic editing. Pre-transfer editing involves the hydrolysis of a non-cognate aminoacyl adenylate, resulting in the release of the amino acid from the active site of an aaRS (169,170). Post-transfer hydrolysis of an amino acid from a misacylated tRNA can be accomplished by one of ten aaRSs containing an editing domain, or by a trans-editing enzyme (21,171).

It should also be recognized that independent of tRNA misacylation, misincorporation of amino acids occurring at the ribosome during mRNA decoding is not uncommon. Variation in cellular concentration of different tRNAs correlates with codon decoding times (172). Delayed decoding of a codon by a low abundance tRNA provides an opportunity for codon misreading with near-cognate aminoacyl-tRNA species, as demonstrated by the two-fold reduction in the misreading of Arg codons upon overexpressing the low abundant tRNA<sub>Arg</sub> in *E. coli* (1). Despite mechanisms that discriminate between aminoacyl-tRNAs at the ribosome, mistranslation by near-cognate aminoacyl-tRNAs at codons decoded by low abundance tRNAs can be as high as 3.7 x 10<sup>-3</sup> (1).

Identity elements of a tRNA are positive determinants for aminoacylation by a specific aaRS and consist of single nucleotides, nucleotide pairs, and structural motifs (173). Since the anticodon of a tRNA links an amino acid to the genetic code, many of the identity elements fall within the anticodon (174). However, identity sets are also found within the acceptor stem near the 3’-end CCA (175), which largely influences catalytic turnover rather than binding affinity. For example, variation of the G3:U70 identity pair in tRNA<sub>Ala</sub> causes the 3’-end CCA sequence to be orientated away from the catalytic site while bound to AlaRS, resulting in a 100-fold decrease in $k_{cat}$ while having no significant effect on binding affinity (176). Mutations in tRNAs can cause a switch in identity and allow for aminoacylation by a non-cognate aaRS. Introduction of the G3:U70 base pair into tRNA<sub>Cys</sub>, tRNA<sub>Phe</sub>, and tRNA<sub>Lys</sub>, is sufficient to confer charging
with alanine (177-180). Thus, mutation of a single nucleotide pair can be the only requirement for changing tRNA identity, causing ambiguous decoding and an increase in mistranslation.

Mutations causing a change in the anticodon can switch the identity of a tRNA (181,182), and have been documented during the evolution of various organisms (183-186). These identity switches would typically not be detrimental to the proteome since the anticodon would still link the correct amino acid with its codon, and tRNA genes are often redundant. However, changing the anticodon without switching tRNA identity, and thus aminoacylation, can cause ambiguous decoding, and in some cases, codon reassignment. Examples of this have been identified in nature. A bioinformatic analysis has identified selenocysteine tRNA species from microbial genomes that have anticodon sequences to recognize all stop codons, as well as ten sense codons (187). In *Candida albicans* a tRNA$^{\text{Sec}_{CAG}}$ variant decodes Leu CTG codon as serine (188,189). Such anticodon switches can lead to detrimental effects on the proteome. Initially, expression of the tRNA$^{\text{Sec}_{CAG}}$ anticodon variant causes mistranslation at levels that lead to significant fitness costs. However, if ambiguous decoding or codon reassignment offers an advantage, cells will adapt to the added proteome stress. Adaptation to such stress includes upregulation of genes involved in translation and the ubiquitin-proteasome system in order to increase protein synthesis and protein turnover (2). Mistranslation levels are also manipulated by altering cytoplasmic tRNA levels through upregulating pathways involved in tRNA surveillance and degradation (2).

While mistranslation has been commonly viewed as harmful translational errors, it can be an adaptive response. In some species, aaRS editing activity has been lost during evolution or is dispensable without significant effects on growth (11,190). *E. coli* can accommodate a proteome consisting of ~10% mistranslated proteins without affecting growth (11). This suggests that cells have efficient mechanisms to manage low levels of mistranslation to maintain optimal growth and fitness. Interestingly, *E. coli, S. cerevisiae*, and HeLa cells increase mis-aminoacylation by methionyl-tRNA synthetase (MetRS) under oxidative stress (3,191,192). The increase in methionine residues incorporated during translation protects cells against toxic oxygen species.
In addition to mistranslation, there are numerous other factors that cause protein misfolding and proteome instability. These include mutations, environmental stress conditions, and changes in cell metabolism. Destabilizing mutations leading to protein aggregation are a hallmark of neurodegenerative disease such as Alzheimer, Huntington’s, and Parkinson’s Disease (193,194). Increases in misfolded proteins correlates with the increase in mutation rates of cancer cells, and coincides with the overexpression of heat shock proteins (195). Environmental stress conditions that affect protein stability consist of increased temperatures, osmotic stress, and exposure to different chemicals such as mutagens, oxidants, and electrophiles (196-199). Moreover, reactive oxygen species accumulation can change the cell environment and disrupt disulfide bonds, metal binding sites, react with aldehydes, and add oxidative species to amino acid side chains (200-202). Metabolic fluctuations that cause protein stress are related to a change in nutrient intake, an increase in reactive oxygen species (ROS), or mitochondrial dis regulation (203,204).

1.5 Protein Quality Control

In addition to their function in protein folding, chaperones and co-chaperones are important for targeting misfolded proteins to the proteasome. The ubiquitin-proteasome system, in general terms, involves tagging misfolded proteins with ubiquitin for targeting to the 26S proteasome (205). In some cases, chaperones and co-chaperones recruit ubiquitin-conjugating enzymes and E3 ubiquitin ligases to misfolded peptides. For example, the mammals co-chaperone CHIP (carboxy terminus of Hsp70-interacting protein) is an E3 ubiquitin ligase that interacts with the C-terminus of both Hsp70 and Hsp90 via TPR repeats (206,207). CHIP contains a U-box domain that binds the ubiquitin-conjugating enzyme UbcH5, mediating ubiquitin ligase activity (206). Mammalian BAG-1 links Hsp70/Hsc70 to the proteasome. Induction of BAG-1 increases association of Hsp70/Hsc70 with the proteasome and is targeted by an N-terminal ubiquitin-like domain (208). Tethering Hsp70/Hsc70 to the proteasome may facilitate the transfer of ubiquitinated clients for their degradation.

Overexpression of heat shock proteins reduces toxicity caused by protein aggregates associated with human disease (209). Extracting misfolded proteins from
aggregates and mediating their refolding is also accomplished by chaperones classified as disaggregases. The AAA+ ATPase Hsp104 in yeast forms hexameric ring structures that remove denatured proteins from aggregates by threading peptides through the ring channel lined with aromatic residues (210,211). The extracted proteins undergo refolding or are targeted to the proteasome; however, the decision to do so is not fully understood.

Autophagy is a eukaryotic cellular response that delivers protein aggregates, organelles, as well as invading bacteria and viruses to the lysosome for degradation (212-216). The autophagy-lysosome response functions by the formation of a doublemembranated autophagosome, recognition and encapsulation of cytoplasmic targets, and fusion with the lysosome where protease digestion occurs (212). It is tightly linked to the ubiquitin-proteasome system and when one is impaired the other is upregulated for compensation (217). Autophagy also works in parallel with the endoplasmic reticulum associated degradation (ERAD) pathway. The ERAD pathway exports misfolded peptides from the ER to the cytoplasm for degradation by the proteasome. Autophagy is required for the elimination of higher molecular weight aggregates that cannot be managed by ERAD. For example, the autophagy-lysosome pathway is required to eliminate procollagen aggregates that form in the ER, since knock-down of autophagy-related genes leads to procollagen aggregate accumulation (218).

Aggresomes are compartments that sequester and store misfolded proteins or aggregates when the ubiquitin-proteasome system is overburdened (219,220). Aggresomes are encompassed by an intermediate filament protein, vimentin, and co-localize with centrosomal proteins where misfolded proteins are trafficked by microtubules (221,222). Studies indicate a beneficial role for aggresomes. Sequestration of toxic huntingtin proteins to aggresomes improves neuronal cell survival (223). Moreover, inhibition of aggresome formation increases the cellular toxicity caused by a mutant androgen receptor aggregate, normally trafficked to the aggresome (224). It is proposed that in yeast and mammals, aggregates can be deposited either near the nuclear membrane, termed the juxtanuclear quality-control (JUNQ) compartment, or next to the vacuole (or lysosome), known as the insoluble protein deposit (IPOD) (225). The JUNQ compartment typically contains either ubiquitinated proteins destined for the proteasome,
or soluble misfolded proteins that can be refolded by chaperones. Conversely, IPOD contains insoluble, irreversible misfolded proteins, such as huntingtin or prions, and associates with autophagy-related proteins (225,226).

Protein misfolding is pervasive to all organisms and its management by degradation, folding, and storage systems is essential for disease prevention and viability. Native conformations of proteins are destabilized by mistranslation, mutations, and conditions of stress. Fortunately, cells have evolved mechanisms to maintain the integrity of the proteome by controlled folding and refolding of peptides, disrupting and degrading toxic aggregates or misfolded proteins, and by directing toxic species to distinct compartments for isolation from cell constituents.

1.6 Bibliography


Chapter 2

2  *Saccharomyces cerevisiae* Tti2 regulates PIKK proteins and stress response

2.1 Introduction

The biosynthesis and regulation of phosphatidylinositol 3-kinase-related kinase (PIKK) proteins is crucial for cells to grow, proliferate, and respond to stress conditions. The PIKK family of proteins includes ATM/Te11 (mammalian/yeast), ATR/Mec1, mTOR/Tor1, Tor2, TRRAP/Tra1, and the mammalian proteins DNA-dependent protein kinase (DNA-PKcs) and SMG1. Each has important roles in cell signaling during stress (1) and regulate one or more critical functions such as cell growth and nutrient response (mTOR), DNA damage response (ATM, ATR and DNA-PKcs), and the regulation of gene expression (TRRAP and SMG1) (2-5). The PIKK proteins are large, and share extensive N-terminal helical regions and a C-terminal domain that resembles the phosphatidylinositol-3-kinases (6). Perhaps due to this common structure, biosynthesis and maintenance of the PIKKs are regulated by a common set of proteins. This first became apparent when Takai *et al.* (7) demonstrated that Tel2 was required to maintain the steady state level of ATM, ATR, DNA-PKcs, SMG1, mTOR, and TRRAP. In subsequent work Takai *et al.* (8) demonstrated that Tel2 interacts with newly synthesized proteins, suggesting that it executes co-translational chaperone activity. *TEL2* was also identified in a screen for genes involved in the DNA damage response, likely as a result of its regulation of ATM and ATR (9). In the same screen Hurov *et al.* (9) identified the genes encoding Tti1 (Tel2 interactor) and Tti2. The three proteins were found to mutually associate and co-elute in molecular weight fractions corresponding to a complex they termed the TTT complex. The TTT complex associates with a number of molecular chaperones including Hsp90, Hsp70, Hsp40 and the R2TP/prefoldin-like complex (8,10). The TTT complex is thus considered a co-chaperone, yet the role of each member of the complex, its substrate specificity and its mechanism of action are unknown.

We identified *tti2* as a genetic suppressor of a *tra1* allele, which alters the C-terminal phenylalanine to alanine (11). This allele reduces the stability and nuclear
localization of Tra1, resulting in reduced growth particularly under stress conditions. Two alleles of tti2 (Phe328 to Ser and Ile336 to Phe) suppress the tra1-F3744A mutation. Consistent with a role for Tti2 as a co-chaperone, tti2 suppression of tra1-F3744A reduces its degradation, and enhances the stability and nuclear localization of Tra1-F3744A (11). The tti2 alleles did not, however, suppress the growth defects of a mecl allele with a C-terminal Trp to Ala mutation, suggesting specific interactions between Tti2 and Tra1.

In S. cerevisiae TTI2 encodes an essential protein of 421 amino acid residues. Like Tel2 (8), Tti2 is predicted to be primarily helical, and localizes to both the nucleus and cytoplasm (11). Further emphasizing the importance of Tti2 are findings that it is implicated in human brain development. A missense mutation that converts I436 to N within human Tti2 causes an autosomal recessive disorder defined by microcephaly, short stature, behavioural problems, skeletal abnormalities, and facial dismorphic features (12). Furthermore, A P367L missense mutation in TTI2 has been linked to intellectual disability (13). Individuals with a Tti2 defect show characteristics similar to individuals who are unable to respond to DNA damage, possibly due to inhibition of ATR function (12).

Our goal is to use a molecular genetic approach to define the role of Tti2, characterize its structure/function relationships, as well as its relationship with Tel2 and Tti1. We therefore began this study with a screen for tti2 alleles that would identify features required for the function of the protein. Surprisingly, we identified an allele with an ochre mutation at codon 276. As C-terminal truncations of Tti2 do not support viability, this result suggested that low levels of Tti2 potentially obtained by read-through of the ochre mutation were sufficient for viability. To further characterize the effect of depleting Tti2, we placed it under control of the GAL10 promoter and analyzed the properties of the protein and strain in raffinose and glucose-containing media. We demonstrate that low levels of Tti2 are sufficient for viability in non-stress conditions. Depleting Tti2 does, however, result in temperature sensitivity and impairs the ability of cells to respond to certain conditions of stress related to PIKK function. We show that the steady state levels of Mec1, Tra1 and Tor1 decrease when Tti2 levels are depleted, with
Mec1 and Tra1 being more affected. A significant proportion of both Tra1 and Mec1 mislocalize to foci within the cytosol when Tti2 is expressed at low levels, which does not appear to result from protein aggregation. Furthermore, overexpressing Hsp90, a molecular chaperone involved in PIKK complex assembly (8,10), results in synthetic lethality in cells depleted of Tti2. Tti2 is not induced by heat shock, but its absence induces expression of Hsp42. Taken together, these results support a specialized role for Tti2 in PIKK complex assembly, rather than acting as a general chaperone in protein folding.

2.2 Materials and Methods

2.2.1 Yeast strains and Growth Conditions

Yeast strains were grown in YP (Yeast Peptone) media or synthetic dropout media supplemented with required nitrogenous bases and amino acids. Strains containing YCplac111-GAL10-TTI2 as the sole source of TTI2 were grown in YP or synthetic dropout media containing 2% galactose except as otherwise indicated.

All strains in this study (Table S 2-1) are derivatives of the wild type yeast strains BY4741, BY4742, and BY4743 (14). The tti2 disruption strain CY6032 was generated in the diploid BY4743 strain by integrating a TTI2-Tn10luk-MET5 cassette (described in DNA constructs). This strain was subsequently transformed with YCplac111-DED1-TTI2 (LEU2 centromeric plasmid), sporulated, and URA3/LEU2 haploids selected to give rise to the MATa strain CY6049. The URA3 was then eliminated by counter-selection with 5-fluoroorotic acid (5-FOA; Toronto Research Chemicals Inc.) YCplac33-DED1-TTI2 (URA3 centromeric plasmid) was transformed into CY6049 and YCplac111-DED1-TTI2 lost after repeated growth on leucine containing plates to generate CY6070. This strain was subsequently transformed with YCplac111-DED1-TTI2, YCplac111-DED1-tti2L187P, and YCplac111-DED1-tti2Q276TAA, then plated on 5-FOA to lose YCplac33-DED1-TTI2 and generate strains CY6857, CY6872, and CY6874, respectively. The related MATa strain CY6963 was obtained by sporulation after crossing BY4742 with CY6070. CY6971 and CY6991 were made by transforming CY6070 with YCplac111-GAL10-TTI2 and YEplac181-GAL10-TTI2, respectively, and in each strain the DED1-TTI2 URA3
plasmid was lost by plasmid shuffling on 5-FOA. To generate CY6973, the plasmid copy of \( LEU2 \) was disrupted with \( URA3 \) by integrating pLU12 (15) digested with \( HpaI \) and \( SalI \). CY7000 is a diploid strain containing a disruption of both genomic copies of \( TTI2 \) made by mating CY6971 with CY6963, and subsequently losing the \( URA3 \) on 5-FOA.

CY7086 expressing C-terminal Myc\(^9\)-tagged Tti2 from its endogenous promoter was constructed by transforming BY4742 with the \( SphI-SacI \) fragment of pCB2890 and selecting for growth on medium lacking histidine. Integration of the Myc\(^9\)-tag was confirmed by Western blot. To generate CY7172, pCB2911 was transformed into CY6971, repeatedly grown on leucine containing plates depleted of uracil to lose YCplac111-\( GAL10-TTI2 \), then checked for leucine auxotrophy.

Flag\(^5\)-tagged \( TRA1 \) (CY5919) and Flag\(^5\)-tagged \( MEC1 \) (CY6194) strains have been previously described (11,16). CY6999 and CY7030 were made by integration of the Flag\(^5\)-tagged \( TRA1 \) and \( MEC1 \) containing cassette into CY6971 (11,16). CY6415 containing a Flag\(^5\)-tagged \( TOR1 \) allele was made by integration of a \( URA3 \)-containing cassette (described below) into BY4743 and CY7000.

Strains expressing \( eGFP-TRA1 \) (CY5998 and CY7193) were made by integrating an \( SphI-XbaI \) fragment of pCB2301 (as described in (11)). Similarly, \( eGFP-MEC1 \) strains (CY6306 and CY7189) were made by integrating an \( SphI-EcoRI \) fragment of pCB2395 (16).

CY6857 and CY6872 were transformed with a two micron \( URA3 \)-containing plasmid expressing \( htt25Q \) from the \( GAL1 \) promoter (described in (17)) to generate CY7236 and CY7238, or \( htt103Q \) expressed from the \( GAL1 \) promoter to create CY7237 and CY7239. For an empty vector control, YCplac33 was transformed into CY6872, resulting in CY7241.

Two micron \( LEU2 \) plasmids containing \( CDC37, HSP82, AHA1 \), or \( HSC82 \) expressed from the \( GPD \) promoter were transformed into CY6070 to generate strains CY7370, CY7371, CY7372, and CY7373, respectively and into CY6973 to create strains CY7374, CY7375, CY7376, and CY7377, respectively. Two micron \( URA3 \) plasmids
expressing either HSP42, HSP26, or HSP104 (described in (18)) were transformed into CY6857 to generate CY7323, CY7247, and CY7248, respectively, and into CY6971 to produce CY7324, CY7251, and CY7252, respectively. YCplac33 was transformed into CY6857 and CY6971 to give rise to empty vector control strains CY7245 and CY7249, respectively.

2.2.2 DNA molecules

The tti2 disruption cassette in pCB2312 was created in three steps. First, genomic DNA was used to PCR amplify a SalI-BamHI fragment at the 3’ end of TTI2 extending into noncoding sequence using primers 6085-3 and 6085-4 (see Table S 2-2 for a listing of oligonucleotides), which was cloned into pTZ18r. A 5’ fragment of TTI2 extending into MET5 fragment was amplified using primers 6085-1 and 6085-2 and inserted as a BamHI-EcoRI into the above plasmid. Tn10luk (19) was then inserted into the BamHI site of this molecule. Digestion with SalI and EcoRI allowed insertion into yeast with selection for Ura<sup>+</sup> transformants.

pCB2134 and pCB2319 express Myc<sup>9</sup>-tagged TTI2 from the DED1 promoter in the LEU2 and URA3 centromeric plasmids YCplac111 and YCplac33, respectively (11). The GAL10 promoter was substituted into pCB2134 after cloning a PCR product using oligonucleotides 2764-1 and 2764-2 as a PstI-HindIII fragment to generate pCB2844 (YCplac111-GAL10-TTI2). GAL10-TTI2 from pCB2844 was inserted as a PstI-SacI fragment into YEplac181 to give pCB2862.

The PHO5-lacZ and STRE-lacZ fusion constructs in YCp87 have been previously described (20,21). The HSE-lacZ plasmid has been described by Duennwald & Lindquist (22).

pCB2890, the molecule to integrate a C-terminal Myc<sup>9</sup>-tag into Tti2 was constructed in a cassette using a base oligonucleotide synthesized by Life Technologies Inc. (see Supporting Information for sequence, Figure S 2-1). This molecule was cloned as a SphI-SacI fragment into pTZ19r lacking a HindIII site. The SphI site is at base pair 1076 relative to the TTI2 translational start, followed by the TTI2 coding sequence to the
C-terminus, with the Myc\(^9\)-tag from YCPlac111-DE\(D\)1-TTi2 cloned as a \(HindIII\)-NotI fragment in front of the translational stop codon. The molecule contains a \(BamHI\) site downstream of the translational stop into which \(HIS3\) was cloned, followed by additional downstream sequence and a \(SacI\) site to allow integration into yeast as a \(SphI\)-\(SacI\) fragment. The \(TTi2\) promoter was PCR amplified using primers TD0569 and TD0570 and genomic DNA as template, cloned into YCplac33 as a \(SalI\)-\(HindIII\) fragment in a triple ligation with \(TTi2\) from pCB2134 as a \(SalI\)-\(SacI\) fragment to generate pCB2911.

pCB2425, which contains a cassette to integrate Flag\(^5\)-tagged \(TOR1\) as an \(SphI\)-\(EcoRI\) fragment was adapted from pCB2134 used previously to tag \(TRA1\) (11). \(TOR1\)-specific sequences were added to this molecule as \(SphI\)-\(HindIII\) and \(NotI\)-\(SalI\) fragments after PCR with oligonucleotides 6496-1 and 6496, and 6496-3 and 6496-4. Digestion with \(SphI\) and \(SalI\) allows insertion into at \(TOR1\) after selection for Ura\(^+\) transformants.

\(CDC37\), \(HSP82\), \(HSC82\), and \(AHA1\) were amplified by PCR (refer to Table S 2-2 for oligonucleotide sequences) using genomic DNA as template and cloned into Gateway® \(GAL1\) two micron destination vectors as described in Alberti et al. (23).

The plasmids used for overexpressing Hsp26 and Hsp104 from the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter have been previously described (18). \(HSP42\) along with its promoter (500bp sequence upstream of translation start site) was cloned by PCR using primer TK7578 and TK7579 and genomic DNA as template. The PCR product was ligated into a \(URA3\) two micron plasmid (YEplac195) as a \(NotI\)-\(SacI\) fragment.

\subsection{2.2.3 Screening for defective \(tti2\) alleles}

To generate strains CY6872 and CY6874, a library of randomly mutagenized \(TTi2\) alleles was constructed by PCR using Taq polymerase and primers 5693-1 and 5693-2, and inserting into YCplac111-DE\(D\)1-TTi2 as a \(NotI\)-\(SacI\) fragment. The randomly mutated alleles were transformed into CY6070, and \(TTi2\) on the \(URA3\) plasmid was shuffled out by plating on 5-FOA. These strains were patched onto a YPD plate and screened for slow growth on a YPD plate containing 6\% ethanol or when grown at 37\(^\circ\)C. Plasmids were
recovered (24) and transformed back into CY6070 to confirm the phenotype. Alleles resulting in slow growth were sequenced to identify mutations.

### 2.2.4 Growth assays

Stress sensitivity assays were performed at 30°C or 37°C on YP plates containing 2% raffinose or glucose and either 6% ethanol, 1 nM rapamycin (LC Laboratories), 0.1 M hydroxyurea (Sigma-Aldrich), 8 μg/ml Calcofluor white (Sigma-Aldrich), 10 mM caffeine (Sigma-Aldrich) or 1 M sodium chloride (EMD Chemicals Inc.). Strains were grown to stationary phase, their optical density at 600 nm normalized, and spotted in ten-fold serial dilutions onto each plate. Growth curves were performed in YP media with either 2% glucose, 2% galactose, or 2% raffinose as the carbon source after growth of a starter culture in YP containing 2% raffinose.

### 2.2.5 Western blotting

Yeast strains were grown to mid-logarithmic phase then lysed with glass beads to harvest protein. Western blotting was performed using PVDF membranes and anti-Flag (M2; Sigma-Aldrich) and anti-Myc (9E10; Sigma-Aldrich) monoclonal antibodies as previously described (20,21). Anti-Hsp42 polyclonal antibody was kindly provided by Johannes Buchner (Technische Universität München) and was used as previously described (25).

### 2.2.6 Half-life of Tti2

Yeast strains CY6971 and BY4742 were grown to stationary phase in YP medium containing galactose, diluted 1:100 in the same media, then grown to an optical density of 2.0 before adding 35 μg/ml of cycloheximide. 10⁸ cells were harvested before adding cycloheximide, and at two, four, six, and eight hours thereafter. Protein was extracted as described in von der Haar (26) in the presence of protease inhibitors (working concentrations: 10 μM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 10 μM benzamidine, 500 μg/ml trypsin inhibitor, and 5 μg/ml leupeptin), and the lysates were separated by SDS-PAGE and Western blotted with anti-Myc antibody. The bottom
quarter of the gel was stained with Coomassie Brilliant Blue and shown as a control for equal loading.

2.2.7 β-galactosidase assays

LacZ reporter plasmids were assayed in strains CY6070 and CY6973. For the PHO5-lacZ reporter, strains were grown to stationary phase, washed three-times with sterile water, diluted 20-fold in YPD medium depleted of phosphate, and grown for eight hours. Cell densities were normalized and β-galactosidase units were determined using o-nitrophenol-β-D-galactosidase as substrate as described in Mutiu et al. (21). Strains containing the STRE-lacZ reporter were diluted 1:20 from stationary culture and grown in medium containing 2% glucose and 6% ethanol for eight hours prior to the assay. When assaying the heat shock element, strains were grown to mid-logarithmic phase in YPD then heat shocked at 42°C for 30 minutes. Strains containing PGK-lacZ were grown to stationary phase in medium with raffinose as the sole carbon source, then diluted 1:20 in YPD and grown for eight hours.

2.2.8 Fluorescence microscopy

Yeast strains CY5998, CY7193, CY6306, and CY7189 were grown to stationary phase in minimal medium lacking uracil, diluted 1:20 and grown to an optical density of 0.8 at 600 nm. Cells were concentrated 10-fold and 33258 Hoechst bisbenzimidazole dye (Sigma-Aldrich) was added to each culture at 1.0 µg/ml one hour prior to imaging. Cells were washed twice in one ml of sterile water and imaged using a Zeiss Axioskop 2 microscope driven by Image J 1.41 software (National Institutes of Health) and a Scion CFW Monochrome CCD Firewire camera (Scion, Frederick MD) with bright field, DAPI and GFP filter sets. Image J 1.48 software was used for quantifying GFP signal intensities. For each cell counted, the elliptical tool was used to trace each cell on bright field images and the measure function was used to calculate signal intensity per unit area from GFP images. The nucleus was defined as the top 10% of pixels with the highest intensity values within the outlined cell from images taken with the DAPI filter and was determined using the threshold function. This area was outlined using the magic wand tool and the traced area was used to measure GFP signal intensity in the nucleus.
Background noise was subtracted from the whole cell and nuclear intensity values and was measured in the area adjacent to the cell. Percent intensity in the nucleus was calculated by dividing the background subtracted intensity values of the nucleus by the background-subtracted values of the whole cell. Percent nuclear intensity calculations represent an average of individual cell ratios (nuclear GFP intensity: whole cell GFP intensity) taken across 20 cells for each strain.

2.2.9 Semi Denaturing Detergent-Agarose Gel Electrophoresis (SDD-AGE)

Aggregation of Mec1 was analyzed using SDD-AGE as described in Kryndushkin et al. (27) with the following modifications. Yeast strains CY6194 and CY7030 were grown to stationary phase in minimal medium lacking uracil and containing 2% raffinose. BY4742 was grown in YPD to stationary phase. All strains were diluted 1:20 in YPD and grown to mid-logarithmic phase. Protein was extracted from cells by glass bead lysis in buffer containing 100 mM Tris pH 7.5, 200 mM NaCl, 1.0 mM EDTA, 5% glycerol, 1.0 mM DTT, and protease inhibitors (working concentrations: 10 μM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 10 μM benzamidine, 500 μg/ml trypsin inhibitor, and 5 μg/ml leupeptin). Protein lysates were separated on a 1.8% agarose gel then transferred to PVDF overnight by capillary transfer in Tris buffered saline pH 7.5. The PVDF membrane was Western blotted using anti-Flag primary and mouse secondary antibodies as described previously (20,21). Ponceau S (Sigma-Aldrich) was used to stain the membrane after capillary transfer to document equal loading.

2.3 Results

2.3.1 Identification of stress-sensitive tti2 alleles

To initiate studies into the structure/function relationships of Tti2 we selected for randomly created mutations that confer slow-growth under stress conditions. A library containing random mutations in TTI2 generated by PCR with Taq polymerase was transformed into CY6070, a strain with the genomic copy of TTI2 deleted and with TTI2 on a URA3-containing plasmid. After plasmid shuffling on 5-fluroorotic acid, we
screened colonies for slow growth on YPD plates containing 6% ethanol and/or when grown at $37^\circ$. Two strains were identified. The plasmids from each strain were then sequenced and the phenotype confirmed after retransformation. One $tti2$ allele contained a mutation converting leucine 187 to proline; the second allele, $tti2_{Q276TAA}$, contained an ochre mutation at codon 276. As shown in Figure 2-1, the L187P mutation causes a slight reduction in growth on YPD at $30^\circ$, and more severely reduced growth on medium containing 6% ethanol, and at $37^\circ$. The $tti2_{Q276TAA}$ allele resulted in slow growth under each condition with severely reduced growth on medium containing the cell wall binding compound Calcofluor white and at $37^\circ$. 
Figure 2-1. Selection of randomly created \textit{tti2} mutations causing slow growth.

\textit{CY6070 (TTI2)}, \textit{CY6872 (tti2_{L187P})}, and \textit{CY6874 (tti2_{Q276TA})} were grown to saturation in YPD medium then spotted in 10-fold serial dilutions onto YPD, YPD containing 6\% ethanol or 8 \(\mu\text{g/ml}\) Calcofluor white and grown at 30\(^{\circ}\), and on YPD grown at 37\(^{\circ}\).
2.3.2 Low levels of Tti2 support viability but cause slow growth

As the tti2 alleles were Myc\(^9\)-tagged we were able to examine their expression by Western blotting (Figure 2-2a). Tti2\(_{L187P}\) is expressed at a level approximately two-fold less than the wild type protein. tti2\(_{Q276TAA}\) is expressed at a reduced level, and as expected is truncated. The ability of tti2\(_{Q276TAA}\) to support viability may indicate that the C-terminal sequence of Tti2 is not essential for function, or that the ochre mutation is read-through (or skipped) at a low frequency, which is sufficient to support viability. To test if the C-terminus of Tti2 is essential we used plasmid shuffling to examine whether C-terminal truncations to residue 356 and 321 support viability. As shown in Figure 2-2b, neither truncation allele supports viability. We therefore conclude that C-terminal sequences are essential, and hypothesize that tti2\(_{Q276TAA}\) supports viability due to a low level of read-through of the ochre mutation.
Figure 2-2. Characterization of Tti2 mutations.
A. Yeast strains CY6872 (lanes 1, 2, and 3), CY6070 (lanes 5, 6, and 7), CY6874 (lanes 9, 10, and 11), and BY4742 (lane 13), were grown to stationary phase in minimal medium, diluted 1:20 in YPD and grown for 8 hours at 30º. Protein was harvested by grinding with glass beads and the indicated amount of lysate separated by SDS-PAGE (8%) and Western blotted with anti-Myc antibody.
B. CY6070 was transformed with plasmids containing full length TTI2, or derivatives with C-terminal deletions to residues 350 (Δ351-421) or 325 (Δ326-421). Individual transformants were grown in YPD and spotted serially on a minimal plate containing 5-FOA.
C. Strains CY6070 (DED1-TTI2) and CY6971 (GAL10-TTI2) were grown to stationary phase, then spotted in 10-fold serial dilutions onto YP plates containing 2% galactose, raffinose, or glucose, and grown for three days at 30º.
To test the effect of reducing *TTI2* expression, we placed it under the control of the *GAL10* promoter on a *LEU2* centromeric plasmid in a strain lacking the genomic copy (CY6971). Comparisons were made between *TTI2* expressed from the *DED1*, the promoter of a constitutively expressed RNA helicase, and the *GAL10* promoter. When grown on galactose-containing medium *GAL10-TTI2* supported growth at a rate comparable to *DED1-TTI2* (Figure 2-2c). To test for growth on raffinose and glucose-containing plates, conditions that repress *GAL10* transcription, cells were first grown in liquid culture containing the respective carbon source. Similar to what was observed with *tti2Q276TA*, *GAL10-TTI2* supported viability but showed reduced growth when cells were grown with these carbon sources. These results were reproduced in liquid media where growth of the *GAL10-TTI2* strain in glucose and raffinose decreased the doubling time of cells in logarithmic phase by approximately three and 1.5-fold (relative to *DED1-TTI2*), respectively (Table 2-1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Galactose</th>
<th>Raffinose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY6070 (<em>DED1-TTI2</em>)</td>
<td>2.0 +/- 0.1</td>
<td>2.8 +/- 0.1</td>
<td>1.50 +/- 0.01</td>
</tr>
<tr>
<td>CY6973 (<em>GAL10-TTI2</em>)</td>
<td>2.5 +/- 0.1</td>
<td>4.1 +/- 0.1</td>
<td>4.4 +/- 0.4</td>
</tr>
</tbody>
</table>

To ensure that the observed doubling times were not influenced by excess Tti2 remaining in the cells from their prior growth in galactose, we determined the protein’s half-life. The *tti2* disruption strain containing *GAL10-TTI2* (CY6971) was grown to mid-logarithmic phase with galactose as the carbon source and translation was blocked with cycloheximide. Tti2 levels decreased approximately two-fold after six hours (Figure 2-3a; compare lanes 2 and 7). We conclude that by growing cells in raffinose or glucose containing media over two days, we can deplete the cellular levels of Tti2 and assess the effect on growth.
Figure 2-3. Depleting Tti2 below endogenous levels supports viability.

A. Yeast strains CY6971 and BY4742 were grown to saturation in YP medium containing galactose then diluted 1:100 in the same media and grown for 16 hours to an optical density of 2.0 at 600 nm. 10^8 cells were then harvested before blocking translation and two, four, six, and eight hours after adding 35 µg/ml of cycloheximide. For each time point, cell pellets were washed with lysis buffer then immediately stored at -80º. Total protein was extracted from each cell pellet using an optimized extraction protocol (von der Haar, 2007) and in the presence of protease inhibitors. 1.0 µl of lysate was loaded per time point and separated by SDS-PAGE. Tti2 was detected by Western blotting with anti-Myc antibody and the bottom of the gel was stained with Coomassie Brilliant Blue for a loading control. B. GAL10-TTI2 expression in glucose-containing medium. Yeast strain CY6070 (lanes 1, 2, and 3), CY6971 (lanes 5 and 8), and BY4742 (no tag control; lane 4) were grown to stationary phase in glucose-containing YP, diluted 1:20 in fresh medium and grown for 8 hours before harvesting. Protein was extracted with glass beads and the indicated amounts separated by SDS-PAGE and Western blotted with an anti-Myc antibody to detect tagged Tti2. Lane numbers are listed between the blot and the bottom of the gel, which was stained with Coomassie Brilliant Blue for a loading control (CBB). Lane 6 was left open to separate galactose control lanes. C. CY7086 (genomically encoded C-terminal Myc^0-tagged TTI2, WT-TTI2; lanes 1, 2, 3, and 7), CY6971 (GAL10-TTI2; lanes 4, 5, 6, and 8), and BY4742 (no tag; lane 9) were grown to saturation in YP media containing either raffinose or galactose, then diluted 1:20 in the same media and grown for 8 hours. Protein was extracted by bead lysis and the indicated amounts
separated by SDS-PAGE and Western blotted as described above. The bottom portion of
the gel was stained with Coomassie Brilliant Blue. D. CY6971, CY7172 (YCplac33-
TTI2-TTI2), and BY4742 were grown to stationary in media depleted of leucine and
containing either raffinose or galactose, diluted 1:20 in the same media, and grown for
eight hours before harvesting. Protein extraction and Western blotting was performed as
in (B).
We then examined the steady state expression of Tti2 in different media by Western blotting. The amount of Tti2 was similar when expressed from the GAL10 or DED1 promoter when cells were grown in galactose-containing medium (Figure S 2-2a). In contrast, Tti2 was not detected in 100 µg of protein extract when the GAL10-TTI2 allele was expressed in glucose (Figure 2-3b; lane 5) or raffinose media (Figure S 2-2b; lane 5). In these same conditions DED1-TTI2 expression was detected in one microgram of extract (Figure 2-3b and Figure S 2-2b; lane 2). To provide an indication of the relative expression of GAL10-TTI2 in raffinose and glucose media we engineered the constructs in the two micron plasmid YEplac181. The level of expression from the two micron plasmid supported robust growth on glucose-containing plates (Figure S 2-3a). GAL10 expressed Tti2 from the two micron plasmid was detected in raffinose and glucose-containing media with the expression in raffinose being approximately five-fold higher than in glucose (Figure S 2-3b; compare lanes 4 and 7), a fold-change consistent with what we routinely observe with LacZ-fusions to the GAL10 promoter.

Two approaches were taken to demonstrate that depleting Tti2 using the GAL10 promoter results in expression below endogenous levels. First, the expression of an integrated C-terminally Myc9-tagged tti2 allele was compared to GAL10-TTI2 expression from a centromeric plasmid in raffinose containing medium. Again, Tti2 expressed from the GAL10 promoter was not detected with 100 µg of protein loaded (Figure 2-3c, lane 4). In comparison the endogenous allele allowed detection at 10 µg (lane 2), suggesting a minimum difference of 10-fold in raffinose and by extrapolation 50-fold in glucose medium. Second, we analyzed Tti2 expression regulated by the TTI2 promoter on a centromeric plasmid. Tti2 was detected when expressed from its promoter at 37.5 µg of protein extract (Figure 2-3d, lane 3), while expression from the GAL10 promoter in raffinose was undetectable in 150 µg of protein loaded (lane 4). Taken together, these results indicate that expression of Tti2 below endogenous levels supports viability but lead to slow growth.

2.3.3 Depleting Tti2 levels causes sensitivity to stress conditions

Strains containing YCplac111-GAL10-TTI2 were analyzed to determine whether depleting Tti2 would affect growth under stress conditions, possibly by regulating PIKK
expression. Serial dilutions of the GAL10-TTI2 and DED1-TTI2 containing strains were spotted onto plates with raffinose as the carbon source, and also on raffinose containing either hydroxyurea (ribonucleotide reductase inhibitor), ethanol, Calcofluor white (cell wall integrity), rapamycin (mTOR inhibitor), caffeine, and sodium chloride (osmotic stress) (Figure 2-4a). Growth at 37°C was also examined. Slow growth was observed in all of these stress conditions. For conditions that had more moderate effects on growth we repeated the assay on plates with glucose as the carbon source. In each case (ethanol, Calcofluor white, and rapamycin) this resulted in a more severe growth defect (Figure 2-4b). The pattern resembled that seen for the tti2_276TAA allele, however the GAL10-TTI2 strain was more sensitive to rapamycin and slightly less sensitive to Calcofluor white. Taken together, these results indicate that low levels of Tti2 cause stress sensitivity to conditions that perturb pathways regulated by PIKK proteins, including the DNA damage response, cell wall integrity pathway, nutrient limitation, and osmoregulation.
Figure 2-4. Stress sensitive phenotypes caused by reduced expression of TTI2.

A. CY6070 (DED1-TTI2), CY6971 (GAL10-TTI2), and mec2-1 (included as a control for sensitivity to hydroxyurea; (28)), were grown to stationary phase in YP medium containing raffinose then 10-fold serial dilutions spotted onto YP plates containing raffinose or with raffinose and either 0.1 M hydroxyurea, 6% ethanol, 8 μg/ml Calcofluor white, 1.0 nM rapamycin, or 1 M NaCl and grown for 3 days at 30º or 37º. B. CY6070, CY6971 and CY6874 (tti2Q276TAA) were grown to stationary phase in YPD medium then 10-fold serial dilutions spotted onto YPD plates, or YPD with either 6% ethanol, 8 μg/ml Calcofluor white, 1.0 nM rapamycin, or 10 mM caffeine and grown at 30º for 3 days.
2.3.4 Depleting Tti2 reduces PIKK steady state levels

The nature of the stress-related growth defects following reduced levels of Tti2 suggested that PIKK proteins are affected. We previously found that reduced levels of Tra1 alter the expression of genes involved in stress response (20). To address whether Tra1 was affected by depleting Tti2, we analyzed transcription regulated by a stress response element (STRE), a heat shock element (HSE) and the PHO5 promoter. Tti2 levels were depleted in the GAL10-TTI2 strain by growing in medium containing glucose and β-galactosidase activity was compared to that of the DED1-TTI2 containing strain. The STRE promoter was activated by growth in medium containing 6% ethanol. The PHO5 promoter was activated by growth in phosphate-depleted medium, and the heat shock element by growth at 42º. Activity of the PGK promoter was analyzed under steady state conditions and used as a negative control. As shown in Figure 2-5, expression decreased approximately three-fold in the GAL10-TTI2 strain for each promoter except PGK, suggesting that Tti2 is required for transcription at Tra1-regulated promoters.
Yeast strains CY6070 (DED1-TTI2) and CY6973 (GAL10-TTI2) were transformed with either a PHO5-lacZ fusion, the stress response element (STRE)-regulated lacZ fusion, the heat shock element (HSE)-regulated lacZ fusion, or PGK-lacZ on a LEU2 centromeric plasmid. For the PHO5 promoter, strains were grown to stationary phase in raffinose medium depleted of leucine, cells were washed three times with water before being diluted 1:20 in YPD medium depleted of phosphate and grown for 8 hours. Cell densities were normalized and β-galactosidase activity determined. The β-galactosidase units are the average of three replicates, with the standard deviation shown by error bars. Average enzyme units are compared as the percentage of GAL10-TTI2 to the DED1-TTI2 strain.

Strains containing STRE-lacZ fusions were grown to stationary phase in raffinose medium depleted of leucine, diluted 1:20 in YPD media containing 6% ethanol and grown for 16 hours. HSE-lacZ expression was analysed in strains grown to stationary phase in raffinose medium depleted of leucine, diluted 1:20 in YPD media, grown for 8 hours then heat shocked at 42º for 30 minutes. PGK-lacZ was analyzed after growing cells to stationary phase in raffinose medium depleted of leucine, diluting 1:20 in YPD and growing for 8 hours.
To directly test if reduced levels of Tti2 alter the expression of PIKK proteins, we examined the levels of Flag\(^5\)-Tra1, Mec1 and Tor1 in cells containing *GAL10-TTI2*, comparing to a strain expressing *TTI2* from the *DED1* promoter. Tra1 expression was similar for cells grown in galactose (Figure 2-6a, lanes 7 and 8), but reduced approximately eight-fold for cells grown in glucose (for example compare lanes 3 and 6 in Figure 2-6a). Mec1 expression (Figure 2-6b) was similarly reduced in the *GAL10-TTI2* strain when cells were grown in glucose (densitometry indicated an 8.9-fold change). Tor1 expression was affected to a somewhat lesser extent compared to Tra1 and Mec1 after depleting Tti2, having an approximate 2.5-fold decrease in levels (Figure 2-6c).
Figure 2-6. Expression of Tra1, Mec1, and Tor1 are reduced when Tti2 is depleted.

A. Yeast strains CY5919 (Flag$^5$-TRA1 TTI2; lanes 1, 2, 3, and 7), CY6999 (Flag$^5$-TRA1 tti2::Tn10 luk GAL10-TTI2; lanes 4, 5, 6, and 8) and BY4742 (wild type no tag; lane 9) were grown to stationary phase in media containing glucose or galactose (indicated above each blot), diluted 1:20 and grown for 8 hours before extracting protein by bead lysis. The indicated amount of protein (in micrograms) was separated by SDS-PAGE. Anti-Flag (M2) antibody was used to Western blot the top portion of each gel and the bottom was stained with Coomassie Brilliant Blue (CBB).

B. Protein lysates from strains CY6194 (Flag$^5$-MEC1 TTI2; lanes 1, 2, 3, and 7), CY7030 (Flag$^5$-MEC1 tti2::Tn10luk GAL10-TTI2; lanes 4, 5, 6, and 8), and BY4742 (wild type no tag; lane 9) were prepared and Western blotted as described in (A).

C. Protein lysates from strains CY6415 (Flag$^5$-TOR1/TOR1 TTI2/TTI2; lanes 1, 2, 3 and 7), CY7035 (Flag$^5$-TOR1/TOR1 tti2::Tn10luk/tti2::Tn10luk GAL10-TTI2; lanes 4, 5, 6, and 8), and BY4742 (wild type no tag; lane 9) were prepared and Western blotted as described in (A).
2.3.5 Mislocalization of Tra1 and Mec1 in the absence of Tti2

Tra1 and Mec1 normally localize to the nucleus, however when the stability of each protein is compromised by mutations or truncations at the C-terminus, mislocalization to the cytoplasm occurs (11,16). Since the absence of Tti2 may affect the stability or folding of PIKKs, we determined if depleting Tti2 levels causes mislocalization of Tra1 and Mec1. We integrated an eGFP tag at the N-terminus of Tra1 and Mec1 into a wild type strain and in a strain expressing TTI2 from the GAL10 promoter. Upon depleting Tti2 levels, both Tra1 and Mec1 mislocalized to foci outside the nucleus. eGFP signal was also dispersed throughout the cytoplasm (Figure 2-7). When grown in minimal medium containing glucose, 38% of the fluorescent signal was localized to the nucleus in the eGFP-Tra1 strain expressing abundant Tti2, whereas 18% was nuclear when depleting Tti2. For eGFP-Mec1, 26% of the signal intensity was detected in the nucleus in wild type cells compared to 14% when Tti2 levels were depleted. These results indicate that low levels of Tti2 cause mislocalization of Tra1 and Mec1 into foci and other areas throughout the cytoplasm.
Figure 2-7. Localization of Tra1 and Mec1 upon depleting Tti2.

Strains CY5998 (eGFP-TRA1 WT-TTI2), CY7193 (eGFP-TRA1 GAL10-TTI2), CY6306 (eGFP-MEC1 WT-TTI2), and CY7189 (eGFP-MEC1 GAL10-TTI2) were grown to stationary phase in synthetic complete medium containing raffinose, diluted 1:20 in synthetic complete medium containing glucose and grown to mid-logarithmic phase and stained with Hoechst stain. Cells were harvested and washed twice in sterile water then imaged using fluorescence microscopy.
We then examined if the cytoplasmic foci of eGFP-Mec1 seen upon depleting Tti2 resulted from the formation of Mec1 aggregates within the cell. To detect protein aggregation, we performed Semi-Denaturing Detergent-Agarose Gel Electrophoresis (SDD-AGE), comparing the migration of Flag-tagged Mec1 from strains having either wild type or depleted levels of Tti2. The same molecular weight product for Flag-Mec1 was detected for both strains indicating no change in protein aggregation (Figure S 2-4). A protein product of a Huntingtin's disease allele was used as a positive control for aggregation under the same semi-denaturing conditions.

2.3.6 Overexpressing Hsp90 chaperones and co-chaperones is lethal when Tti2 is depleted

Tti2, along with Tel2 and Tti1, share interactions with Hsp90 and inhibiting Hsp90 prevents the association of Tel2 with PIKK proteins (8). We tested if Hsp90 overexpression could rescue slow growth caused by Tti2 depletion, possibly through an increase in the recruitment of Tti2 to PIKK peptides. We expressed Hsp90 chaperones Hsp82 and Hsc82, and co-chaperones Cdc37 and Aha1, from multicopy plasmids in tti2 disruption strains containing either DED1-TTI2 or GAL10-TTI2. Interestingly, when Tti2 was depleted by growing the GAL10-TTI2 cells in glucose-containing medium, overexpression of each chaperone or co-chaperone resulted in synthetic lethality (Figure 2-8). This effect depended on depleting Tti2 levels as the GAL10-TTI2 strains were viable on galactose-containing plates (Figure 2-8). Together with the results from Takai et al. (8), these findings suggest that altered stoichiometry of protein interactions between Hsp90 chaperones, Hsp90 co-chaperones, and TTT complex members is detrimental to the cell, possibly through an effect on PIKK assembly.
Figure 2-8. Overexpression of Hsp90 chaperones and co-chaperones results in synthetic lethality in strains depleted for Tti2.

Yeast strains CY7245 (DED1-TTI2), CY7249 (GAL10-TTI2), CY7370 (DED1-TTI2 GPD-CDC37), CY7371 (DED1-TTI2 GPD-HSP82), CY7372 (DED1-TTI2 GPD-AHA1), CY7373 (DED1-TTI2 GPD-HSC82), CY7374 (GAL10-TTI2 GPD-CDC37), CY7375 (GAL10-TTI2 GPD-HSP82), CY7376 (GAL10-TTI2 GPD-AHA1), CY7377 (GAL10-TTI2 GPD-HSC82) were grown to stationary phase in minimal medium containing 2% raffinose and lacking leucine. Cell densities were normalized then cells spotted in ten-fold serial dilutions onto both YPD and YP-galactose plates and grown at 30º for two days.
2.3.7 Tti2 is required for stress responses

Considering Tti2’s proposed role as a co-chaperone, and that depleted or mutant Tti2 strains grow slowly at 37°, we hypothesized that Tti2 plays a role in responding to protein stress and managing misfolded proteins. To test this, we examined if exon 1 of the human Huntingtin gene containing a 103 residue polyQ (polyglutamine) sequence caused synthetic slow growth when expressed in the Tti2L187P-containing strain. The 103 residue polyQ sequence caused slow growth in both wild-type TTI2 and tti2-L187P strains when expressed from the GAL10 promoter on a two micron (Figure 2-9a) or centromeric plasmid (Figure S 2-5), whereas a 25 residue polyQ sequence did not. Growth was more severely compromised in the tti2L187P background, suggesting a role for Tti2 in responding to protein stress. To determine whether reduced Tti2 function induces a heat shock response, we analyzed the expression of the heat inducible Hsp42 (25) in strains containing DED1-TTI2 or GAL10-TTI2 when grown at 30° in glucose containing medium. Steady state levels of Hsp42 were increased 3.8 fold when Tti2 was depleted (Figure 2-9b; average signal intensity was calculated using lanes 1, 3, and 5 for DED1-TTI2, and lanes 2, 4, and 6 for GAL10-TTI2). However, as shown in Figure 2-9c Tti2 is not heat inducible, with the expression of endogenous Tti2 slightly decreased after shifting yeast strain CY7086 (WT-TTI2-MYC) from 30° to 42° for 30 minutes, conditions in which Hsp42 was induced (Figure 2-9d). These results suggest that Tti2 does not play a general role in the heat shock response, however depleting Tti2 results in protein stress.
Figure 2-9. Tti2 is required for stress response.

A. Yeast strains CY7245 (DED1-TTI2 YCplac33), CY7236 (DED1-TTI2 GAL1-htt25Q), CY7237 (DED1-tti2L187P GAL1-htt25Q), CY7238 (DED1-TTI2 GAL1-htt103Q), CY7239 (DED1-tti2L187P GAL1-htt103Q), and CY7241 (DED1-tti2L187P YCplac33) were grown to stationary phase in minimal medium containing 2% galactose and lacking uracil. Cell densities were normalized then cells were spotted in 10-fold serial dilutions onto a YP plate containing galactose and grown at 32°C for two days.

B. CY6070, C6973, and a Δhsp42 strain were grown to stationary phase in YP medium containing raffinose, diluted 1:20 in YPD, grown for eight hours at 30º then harvested. Protein was extracted by bead lysis and 10 µg of protein loaded for each sample. Western blotting was performed with anti-Hsp42 antibody. Lane 7 was left blank to avoid overflow into the Δhsp42 lane and the bottom of the gel was stained with CBB for a loading control.

C. Yeast strain CY7086 (WT-TTI2) and BY4742 (no tag control) were grown to stationary phase in YPD, diluted 1:20, grown for eight hours then heat shocked at 42º for 30 minutes. Protein extraction and Western blotting were done as described in (B), except with anti-Myc antibodies. The bottom of the gel was stained with Coomassie Brilliant Blue (CBB) and shown for a loading control. D. The same CY7086 cell lysate from (C) was separated a second time by SDS-PAGE. Protein lysates were then Western blotted with anti-Hsp42 antibody. Protein extract from a Δhsp42 strain (Cashikar et al. 2005) was used as a negative control.
As Hsp42 levels increase in the Tti2-depleted strain, we examined whether its overexpression, or overexpression of Hsp26 or Hsp104, could suppress slow growth and stress sensitivity caused by low levels of Tti2. Each of these heat shock proteins has been characterized for their role as molecular chaperones that bind unfolded or misfolded proteins, preventing or disrupting aggregation and mediating protein reactivation (25,29-31). Hsp26, Hsp42, and Hsp104 were expressed from multicopy plasmids in tti2 disruption strains containing either DED1-TTI2 or GAL10-TTI2. Their overexpression did not result in the synthetic slow growth observed with overexpression of the Hsp90 chaperones and co-chaperones, but also did not suppress slow growth caused by depleting Tti2 on any of the conditions tested (Calcofluor white, hydroxyurea, ethanol, and growth at 37º) (Figure S 2-6).
2.4 Discussion

Tti2 was initially characterized through its interaction with Tel2 and Tti1 in mammalian cells and *S. pombe* (8,10,32). The studies in mammalian cells identified a role for Tel2 in the cellular level of PIKK proteins. The association of Tel2 with nascent PIKK peptides, and interactions of Tti2, Tel2 and Tti1 with chaperones and co-chaperones including, Hsp70, Hsp90, Hsp40, and R2TP subunits (8,10), suggested they were involved in the biosynthesis of members of the PIKK protein family. In *S. cerevisiae* mutation of *tel2* results in shortened telomeres (33), a telomere position effect (34) and defects in DNA repair (35). This is likely due to reduced levels of Tel1 (35), an effect also observed with mutation of *tti2* (36). The effect of individual *S. cerevisiae* TTT proteins on cellular phenotypes and on the PIKK family members has not been well characterized. This is likely in part because gene knockouts are inviable and the limited availability of conditional alleles.

Our screen for alleles of *tti2* that resulted in growth defects identified two alleles, *tti2*<sub>L187P</sub> and *tti2*<sub>Q276TAA</sub>. We have found that with more extensive mutagenesis, additional alleles can be obtained with multiple mutations. The protein is predicted to be rich in alpha helices, and L187 is found within a predicted helix. The proline mutation may destabilize this helix leading to partial loss of function. We have also analyzed numerous N and C-terminal truncations of Tti2 and find that only short truncations at the C-terminus are viable. We were therefore initially surprised to see that the *tti2*<sub>Q276TAA</sub> allele supported viability. Although we do not see full-length protein by Western blotting some read through is likely because ochre mutations are read through by tRNA<sub>Gln</sub> (37,38). Such read through is enhanced if the context of the stop codon is not optimal (39). This has been observed previously for nonsense mutations (40), and for native stop codons and may be a regulated event (41).

The *GAL10*-expressed *TTI2* allele confirmed that low level expression of Tti2 is sufficient for viability. Further characterization revealed that the reduced expression leads to slow growth in many stress conditions. Many of these phenotypes are shared with mutations of PIKK encoding genes (e.g. sensitivity to rapamycin, Calcofluor white, ethanol, hydroxyurea, and caffeine; see Table 2-2 for a list of the phenotypes examined).
In addition, the expression of Tra1-regulated genes decreases upon Tti2 depletion. This further suggests that Tra1 is affected by depleting Tti2 as we have found hypomorphic alleles of TRAI have these same characteristics (20). Indeed, the protein levels of epitope-tagged versions of Tra1, Mec1 and Tor1 were reduced in response to Tti2 depletion, with Tra1 and Mec1 steady state levels being decreased approximately 8-fold. Together with the result of Stirling et al. (36) that Tel1 is reduced in a conditional tti2 strain, our findings suggest that Tti2 is required for the expression of all of the PIKK proteins. The ability of Tti2 to influence the PIKK proteins places it at a strategic position to regulate cell function. The PIKK proteins are themselves regulators of gene expression, chromosomal stability, DNA damage response and protein metabolism. Factors that influence Tti2 will thus have a profound effect on cell survival.

**Table 2-2. Growth phenotypes of the GAL10-TTI2 strain (CY6971) under stress conditions.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Galactose</th>
<th>Raffinose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>6% ethanol</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>8 µg/ml Calcofluor white</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>0.1 M hydroxyurea</td>
<td>++</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>37°C</td>
<td>++</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1 nM rapamycin</td>
<td>++</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>10 mM caffeine</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(++) wild type growth, (+) slow growth, (+/-) severe slow growth

Our results support a role for Tti2 in protein biosynthesis, though perhaps more complex than acting exclusively in protein folding. As expected for a co-chaperone, Tra1, Mec1 and to a lesser extent Tor1 levels are diminished upon Tti2 depletion. Nuclear localization of Tra1 and Mec1 is reduced and cytoplasmic foci apparent; potentially suggesting that Tti2 has a role in protein trafficking. The nature of the Mec1 and Tra1 foci is unclear but they do not appear to result from aggregation. Consistent with Tti2 being required for proteostasis, its depletion induces expression of Hsp42, and the stress sensitive tti2L187P allele causes synthetic slow growth with overexpression of the
Huntingtin gene PolyQ repeat sequences.

The TTT complex is associated functionally with Hsp90 and its co-chaperones. Takai et al. (8) demonstrated that the maturation of PIKK complexes is disrupted by depleting Tel2 or by inhibiting Hsp90. Furthermore, Tel2 links the R2TP/prefoldin like complex with Hsp90 and the PIKKs (8,10,42). Tti2 shares Hsp90 and R2TP interactions with Tel2, and the binding of Tel2 with Tti1-Tti2 is important for PIKK levels (8,9,36). These findings suggest that the toxicity we observe when Hsp90 or its co-chaperones are overexpressed in strains depleted for Tti2, is due to a role for Tti2 with Tel2 and Tti1 in mediating the association of PIKK proteins with Hsp90 and R2TP/prefoldin. We favor the idea that the toxicity is caused by Hsp90 or the co-chaperones sequestering the low level of Tti2 into nonproductive complexes, though we cannot exclude that Tti2 normally functions to negatively inhibit what are detrimental effects of the Hsp90 chaperones.

The finding that cells can survive in exceedingly low levels of Tti2 does suggest however that Tti2 may have a specialized role. This agrees with the limited number of Tti2 client proteins identified to date (only the PIKK proteins), the finding that Tti2 is not a heat shock protein, its relatively low cellular abundance (estimated to be up to 100-fold less than most of the heat shock proteins with the exception of Hsp42; Ghaemmaghami et al. (43); refer to Table 2-3), and that overexpressing Hsp26, Hsp42 or Hsp104 fails to compensate for Tti2 depletion.

The nature of tti2 alleles that suppress slow growth due to mutations in Tra1’s FATC domain also suggests that the role for Tti2 in Tra1 biosynthesis is more complex. The C-terminal FATC domain of the PIKK proteins is integral to the kinase domain (44). Substitution of the terminal phenylalanine of Tra1 to alanine reduces both the level of the protein and its nuclear localization, particularly under stress. We described two alleles of tti2 that almost completely reverse this effect (11). The suppressing alleles are partially dominant, but since we have subsequently found numerous other suppressing alleles, many with multiple mutations, we believe that suppression is due to a loss of Tti2 function. If as predicted, partial loss of a Tti2 function restores the stability and localization of defective Tra1 derivatives, Tti2 would seem to have a role outside of
protein folding. In this regard, the relationship between Tti2, Tel2, and Tti1 needs further investigation. Interestingly, our random selections for suppressors of \textit{tra1}_{F3744A} have identified numerous alleles of \textit{tti2}, but not alleles of \textit{tel2} or \textit{tti1}.

Table 2-3. Relative cellular abundance of TTT members to other heat shock proteins and the R2TP components (43).

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td>Tel2</td>
<td>638</td>
</tr>
<tr>
<td>Tti1</td>
<td>721</td>
</tr>
<tr>
<td>Tti2</td>
<td>1470</td>
</tr>
<tr>
<td>Ssa1 (Hsp70)</td>
<td>269000</td>
</tr>
<tr>
<td>Hsp82</td>
<td>445000</td>
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<tr>
<td>Hsc82</td>
<td>132000</td>
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<td>32800</td>
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<td>19300</td>
</tr>
<tr>
<td>Hsp42</td>
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</tr>
<tr>
<td>Tah1</td>
<td>1660</td>
</tr>
<tr>
<td>Pih1</td>
<td>2610</td>
</tr>
<tr>
<td>Rvb1</td>
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<tr>
<td>Rvb2</td>
<td>3030</td>
</tr>
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</table>

The possibility that the PIKK proteins are the only known clients of the TTT complex has implications with regard to the function of the TTT complex. It also may further define the significance of the PIKK proteins, which may warrant their exclusive regulation by the TTT complex. What targets the PIKK proteins for regulation by Tti2 is unclear? Our earlier study highlighted a genetic interaction between the C-terminal FATC domain of Tra1 and Tti2 (11). The FATC domain is shared amongst the PIKK proteins, but is not likely to be solely responsible because suppression is not allele specific, and similar mutations in \textit{mec1} are not suppressed (11). To further address if there are additional clients of the TTT complex, we have analyzed two-dimensional gels before and after Tti2 depletion. Of the ~500 proteins visible, we could detect none with a change in expression comparable to the Tra1 and Mec1.
2.5 Bibliography


2.6 Supporting Information

2.6.1 Tables

Table S 2-1. Yeast strains used in this study.

<table>
<thead>
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<th>Strain</th>
<th>Genotype</th>
<th>Plasmid (plasmid-promoter-gene)</th>
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Table S 2-2. Oligonucleotides used in this study.

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2.6.2 Figures

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Figure S 2-1. Sequence of oligonucleotide to integrate C-terminal *TTI2* Myc$^9$-tag.
Figure S 2-2. GAL10-TTI2 expression in galactose and raffinose containing media.

A. GAL10-TTI2 expression in galactose-containing medium. Yeast strain CY6070 (lanes 1, 2, and 3), CY6971 (lanes 5, 6, and 7), and BY4742 (no tag control; lane 4) were grown to stationary phase in galactose-containing YP, diluted 1:20 in fresh medium and grown for 8 hours before harvesting. Protein was extracted with glass beads and the indicated amounts separated by SDS-PAGE and Western blotted with anti-Myc antibody to detect tagged Tti2. Lane numbers are listed between the blot and the bottom of the gel, which was stained with Coomassie Brilliant Blue for a loading control (CBB). B. GAL10-TTI2 in raffinose medium. Strains CY6070, CY6971 and BY4742 were prepared for Western blotting as in (A), with the exception that galactose was substituted by raffinose.
Figure S 2-3. Relative levels of constitutive and depleted Tti2 expression.

A. CY6070, CY6971 (CEN GAL10-TT12), and CY6991 (2µ GAL10-TT12) were grown to stationary phase in YPD medium, cell densities were normalized, then spotted in 10-fold serial dilutions onto a YPD plate and grown for three days at 30º. B. Yeast strains CY6070 (lanes 1, 2, and 3), CY6991 (lanes 4-9), and BY4742 (no tag control; lane 10) were grown to stationary phase in YP media containing the indicated carbon source, diluted 1:20 in the same media, then grown for 8 hours. Cells were lysed using glass beads and protein extracts separated by SDS-PAGE. Western blotting was then performed using an anti-Myc antibody. The bottom of the gel was stained with Coomassie Brilliant Blue and shown as a loading control.
Figure S 2-4. Depleting Tti2 does not result in aggregation of Mec1.

Yeast strain CY6194, CY7030, and BY4742 were grown in raffinose containing medium then diluted 1:20 in YPD and grown for 8 hours. Protein was extracted by bead lysis and the indicated amounts separated on a 1.8% agarose gel containing 0.1% SDS. Protein was transferred onto a PVDF membrane by capillary transfer and Western blotted using an anti-Flag antibody (left). A W303 yeast strain expressing human huntinton exon I variant containing a 72 residue polyQ region was used as a positive control (described in Duennwald et al. 2006). The membrane was stained with Ponceau S prior to Western blotting and shown for a loading control (right).
Figure S 2-5. Synthetic slow growth due to exon 1 of the human Huntingtin gene containing a 103 residue polyQ sequence.

The wild-type (CY6857) and tti2L187P (CY6872) strains were transformed with YCplac33 or CEN-GAL1-htt103Q, and grown to stationary phase in minimal medium containing 2% glucose and lacking uracil. Cell densities were normalized then cells spotted in 10-fold serial dilutions onto a YP plate containing 2% galactose and grown at 30°C for two days.
Figure S 2-6. Overexpression of HSP26, HSP42, or HSP104 does not compensate for depleting Tti2.

Strains CY7245 (DED1-TTI2 YCplac33), CY7249 (GAL10-TTI2 YCp33), CY7247 (DED1-TTI2 2µ-GPD-HSP26), CY7251 (GAL10-TTI2 2µ-GPD-HSP26), CY7248 (DED1-TTI2 2µ-GPD-HSP104), CY7252 (GAL10-TTI2 2µ-GPD-HSP104), CY7323 (DED1-TTI2 2µ-HSP42-HSP42), and CY7324 (GAL10-TTI2 2µ-HSP42-HSP42) were grown to stationary phase in medium lacking uracil and containing raffinose. Cell densities were normalized and then 10-fold serial dilutions spotted onto YPD plates grown at either 30º or 37º, and onto YPD plates containing 6% ethanol, 8 µg/ml Calcofluor white, or 100 mM hydroxyurea.
Chapter 3

3 Genetic selection for mistranslation rescues a defective co-chaperone in yeast

3.1 Introduction

Protein synthesis is a high fidelity process that ensures accurate production of the proteome as specified by the sequence of codons in protein coding genes. Overall error rates for translation indicate one in every ~5000 amino acids are mis-incorporated (1). Faithful interpretation of the genetic code requires that aminoacyl-tRNA synthetases (aaRSs) specifically recognize and ligate their cognate tRNAs with the appropriate amino acid (2). Accurate recognition is determined by specific nucleotides in the tRNA called identity elements that allow the aaRSs to discriminate between tRNA species (3,4). Altering identity nucleotides can cause loss of amino acid charging, reduced substrate affinity, or a decrease in turnover (5-8). Certain changes or modifications to identity nucleotides switch tRNA identity, causing mis-aminoacylation of a tRNA by a non-cognate aaRS (9,10). Ten of the aaRSs have editing activities that prevent the formation of mis-aminoacylated tRNA species (11). While cells also have quality control mechanisms to cope with increased rates of mistranslation and protein mis-folding (12-14), defects in the editing activity of aaRSs can result in cancer and neurodegeneration (15,16), and lead to a cardiac disease phenotype in mouse models (17).

In an apparent contradiction, cells are able to tolerate large amounts of amino acid mis-incorporation with respect to the standard codon assignments (18). It is estimated that *Escherichia coli* tolerates a proteome with 10% of proteins made incorrectly by activating a compensatory heat shock response to eliminate mis-folded proteins (13). Under certain conditions tRNA mis-aminoacylation and the resulting mistranslation is an adaptive response (13,19-22). Despite the loss in translational fidelity, altering tRNA identity can allow cells to survive otherwise lethal mutations. Such mutant tRNAs are known to suppress missense, frameshift, and nonsense mutations (23-25).

We identified a novel missense suppressor tRNA\textsubscript{Pro} and elucidated a mechanism that induces yeast cells to mistranslate Pro codons. We selected suppressor mutations for
a stress-sensitive Leu (CTA) to Pro (CCA) substitution at residue 187 in the
Saccharomyces cerevisiae co-chaperone Tti2. Tti2 is involved in the folding and
regulation of phosphatidylinositol 3-kinase related kinases (PIKKs) (26-29). The
defective tti2 allele results in loss of function and an inability to grow under stress
conditions. We demonstrate that Pro to Ala mistranslation in vivo restores wild-type like
growth in the tti2L187P-containing suppressor strains, and that yeast cells tolerate this
proline to alanine mis-incorporation. As suppression can also be engineered with
tRNAProAGG, we have identified a general mechanism to direct proteome-wide missense
Ala mutations at all Pro codons.

3.2 Material and Methods

3.2.1 Yeast strains and growth conditions
Yeast strains were grown in Yeast Peptone media containing 2% glucose (YPD) or
synthetic media supplemented with nitrogenous bases and amino acids as required. For
spot plate assays, strains were grown to stationary phase, normalized to cell density, then
spotted in 10-fold serial dilutions onto YPD plates or YPD plates containing 6% ethanol
or 6 µM tunicamycin. Cells were grown at 30°C unless otherwise indicated. For growth
curves, strains were grown to stationary phase in selective media diluted 1:100 in YPD
media and grown at 30°C. Optical density measurements were taken every hour and
average growth rates and doubling times were calculated using three biological replicates
per strain.

All strains used (Table S 3-1) were derived from either BY4741, BY4742, or
BY4743 (30). The tti2 disruption strains CY6070 and CY6857 have been described (31).
YCplac111-DED1-tti2Q276TAA and YCplac111-DED1-tti2L50P were transformed into
CY6070, and YCplac33-DED1-TTI2 was lost by plating on 5-fluoroorotic acid to
generate CY6874 and CY6944, respectively. CY6070 was crossed with BY4742, giving
rise to the diploid strain CY6945. Haploid spore colonies of CY6945 were genotyped to
identify MATa and MATα strains CY6963 and CY6965, respectively. YCplac111-DED1-
tti2L187P was transformed into CY6963 and YCplac33-DED1-TTI2 lost by plating on 5-
fluoroorotic acid to generate CY7020. YCplac111-DED1-tti2L187A was plasmid shuffled
into CY6963 to give CY7369. CY7093, CY7105, CY7106, and CY7108 were selected as spontaneous suppressors of the slow growth of CY7020 and contain \( tP(UGG)N1_{G3:U70} \), \( tP(UGG)N2_{G3:U70} \), \( SUF8_{G3:U70} \), and \( SUF9_{G3:U70} \), respectively. CY7222 was obtained after mating CY7108 with CY6965 and selecting a Leu+ ethanol resistant spore colony. To generate CY7243, YCplac33-DED1-TTI2 was transformed into CY7222 and the \( tti2_{L187P} \) allele lost by repeated growth on media containing leucine. This strain was crossed to CY7093. The resulting diploid strain was sporulated and Ura+ strains CY7286 containing \( SUF9_{G3:U70} \) and \( tP(UGG)N1_{G3:U70} \), CY7287 containing \( SUF9 \) and \( tP(UGG)N1 \), and CY7288 containing \( SUF9_{G3:U70} \) and \( tP(UGG)N1 \) were identified. CY7093 was transformed with YCplac33-DED1-TTI2 and YCplac111-DED1-TTI2\(_{L187P} \) lost by repeated growth on media containing leucine to generate CY7355.

Yeast strain CY7416 was purchased from GE Healthcare (described in (32)). The Sik1-RFP strain used for fluorescence microscopy was provided by Martin Duennwald and has been described (33). Yeast strains CY2423, CY7450, and CY1217 contain deletions of \( rpn4 \), \( atg8 \), and \( ire1 \), respectively. Each strain is derived from a spore colony of the yeast magic marker strain in the BY4743 diploid background (34).

### 3.2.2 Plasmid constructs

\( SUF9 \) (pCB2957) and \( SUF9_{G3:U70} \) (pCB2948) including 500bp of upstream sequence were amplified by PCR using primers TH4224 and TH4225 (Table S 3-2) and wild-type or CY7108 genomic DNA, respectively. \( SUF9 \) alleles were cloned as a HindIII fragment into YCplac33 and YCplac111.

\( SUF2 \) was amplified by PCR using genomic DNA as template with primers UA9497 and UA9498, then ligated into YCplac33 as a BamHI/EcoRI fragment (pCB3015). To introduce the suppressor mutation into the 3'-end acceptor stem, YCplac33-SUF2 was used as template in a PCR with primers UA9498 and UB0181. This product was cloned as a SmaI/SalI fragment into YCplac33-SUF2 (pCB3028).

A two-step PCR was used to remove the \( SUF9 \) intron and introduce the intron into \( SUF2 \). \( SUF9 \) was amplified using primer TH4224 with UF4196 and TH4225 with
UF4197. The PCR products were the template in a second round of PCR with primers TH4224 and TH4225. Primers UA9497 with UF4199 and UA9498 with UF4198 were used to amplify $SUF2$ in the first step, and primers UA9497 and UA9498 in the second step. $SUF9$ was cloned into YCplac33 as a HindIII fragment, $SUF2$ as a BamHI/EcoRI fragment.

$TTI2$ (pCB2595) and $tti2_{L187P}$ (pCB2599) expressed from the $DED1$ promoter and containing TAP and Flag tags (35) were cloned in the $URA3$ two micron plasmid YCplac195 as NotI/SacI fragments. The $tti2_{L187P}$ allele has been described (31). $tti2_{L50P}$ was isolated in an identical screen. $tti2_{L187A}$ was created by a two-step primer extension PCR. Using primers 5693-1 with TK7291, and 5693-2 with TK7290 in the first step and primers 5693-1 and 5693-2 in the second step. This product was cloned as a NotI/SacI fragment into YCplac111 (pCB3020).

Yeast AlaRS gene ($ALAI$) was amplified by PCR with primers TI5697 and TI5698. The PCR product was digested with NcoI/EcoRI and EcoRI/SacI, and triple ligated into NcoI/SacI cut pP$_{RO}$EX$^TM$ HTa (Invitrogen; pCB2975).

eGFP$_{D129P}$ (CCA) was generated by QuikChange (Agilent) using eGFP expressed from the $DED1$ promoter on a $URA3$ centromeric plasmid (36) as template and oligonucleotides UD2159 and UD2160. $SPT7$ was subsequently cloned into this vector NotI/EcoRI from YCp88-$myc^9$-$SPT7$ (35) giving $eGFP_{D129P}$-$SPT7$ (pCB3057). Primers UF4570 and UF4571 were similarly used to create $eGFP_{D129A}$-$SPT7$.

Centromeric $URA3$-containing plasmid expressing $htt103Q$ from the $GAL1$ promoter (described in (37)), as well as the $URA3$-containing plasmid expressing $HSE$-$eGFP$, were kindly provided by Martin Duennwald.

### 3.2.3 Selection of suppressor mutations of $tti2_{L187P}$

CY7020 was grown to stationary phase in YPD medium and approximately $10^6$ cells from independent cultures plated onto each of ten YPD plates containing 6% ethanol. Colonies with restored growth were confirmed after growth under non-selective conditions. Suppressor strains where Western blotting indicated an increase in Tti2
expression or where plasmid shuffling indicated that the suppressor mutation was plasmid borne were eliminated. Four of ten colonies were taken for further characterization.

3.2.4 Preparation of genomic DNA

Yeast strains were grown in 25 ml YPD medium to stationary phase, harvested, washed in 10 ml of sterile water, and suspended in 5 ml of water containing 100 µl of β-mercaptoethanol. After 15 minutes cells were harvested and cell pellets incubated at 37ºC for 1 hour in 5 ml of 1 M sorbitol, 100 mM sodium citrate (pH 5.8), 10 mM EDTA, and 1 mg of lyticase. Cells were harvested and suspended in 1.2 ml of 150 mM NaCl, 100 mM EDTA, and 0.1% SDS. A half volume of buffered phenol was added and mixed gently for 30 minutes before adding a half volume of chloroform. The aqueous phase was collected and the extraction repeated. Nucleic acids were precipitated with two volumes of ethanol and incubating at -20ºC for 15 minutes. Precipitated nucleic acids were pelleted, washed with 80% ethanol, and suspended in 500 µl of Tris-EDTA buffer. RNase was added to 20 µg/ml and incubated at 37ºC for 1 hour. Nucleic acids were purified using phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform:isoamyl alcohol (24:1) extraction and precipitated with 2.5 volumes of 95% ethanol. Genomic DNA was washed in 75% ethanol, air dried, then resuspended in 50 µl of H₂O.

3.2.5 Genomic sequencing and analysis

Samples were processed at the London Regional Genomics Centre (http://www.lrgc.ca) using the Illumina MiSeq (Illumina, Inc.). High molecular weight DNA was quantified using the Qubit DNA HS reagent (Life Technologies) and 1 ng from each sample processed as per the Nextera XT DNA Library Preparation Guide (Illumina, PN: 15031942 Rev. E). Briefly, samples were tagmented, amplified (via PCR with indexed primers to permit sample pooling), cleaned-up and equimolar pooled into one library. The pooled library was analyzed on an Agilent High Sensitivity DNA Bioanalyzer chip (Caliper Life Sciences) to assess size distribution. The quantity of the library was assessed via qPCR (Kapa Biosystems, Inc.). The library was sequenced on an Illumina
MiSeq using 2 x 150 paired end run. Approximately 18 million reads (150 bp paired end) that mapped to the S288c genome were obtained. Data analysis was performed using CLCbio Genomics Workbench, v8.1. Paired end reads (150 bp) were mapped to the Saccharomyces cerevisiae S288c reference genome using the local alignment tool and duplicate reads removed. The fixed ploidy variant detection tool was used with a ploidy of one, required variant probability of 90%, a minimum coverage of 10 reads, and a minimum read quality score of 20. All variants that were detected in the control strain, CY7020, were removed from the analysis of each suppressor strain using the compare sample variants tool.

### 3.2.6 Preparation of tRNAs

Oligonucleotides encoding a T7 promoter and yeast tRNA genes (SUF9-1 and SUF9-2 for tRNA<sup>Pro</sup><sub>UGG</sub>, SUF9<sub>G3;U70</sub>-1 and SUF9<sub>G3;U70</sub>-2 for tRNA<sup>Pro</sup><sub>G3;U70</sub>, and tA(AGC)D-1 and tA(AGC)D-2 for tRNA<sup>Ala</sup><sub>AGC</sub>) were obtained from Sigma-Aldrich. Complementary oligonucleotides for each tRNA gene were phosphorylated with polynucleotide kinase (Roche Inc) at 37°C for 1 hour and hybridized in T4 DNA ligase buffer (New England Biolabs) by boiling for 5 minutes at 95°C then gradual cooling to room temperature over 2 hours. Hybridized products were ligated into pTZ19r and verified by sequencing.

Each tRNA gene was amplified in a 50 µl PCR reaction, using primers UA9593 with UA9594 for SUF9, UA9595 with UA9596 for tA(AGC)D, and UA9597 with UA9598 for SUF9<sub>G3;U70</sub> (Table S 3-2). PCR products were gel purified then used as template for in vitro transcription. Transcription reactions were carried out in 40 mM Hepes/KOH pH 8.0, 22 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 1 mM spermidine, 4 mM of each NTP, 30 nM T7 RNA polymerase and 10 µg of template DNA at 37°C for 3 hours. RNA transcripts were isolated on a 12% polyacrylamide gel, extracted in 3 M sodium acetate and precipitated in an equal volume of isopropanol at -80°C. tRNAs were folded by heating to 95°C for 5 minutes, then gradually cooling to room temperature. MgCl<sub>2</sub> (10 mM) was added at 65°C. For 3'-end labelling, 2 µg of folded tRNA was incubated at 37°C for 1 hour with CCA adding enzyme and [α-<sup>32</sup>P] ATP (PerkinElmer) as described in (38). Labelled tRNAs were purified using BioSpin30 columns (Bio-Rad Laboratories, Inc.).
3.2.7 Protein purification

Yeast strains expressing YEplac195-TAP-Flag-tti2187P (URA3-containing two-micron plasmid) and either SUF9 or SUF9G3:U70 expressed from a LEU2 centromeric plasmid (YCplac111) were grown to stationary phase in minimal medium lacking uracil and leucine, diluted 1:1000 in YPD medium and grown to an $OD_{600nm} = 2.5$. Cells were harvested and protein extracted by grinding cells in liquid nitrogen. Lysate was cleared by ultracentrifugation at 110000 x g for 1 hour. TAP-tagged Tti2 was purified using the two-step tandem affinity purification protocol as described in (39).

Yeast strain Y258 over-expressing yeast Protein A-tagged ProRS (BG1805-YHR020W, GE Healthcare) was grown for 48 hours in minimal media lacking uracil and containing 3% ethanol, 3% glycerol, and 0.05% glucose. Cells were diluted 1:25 into the same medium and grown for another 48 hours. 25 mL of cells were diluted into 500 mL of YP medium containing 2% galactose. At $OD_{600nm} = 3.0$, cells were harvested by centrifugation. Cell pellets were lysed by grinding in liquid nitrogen. ProRS was purified by binding to IgG resin (Sigma-Aldrich) in buffer containing 10 mM Tris-HCl pH 8.0 and 150 mM NaCl for 2 hours. The resin with bound protein was washed three times with 4 ml of binding buffer and purified protein was cleaved from the resin with Protease 3C (a kind gift from Elton Zeqiraj in Dr. Frank Sicheri’s lab) in buffer containing 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol. This was dialyzed into the same buffer with 40% glycerol and stored at -20°C.

*E. coli* expressing pPROEX HTa-ALA1 was grown to stationary phase in 2 ml of LB medium containing 25 µg/ml chloramphenicol and 100 µg/ml ampicillin. Cells were diluted 1:1000 into 500 ml of the same LB medium and grown to an $OD_{600nm} = 0.6$. Isopropyl β-D-1-thiogalactopyranoside was added to 1 mM and cells induced overnight at room temperature. Harvested cells were lysed in 25 mM Hepes pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.5 mg/ml lysozyme. Histidine-tagged AlaRS was purified using TALON® resin (Clontech Laboratories, Inc.) as described by the manufacturer, and eluted with 100 mM imidazole. Purified protein was dialyzed overnight into 50 mM Hepes (pH 7.5), 300 mM NaCl, 40% glycerol and stored at -20°C.
3.2.8 Aminoacylation assay

Aminoacylation reactions were performed, as described in (40), with 3 µM tRNA, 300 nM $^{32}$P-labelled tRNA, 10 mM amino acid, 5 mM ATP (pH 7.0), and either 1 µM ProRS, 100 nM AlaRS, or 500 pM AlaRS. Two microlitres of each reaction was spotted onto polyethyleneimine-cellulose thin layer chromatography plates (EMD Millipore) and developed in 5% acetic acid and 100 mM ammonium acetate. TLC plates were exposed to phosphor screens and imaged using a Storm 860 Phosphorimager (GE Healthcare Life Sciences). Densitometry analysis of the image was done using ImageJ 1.49i. Enzyme activities were calculated across three technical replicates.

3.2.9 Fluorescence microscopy and GFP reporter assay

Yeast strains were grown to stationary phase in selectable media, diluted 1:20, and grown for 5 hours before harvesting. Cells were imaged using a Nikon eclipse Ti confocal microscope coupled with a Nikon DS-Qi1Mc camera head and using differential interface contrast (DIC), GFP, and RFP filters. Exposure time and gain settings were left constant across all GFP and RFP images. Cell measurements were taken using Image J 1.50i with the following protocol. The DIC, GFP, and RFP images for each field of view were merged by selecting Image-> Color -> Merge Channels and assigning each image to a different channel. RFP-Sik1 was used to identify the nucleus which was circled with the oval selection tool for each cell. The intensity of the GFP signal was calculated using the measure tool. Background intensity was calculated from an open area adjacent to the cell. Integrated density values were divided by the area of the oval selection and the background integrated density values subtracted from each cell measurement. An average and median value of integrated density per unit area was calculated across 150 cells per strain. R version 3.2.0 along with ggplot2 (41) was used to generate the box plot.

For the HSE-eGFP reporter assay, yeast strain BY4742 containing HSE-eGFP and SUF9 or HSE-eGFP and SUF9G3:U70 were grown to stationary phase in medium lacking leucine and uracil, diluted 1:100 in the same medium, and grown for six hours at 30ºC. BY4742 containing HSE-eGFP was heat shocked at 42ºC for one hour and used to determine the heat shock induced eGFP signal intensity. All cell densities were
normalized by optical density at 600 nm prior to measuring fluorescence. Fluorescence
was measured with a BioTek Synergy H1 microplate reader at an emission wavelength of
528 nm using Gen5 2.08 software. A BY4742 strain lacking HSE-eGFP, and containing
either SUF9 or SUF9<sub>G3:U70</sub>, was used to subtract background fluorescence from each
respective experimental strain. The average signal intensity was calculated across three
technical and three biological replicates for each strain.

### 3.2.10 Mass spectrometry

Sample preparation was performed at the Functional Proteomics Facility (University of
Western Ontario, [http://www.uwo.ca/biochem/ fpf/](http://www.uwo.ca/biochem/ fpf/)). TAP-Tti2 was tandem affinity
purified from BY4742 expressing either SUF9 or SUF9<sub>G3:U70</sub>. Tti2 was picked from a
10% polyacrylamide gel stained with Coomassie Brilliant Blue using the Ettan™ Spot
Picker robotic system. Gel pieces were destained in 50 mM ammonium bicarbonate and
50% acetonitrile, reduced in 10 mM dithiothreitol (DTT), alkylated using 55 mM
iodoacetamide (IAA), and digested with trypsin (prepared in 50 mM ammonium
bicarbonate, pH 8). The Waters MassPREP Station (PerkinElmer) was used for in-gel
digestion. Peptides were extracted in 1% formic acid and 2% acetonitrile, then
lyophilized.

Nano liquid chromatography-coupled tandem mass spectrometry (nano LC–
MS/MS) analysis of the digested protein was performed with approximately 250 ng of
peptides on column. The peptide mixture was separated by C18 reverse-phase
chromatography on an ACQUITY MClass Peptide BEH column (length, 25 cm; inner
diameter (i.d.), 75 μm; particle size, 1.7 μm, Waters Inc.) using a trapping column
(ACQUITY MClass Trap, Symmetry C18, length, 2 cm; i.d., 180 μm; particle size, 5 μm,
Waters Inc.) and a mobile phase gradient from 5 to 40% (v/v) ACN, 0.1% (v/v) FA in 90
min with a flow rate of 300 nL/min on an MClass UHPLC (nano) chromatography
system (Waters Inc.). Peptides were ionized utilizing a nanospray Flex ion source
(Thermo Fisher Scientific) into an Orbitrap Elite Velos Pro mass spectrometer (Thermo
Fisher Scientific) via fused silica emitter tip. FT and IT injection waveforms were
enabled, 1 microscan was acquired for all scan types. FT master scan preview was
enabled, as was charge state screening; singly charged ions were rejected in DDA
analysis. The mass spectrometer was calibrated internally with the \( m/z \) of 445.120024. Peptide ions were fragmented using Collision Induced Dissociation (CID). The overall acquisition was an FT/IT/CID Top 15, DDA targeted scheme using dynamic exclusion in positive ion mode. The targeted ions were the +2 and +3 charged states of the peptide of interest, GVLLAQTLNLHFmNETNDSK, where m is oxidized Methionine and A represents the substituted amino acid.

Data files (raw format, Thermo Scientific) were searched against the Uniprot, Yeast database (6729 sequences), with the Peaks algorithm (v.7.5, Bioinformatics Solutions Inc.) using the Spider node for homology search (single point mutation). For database search, the enzyme was set to trypsin, tolerating three missed cleavages and one non-specific cleavage end. The parent mass tolerance was set to 20 ppm, and the MS/MS fragment mass tolerance, to 0.8 Da. Cysteine carbamidomethylation was set as a fixed modification, and oxidation of methionine and deamidation of Asparagine/Glutamine were tolerated as a variable modification. Global false discovery rate (FDR) was set to 1% with minimum 1 unique peptides required for protein identification. All acquired mass spectra are available upon request.

3.2.11 Western blotting

Yeast strains were grown to mid-logarithmic phase then lysed with glass beads. Western blotting was performed with anti-GFP antibodies (Sigma) kindly provided by Patrick Lajoie (University of Western Ontario), and anti-Myc antibodies (Sigma), and used as previously described (31).

3.3 Results

3.3.1 Identifying second site suppressor mutations for \( tti2_{L187P} \)

Tti2 is an Hsp90 co-chaperone required for the folding and stability of PIKK family proteins including Tra1, Mec1, Tel1, and Tor1 (27-29). We identified \( tti2_{L187P} \) and \( tti2_{Q276TAA} \) (31), and subsequently \( tti2_{L50P} \) in random screens for alleles of \( TTI2 \) that cause stress sensitive growth. As our goal was to identify specific functions of Tti2 and its interactions, we undertook a genetic approach selecting for second-site suppressors of the
slow growth caused by the \( tti2_{L187P} \) allele. CY7020 (\( tti2_{L187P} \)) was grown to stationary phase and approximately \( 10^6 \) cells plated without mutagenesis onto each of ten YPD plates containing 6% ethanol. Spontaneous suppressor strains were colony purified then examined for expression of Tti2_{L187P} by Western blotting. Strains with a near wild-type level of expression of \( Tti2_{L187P} \) were further characterized. Suppression was observed in each of four independent strains, CY7093, CY7105, CY7106 and CY7108 (Figure 3-1a), where 2:2 segregation indicated that single mutations were responsible (not shown).

Whole genome sequencing was performed to reveal potential causative mutations in each strain, comparing to the parent CY7020. Interestingly, all of the strains had a cytosine to thymine point mutation at nucleotide 100 in different \( tRNA^{Pro}_{UGG} \) (proline tRNA molecule with UGG anticodon) genes (Table 3-1).

Table 3-1. Suppressors \( tRNA^{Pro}_{UGG} \) mutations identified by next generation sequencing.

<table>
<thead>
<tr>
<th>tRNA gene names (anticodon in subscript)</th>
<th>Chromosome: Position of Mutation</th>
<th>Mutation Type</th>
<th>Reference Nucleotide</th>
<th>Variant Nucleotide</th>
<th>Reads with Variant</th>
<th>Total Number of Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>( tP(UGG)N1_{UGG} )</td>
<td>XIV:547193</td>
<td>SNV</td>
<td>C</td>
<td>T</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>( tP(UGG)N2_{UGG} )</td>
<td>XIV:568214</td>
<td>SNV</td>
<td>C</td>
<td>T</td>
<td>68</td>
<td>69</td>
</tr>
<tr>
<td>( SUF8_{UGG} )</td>
<td>VIII:388896</td>
<td>SNV</td>
<td>C</td>
<td>T</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>( SUF9_{UGG} )</td>
<td>VI:101475</td>
<td>SNV</td>
<td>C</td>
<td>T</td>
<td>67</td>
<td>67</td>
</tr>
</tbody>
</table>

The CY7108 strain containing \( SUF9_{G3:U70} \) and \( tti2_{L187P} \) was characterized in further detail. To verify that the \( SUF9_{G3:U70} \) mutation was responsible for suppression, its linkage with the suppressor phenotype was analyzed. CY7108 was mated with the \( tti2\)-disrupted strain CY6965, which contains \( TTI2 \) on a Ura3+ centromeric plasmid. The diploid was sporulated to allow independent assortment. The resulting spore colonies were plated on 5-FOA to eliminate the wild-type copy of \( TTI2 \) and select for strains containing \( tti2_{L187P} \). Each spore colony was then examined for growth on medium containing ethanol to determine if there was suppression or a stress sensitive phenotype. We sequenced the \( SUF9 \) allele of 10 spore colonies, including four without and six with
the suppressor phenotype. In each case, $SU9_{G3:U70}$ was linked to suppression whereas slow growth was associated with the wild-type $SU9$ allele, thus indicating that $SU9_{G3:U70}$ was responsible for suppression.

We then introduced $SU9$ or $SU9_{G3:U70}$ from a centromeric plasmid into the $tti2_{L187P}$ strain (CY7020) to determine if the mutant allele was sufficient for suppression. The plasmid expressing $SU9_{G3:U70}$ suppressed slow growth of the $tti2_{L187P}$ strain on an ethanol stress plate (Figure 3-1b). The $SU9_{G3:U70}$ allele did not suppress $tti2_{Q276TAA}$ or $tti2_{L50P}$ (CUC to CCC), indicating that suppression is allele specific and thus $SU9_{G3:U70}$ acts directly by mistranslating P187 (Figure 3-1c). These results suggest that the G3:U70 mutation in tRNA$^{Pro}_{UGG}$ is directly responsible for the suppression of $tti2_{L187P}$ in each of the four sequenced suppressor strains.
Figure 3-1. Suppression of \( tti2_{L187P} \) by \( tRNA^{Pro\,UGG}\ (G3:U70) \) alleles.

A) Yeast strains CY7020 (\( tti2_{L187P} \)), CY7093 (\( tti2_{L187P}
\( tP(UGG)N1_{G3:U70} \)), CY7105 (\( tti2_{L187P}
\( tP(UGG)N2_{G3:U70} \)), CY7106 (\( tti2_{L187P}
\( SUF8_{G3:U70} \)), CY7108 (\( tti2_{L187P}
\( SUF9_{G3:U70} \)) were grown to stationary phase in YPD media. Cell densities were normalized then spotted in 10-fold serial dilutions onto YPD plates and grown at either 30°C or 37°C and onto a YPD plate containing 6% ethanol and grown at 30°C. B) Strain CY6963 containing YCplac33-\( DED1\)-\( TTI2 \) (\( TTI2 \)), and CY7020 (\( tti2_{L187P} \)) containing either YCplac33 (-), YCplac33-\( SUF9 \) (G3:C70), or YCplac33-\( SUF9_{G3:U70} \) (G3:U70) were grown to stationary phase in medium lacking uracil, then spotted in 10-fold serial dilutions onto a YPD plate and a YPD plate containing 6% ethanol and grown at 30°C. C) Strains CY7020 (\( tti2_{L187P} \)), CY6944 (\( tti2_{L50P} \)), and CY6874 (\( tti2_{Q276TAA} \), each containing either YCplac33-\( SUF9 \) (G3:C70) or YCplac33-\( SUF9_{G3:U70} \) (G3:U70), were
grown to stationary phase in medium lacking uracil, then spotted in 10-fold serial
dilutions onto a YPD plate and a YPD plate containing 6% ethanol and grown at 30°C.
3.3.2 Suppression is due to alanine substitution for proline during translation of \( tti_2L_{187P} \)

The suppressor mutation in the tRNA\(^{\text{Pro}}\)\(_{UGG}\) genes results in a G3:C70 to G3:U70 base pair change in the tRNA acceptor stem (Figure 3-2a). The G3:U70 pair is the major identity element for alanyl-tRNA synthetase (AlaRS) (42,43). Introducing this base pair confers alanine accepting identity to tRNA\(^{\text{Cys}}\) and tRNA\(^{\text{Phe}}\) in \( E.\ coli \) (44). We predicted that the G3:U70 base pair in tRNA\(^{\text{Pro}}\)\(_{UGG}\) (C70U) results in aminoacylation with alanine and subsequent mis-incorporation of alanine into Tti2\(_L_{187P}\). Consistent with this model an allele of \( TTI2 \) encoding an Ala in place of the wild-type Leu (\( tti_2L_{187A} \)) grows at near wild-type levels under stress conditions (Figure 3-2b).
Figure 3-2. tRNA secondary structures and stress resistance of $tti2_{L187A}$.

A) Secondary structures of $tRNA^{Pro}_{UGG}$, $tRNA^{Pro}_{UGG}$ (G3:U70), and $tRNA^{Ala}_{AGC}$. Each anticodon and the suppressor mutation position are in bold font and the discriminator base is highlighted in orange. B) Yeast strains CY6857 containing YCplac111-$DED1-TTI2$ ($TTI2$), CY7369 containing YCplac111-$DED1-tti2_{L187A}$ ($tti2_{L187A}$), and CY7020 containing YCplac111-$DED1-tti2_{L187P}$ ($tti2_{L187P}$) were grown to stationary phase in YPD medium. Cell densities were normalized before spotting in 10-fold serial dilutions onto YPD plates and grown at either 30°C or 37°C and onto a YPD plate containing 6% ethanol and grown at 30°C.
We performed *in vitro* aminoacylation assays to determine the amino acid accepting activity of tRNA$^{Pro}$$_{UGG}$, tRNA$^{Pro}$$_{UGG}$ (G3:U70), and tRNA$^{Ala}$$_{AGC}$ with purified yeast AlaRS and prolyl-tRNA synthetase (ProRS). AlaRS charges both tRNA$^{Ala}$$_{AGC}$ and mutant tRNA$^{Pro}$$_{UGG}$ (G3:U70) with alanine (Figure 3-3a and Figure S 3-1a). Aminoacylation of tRNA$^{Pro}$$_{UGG}$ with alanine by AlaRS was not detected (Figure S 3-1a). Ala-tRNA$^{Ala}$$_{AGC}$ formation proceeds at an initial rate of 144 ±15 pmol/min per pmol of AlaRS as compared to 0.6 ± 0.01 pmol/min for Ala-tRNA$^{Pro}$$_{UGG}$ (G3:U70), an approximate 230-fold decrease in apparent catalytic rate. As expected tRNA$^{Pro}$$_{UGG}$ was aminoacylated with proline using ProRS (Figure 3-3b and Figure S 3-1b), however, we did not detect the formation of Pro-tRNA$^{Pro}$$_{UGG}$ (G3:U70) with ProRS (Fig S 3-1b). ProRS showed overall less activity compared to AlaRS, with tRNA$^{Pro}$$_{UGG}$ being aminoacylated at an initial rate of 12 ± 2 fmol/min per pmol of ProRS. Though it is difficult to compare reactions using the different enzymes, AlaRS produced Ala-tRNA$^{Pro}$$_{UGG}$ (G3:U70) with a greater than 50-fold increase in specific activity compared to cognate Pro-tRNA$^{Pro}$$_{UGG}$ production by ProRS. Together the *in vitro* experiments show that mutant tRNA$^{Pro}$$_{UGG}$ (G3:U70) is aminoacylated with alanine but not proline. This provides a mechanistic basis for CCA mistranslation as Ala and indicates that production of the mis-translating Ala-tRNA$^{Pro}$$_{UGG}$ (G3:U70) species in the cell is at a level such that Ala-tRNA$^{Pro}$ competes with Pro-tRNA$^{Pro}$ for insertion at CCA codons.
Figure 3-3. tRNA aminoacylation assays.

A) Measurements of alanine aminoacylated tRNA^{Ala}_{AGC} and tRNA^{Pro}_{UGG} (G3:U70) by AlaRS at 0, 2, 10, 20, and 40-minute time points. Each measurement represents an average across three technical replicates and the error bars indicate one standard deviation. B) Proline aminoacylation measurements of tRNA^{Pro}_{UGG} at 0, 2, 10, 20, 40, and 80-minute time points. Measurements represent an average across three technical replicates with one standard deviation indicated by the error bar.
To test if suppression affected Tt2$_{L187P}$ expression, we analysed the steady state level of Tt2$_{L187P}$ with or without $SUF9_{G3:U70}$ (Fig S2). Tt2$_{L187P}$ was elevated 1.1-fold in CY7108 ($SUF9_{G3:U70}$) as compared to the CY7020 strain ($SUF9$). Though slight this increase is consistent with an alanine for proline substitution stabilizing the mistranslated protein. To confirm if alanine was incorporated into Tt2$_{L187P}$ in vivo, we purified TAP-tagged Tt2$_{L187P}$ from yeast expressing $SUF9_{G3:U70}$. The purified proteins were digested with trypsin and peptides containing alanine substitutions identified by electrospray mass spectrometry. Tti2 peptides were detected containing proline or alanine at residue 187 (Table 3-2). A proline to alanine substitution was also detected at Pro245 (CCA codon) in Tti2 peptide V242-K252. Interestingly, two alanine substitutions were detected at CCT codons at Pro280 and Pro327 (Table 3-2). For each peptide containing amino acid substitutions the wild type peptide was also detected, indicating ambiguous decoding for each Pro codon.
Table 3-2. Alanine substitutions at Pro codons detected by mass spectrometry.

<table>
<thead>
<tr>
<th>Mutated or wild type (wt) residue</th>
<th>Peptide</th>
<th>Sequence Range</th>
<th>Mis-translated Codon</th>
<th>Score$^\dagger$</th>
<th>Mass (Da)</th>
<th># Spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>P187A*</td>
<td>GVLLAQTLLNHTFM*NETNDSDK</td>
<td>183-203</td>
<td>CCA</td>
<td>35.62</td>
<td>2361.16</td>
<td>2</td>
</tr>
<tr>
<td>L187P</td>
<td>GVLLPQTLNNHTFM*NETNDSDK</td>
<td>183-203</td>
<td>CCA</td>
<td>47.4</td>
<td>2371.18</td>
<td>7</td>
</tr>
<tr>
<td>L187P*</td>
<td>GVLPLQTLNNHTFM*NETNDSDK</td>
<td>183-203</td>
<td>CCA</td>
<td>32.76</td>
<td>2387.17</td>
<td>9</td>
</tr>
<tr>
<td>P245A</td>
<td>VVFATIQSLYK</td>
<td>242-252</td>
<td>CCA</td>
<td>47.48</td>
<td>1267.71</td>
<td>7</td>
</tr>
<tr>
<td>P245 (wt)</td>
<td>VVFATIQSLYK</td>
<td>242-252</td>
<td>CCA</td>
<td>48.06</td>
<td>1293.73</td>
<td>315</td>
</tr>
<tr>
<td>P280A*</td>
<td>FM*SEILQNIIA_R</td>
<td>269-281</td>
<td>CCT</td>
<td>25.14</td>
<td>1562.84</td>
<td>4</td>
</tr>
<tr>
<td>P280 (wt)*</td>
<td>FMSEILQNIIIP</td>
<td>269-281</td>
<td>CCT</td>
<td>57.35</td>
<td>1572.87</td>
<td>15</td>
</tr>
<tr>
<td>P327A</td>
<td>NAFYTTFPK</td>
<td>326-334</td>
<td>CCT</td>
<td>35.16</td>
<td>1087.53</td>
<td>7</td>
</tr>
<tr>
<td>P327 (wt)</td>
<td>NPFTYTFPK</td>
<td>326-334</td>
<td>CCT</td>
<td>46.72</td>
<td>1113.54</td>
<td>45</td>
</tr>
</tbody>
</table>

* peptides with oxidized methionine resulting in an added mass of 15.99 Daltons.

$^\dagger$ score value was calculated as the negative log (10) of the p-value of the matched peptide. Peptides were considered significant if the false discovery rate of the peptide-spectrum match was less than 1% and the peptide score value was >15.5.

__underlined amino acids represent the position mistranslated or mutated.__

To estimate the frequency of alanine incorporation at Pro codons by tRNA$_{Pro}^{UGG}$ (G3:U70), we engineered an eGFP reporter protein where mistranslation could be visualized. eGFP was constructed with an Asp to Pro mutation at residue 129 (eGFP$_{D129P}$), a position where proline, but not alanine, disrupts the beta-barrel structure of GFP and eliminates fluorescence. Comparisons were made to a functional eGFP$_{D129A}$ molecule. To localize the GFP signal intensity to the nucleus we fused each GFP molecule to Spt7 (45). The nucleus was identified with RFP-tagged Sik1 (33). eGFP$_{D129P}$-Spt7 or eGFP$_{D129A}$-Spt7 were expressed in strains containing either $SUF9$ or $SUF9_{G3:U70}$. Fluorescence of eGFP$_{D129P}$ is significantly enhanced in the presence of $SUF9_{G3:U70}$ but not wild-type $SUF9$ (Figure 3-4, Figure S 3-3). Calculating the signal across 150 cells from each strain, we estimate a ~ four-fold increase (p= 0.008) in the average eGFP$_{D129P}$
A yeast strain containing Sik1-RFP strain was transformed with either YCplac111-SUF9 (SUF9) or YCplac111-SUF9G3:U70 (SUF9G3:U70), and either YCplac33-eGFP_D129P-SPT7 (eGFP_D129p) or YCplac33-eGFP_D129A-SPT7 (eGFP_D129A). Transformants were grown to stationary phase in medium lacking uracil and leucine, diluted 1:20 and grown for 5 hours before harvesting cells for imaging. Exposure time for GFP and RFP images was 1 second and the gain was kept constant. Contrast was adjusted uniformly across all GFP images shown.
fluorescence in the \textit{SUF9}_{G3:U70} strain compared to the \textit{SUF9} strain (Figure S 3-3). To approximate the frequency of alanine incorporation, we compared the signals of \textit{eGFP}_{D129P} with \textit{eGFP}_{D129A}. In the \textit{SUF9}_{G3:U70} strain, \textit{eGFP}_{D129P} signal intensity averaged 8.9\% of the \textit{eGFP}_{D129A} signal, whereas it was 2.8\% in the wild-type \textit{SUF9} strain. The difference of these percentages suggests that mistranslation of proline CCA codons by tRNA^{Pro}_{UGG} (G3:U70) occurs at a frequency of approximately 6\%. Expression of intact \textit{eGFP}_{D129P}-\text{Spt7} was confirmed by Western blot (Figure S4), and consistent to what was observed with Tti2_{L187P}, \textit{SUF9}_{G3:U70} slightly increased the level of \textit{eGFP}_{D129P}-\text{Spt7} (1.4-fold).

### 3.3.3 Suppression of \textit{tti2}_{L50P} by \textit{SUF2}_{G3:U70}

In our screen for defective alleles of \textit{TTI2} we also identified \textit{tti2}_{L50P}, which contains a non-synonymous mutation that alters a CUC to a CCC Pro codon and results in slow growth under stress conditions. \textit{SUF2} encodes tRNA^{Pro}_{AGG}, which decodes the CCC codon (46). To examine if the mutant G3:U70 base pair would alter the specificity of a second tRNA, we incorporated the appropriate cytosine to thymine point mutation (Figure 3-5a) into a plasmid copy of \textit{SUF2} and assessed whether it could suppress \textit{tti2}_{L50P}. \textit{SUF2}_{G3:U70} suppresses the stress sensitivity caused by \textit{tti2}_{L50P} in media containing 6\% ethanol (Figure 3-5b). This result suggests that tRNA^{Pro}_{AGG} molecules containing the G3:U70 pair can also be mischarged, likely with alanine, at an efficiency sufficient for suppression of \textit{tti2}_{L50P}. 
Figure 3-5. Suppression of tti2<sub>L50P</sub> by SUF2<sub>G3:U70</sub>.

A) Secondary structure of tRNA<sub>Pro<sup>AGG</sup></sub> showing the suppressor tRNA base pair change (G3:U70) and anticodon in bold font and the discriminator base in orange. B) Yeast strains CY6963 (TTI2), and CY6944 (tti2<sub>L50P</sub>) containing either YCplac33 (-), YCplac33-SUF2 (SUF2), or YCplac33-SUF2<sub>G3:U70</sub> (SUF2<sub>G3:U70</sub>) were grown to stationary phase in medium lacking uracil. Cell densities were normalized and cells spotted in 10-fold serial dilutions onto a YPD plate or on a YPD plate containing 6% ethanol and grown at 30°C.
3.3.4 Impact of introns in tRNA\textsuperscript{Pro} (G3:U70) genes on suppression of \textit{tti2} alleles

Many of the yeast tRNAs including all of the nuclear encoded \textit{tP(UGG)} alleles contain introns. Having a screen in which plasmid encoded \textit{tP(UGG)}\textsubscript{G3:U70} is required for growth provided a mechanism to evaluate the role of the intron in \textit{SUF9}. We engineered a mischarging \textit{SUF9} allele lacking the intron (Figure S 3-5). The intronless G3:U70 allele, an intron containing G3:U70 allele, and the wild-type \textit{SUF9} allele encoded on centromeric plasmids were transformed into CY7020 (\textit{tti2\textsubscript{L187P}}). The transformed strains were then analyzed for growth on plates containing ethanol. The intronless and intron containing G3:U70 alleles similarly suppressed \textit{tti2\textsubscript{L187P}} (Figure 3-6a).

We also performed the inverse experiment, inserting an intron into the otherwise intronless \textit{SUF2\textsubscript{G3:U70}} allele (Figure S 3-5). The intron-containing \textit{SUF2\textsubscript{G3:U70}} had a slightly reduced ability to suppress slow growth caused by \textit{tti2\textsubscript{L50P}} (Figure 3-6b). This result suggests that the intron in \textit{tP(UGG)} genes partially reduces tRNA maturation or activity.
Figure 3-6. Suppression of tti2 alleles by intron-containing (+) and intronless (-) tP(UGG) and tP(AGG) alleles.

A) CY7020 (tti2L187P) containing either YCplac33-SUF9 (G3:C70), YCplac33-SUF9G3:U70 (G3:U70), or YCplac33-SUF9G3:U70 (G3:U70) lacking (-) the intron sequence (Δ37-67), were grown to stationary phase in medium lacking uracil, cell densities were normalized and spotted in ten-fold serial dilutions onto a YPD plate and a YPD plate containing 6% ethanol. B) Yeast strain CY6944 (tti2L50P) containing either YCplac33-SUF2 (G3:C70), YCplac33-SUF2G3:U70 (G3:U70), or YCplac33-SUF2G3:U70 with the intron (G3:U70 +) was grown and plated as described above onto a YPD plate and a YPD plate containing 6% ethanol.
3.3.5 Cellular response to mistranslation

There are 10 copies of tRNA\textsuperscript{Pro\_UGG} annotated in S288c derivatives of \textit{S. cerevisiae} (47). As tRNA\textsuperscript{Pro\_UGG} (G3:U70) results in the mis-incorporation of alanine for proline, we examined the effect of increasing the copy number of this tRNA on cell growth. Strains were constructed that contained \textit{SUF9\textsubscript{G3\_U70}} (CY7288), \textit{tP(UGG)N1\textsubscript{G3\_U70}} (CY7355), or both \textit{tP(UGG)N1\textsubscript{G3\_U70}} and \textit{SUF9\textsubscript{G3\_U70}} (CY7286) in a background containing wild-type \textit{TTI2}. A single copy of \textit{tP(UGG)\textsubscript{G3\_U70}}, either \textit{tP(UGG)N1\textsubscript{G3\_U70}} or \textit{SUF9\textsubscript{G3\_U70}}, results in minimal change in growth at 30°C in rich medium or in medium containing 6% ethanol. A reduction in growth was observed for cells grown in 6 µM tunicamycin or under heat stress at 37°C (Figure 3-7). The presence of two copies of the mis-translating tRNA (\textit{tP(UGG)N1\textsubscript{G3\_U70}} and \textit{SUF9\textsubscript{G3\_U70}}) slightly reduced growth at 30°C, and exacerbated the growth defect in the stress conditions (Figure 3-7). In liquid culture, the doubling time during exponential growth increased relative to the wild-type strain by 6 ± 2 % and 15 ± 3 % in single copy (\textit{SUF9\textsubscript{G3\_U70}}) and double copy (\textit{tP(UGG)N1\textsubscript{G3\_U70} SUF9\textsubscript{G3\_U70}}) strains, respectively (growth curves shown in Figure S 3-6). These relatively minor effects on cell growth suggest that the cells have compensation mechanisms to cope with some level of mis-incorporation of alanine at Pro codons (48,49).
Figure 3-7. Growth of suppressor tRNA strains under conditions of stress. CY7287 (SUF9 \(tP(UGG)N1\)), CY7288 (SUF9\(_{G3:U70}\) \(tP(UGG)N1\)), CY7355 (SUF9 \(tP(UGG)N1\) \(G3:U70\)), and CY7286 (SUF9\(_{G3:U70}\) \(tP(UGG)N1\)\(_{G3:U70}\)) were grown to stationary phase in YPD medium. After normalizing for cell densities cells were spotted in 10-fold serial dilutions onto YPD plates and grown at either 30°C or 37°C, and onto YPD plates containing either 6% ethanol or 6 µM tunicamycin and grown at 30°C.
We further analyzed the effect of mistranslation on the cellular response to protein stress. Normally cells compensate for mistranslation through induction of the heat shock response (13), therefore, we compared the fluorescent signal intensity resulting from expression of eGFP fused to a heat shock element in strains containing SUF9 or SUF9_{G3:U70}. A wild type strain grown at 30°C then shifted to 42°C for one hour shows a 5.8-fold increase in eGFP signal intensity after heat shock (Figure 3-8a). When comparing strains containing SUF9 or SUF9_{G3:U70} grown at 30°C, we detected a 3.4-fold increase in eGFP signal intensity caused by SUF9_{G3:U70} (Figure 3-8a). This result suggests that cells compensate for low levels of mistranslation by partially inducing the heat shock response.

We investigated synthetic interactions between SUF9_{G3:U70} and deletions of genes involved in autophagy (ATG8), the unfolded protein response (IRE1), or proteasome regulation (RPN4). SUF9_{G3:U70} was expressed from a URA3 centromeric plasmid that was transformed into each deletion strain (atg8Δ0, ire1Δ0, rpn4Δ0) and the wild-type yeast strain BY4741. Transformants were spotted on agar plates lacking uracil (Figure 3-8b). Expression of SUF9_{G3:U70} in the atg8Δ0 and ire1Δ0 modestly reduced growth, at a level similar to the wild-type strain. Synthetic slow growth was observed upon expressing the mis-translating tRNA in the rpn4Δ0 strain. This observation suggests a role for the proteasome in managing cellular stress caused by mistranslation.

We next tested if mistranslation caused by tRNA_{Pro}^{UGG} (G3:U70) might induce protein quality control pathways that could alleviate or exacerbate the growth defects associated with a disease-causing allele. The human huntingtin gene contains extensive CAG repeats encoding a toxic polyglutamine tract protein that causes an autosomal-dominant neurodegenerative disorder, known as Huntington’s disease (reviewed in (50)). We analyzed the consequence of SUF9_{G3:U70} expression in yeast cells containing human huntingtin exon 1 with a 103 residue polyQ tract (htt103Q). The htt103Q exon was expressed from a galactose inducible promoter on a centromeric plasmid. Wild-type SUF9 or SUF9_{G3:U70} was expressed from a centromeric plasmid in these strains. Induction of htt103Q in galactose-containing medium reduced cell growth (Figure S 3-7). Expression of SUF9_{G3:U70} did not change the toxicity resulting from expression of
*htt103Q*. The tRNA-induced mistranslation was unable to rescue the growth defect, but did not further inhibit growth. The data suggest that mistranslation caused by \textit{SUF9}_{G3:U70} is tolerated in strains containing toxic protein aggregates.
Figure 3-8. Effects of mistranslation on the heat shock response and proteostasis.

A) Yeast strain BY4742 containing HSE-eGFP and SUF9 or HSE-eGFP and SUF9G3::U70 were grown to stationary phase in medium lacking leucine and uracil, diluted 1:100 and grown for six hours at 30°C. Cell densities were normalized and fluorescence was measured at an emission wavelength of 528nm. BY4742 containing HSE-eGFP and SUF9 was heat shocked for one hour at 42°C, cell densities were normalized to the cell densities measured from 30°C growth, and fluorescence was measured at an emission wavelength of 528nm. A BY4742 strain lacking HSE-eGFP, and containing either SUF9 or SUF9G3::U70, was used to subtract background fluorescence from each respective experimental strain. All calculations were done using three biological replicates and three technical replicates for each strain. Error bars indicate one standard deviation. B) Yeast strains BY4741 (-), CY1217 (ire1), CY2423 (rpn4), and CY7450 (atg8) expressing either YCplac33-SUF9 (SUF9) or YCplac33-SUF9G3::U70 (SUF9G3::U70) were grown to stationary phase in medium lacking uracil. Cell densities were normalized and cells spotted in 10-fold serial dilutions onto a minimal medium plate lacking uracil and grown at 30°C.
3.4 Discussion

The \textit{tti2}_{L187P} allele provided an efficient system to select for mistranslation and to identify suppressor tRNA mutations. The L187P mutation has a profound effect on cell growth in stress conditions (31). The structure of Tti2 is unknown, but L187 is situated in a predicted alpha helix, which is likely disrupted by the Pro mutation. In addition, a low level of Tti2 is sufficient to support viability (31). We surmise that the low level of Tti2 required to support normal growth is related to the ability of the L187P to select for mistranslation in yeast. The amount of mistranslation required to restore Tti2 function can thus be at a level that is low enough to not compromise viability yet high enough to provide a sufficient amount of Tti2 for normal growth under stress conditions.

3.4.1 A single nucleotide mutation disrupts the genetic code

The anticipated high fidelity of protein synthesis and the interpretation of the genetic code itself can be disrupted by a single nucleotide mutation. Our genetic screen for suppressors of \textit{tti2}_{L187P} identified four alleles of tP(UGG) with a mutation converting C70U that restored growth in stress conditions. This point mutation changes a G3:C70 base pair in the acceptor stem of tRNA\textsubscript{Pro}_{UGG} to a G3:U70 wobble base pair. We hypothesized that tRNA\textsubscript{Pro}_{UGG} (G3:U70) is charged with alanine based on several previous findings. Introducing a single G:U pair in the acceptor stem can be the sole determinant for charging a tRNA molecule with alanine in \textit{Escherichia coli} (44,51).

Missense suppressors containing C70U in tRNA\textsubscript{Lys} have been identified in \textit{E. coli} (24), and are likely charged with either glycine or alanine (52). Finally, the introduction of G3:U70 into a mini RNA helix derived from tRNA\textsubscript{Tyr} permits charging with alanine (53).

Crystal structures (54) revealed that AlaRS interacts with the minor and major grooves of the acceptor stem of tRNA\textsubscript{Ala} such that the G3:U70 identity element is required for orientation of the 3'-CCA into the active site of AlaRS. Similarity in the acceptor stems of tRNA\textsubscript{Ala} and tRNA\textsubscript{Pro} (Fig 2A), and the extensive interactions between acceptor stem nucleotides and residues of the AlaRS tRNA recognition domain, are plausible reasons for why tRNA\textsubscript{Pro} containing the G3:U70 pair acts as an efficient substrate for aminoacylation by AlaRS (54).
We have shown that single spontaneous mutations can give rise to tRNA molecules with switched identity, mischarging alanine for proline. Similar results in E. coli (44) have led to the idea that tRNA identity elements are part of a ‘second genetic code’ that is non-degenerate and more deterministic than the classic view of the code based on accurate pairing of codon and anti-codon (55). Moreover, genetic diversity found in tRNA genes and the discovery of codon reassignments that can be of selective advantage is consistent with an evolving genetic code, contrary to Crick's “frozen accident” hypothesis (56-60). In Candida albicans, serine is incorporated into 97% of Leu CUG codons, suggesting a naturally evolved CUG codon reassignment from Leu to Ser (61). Alterations to the standard genetic code are also found in yeast mitochondrial organelles, bacteria, archaea, and viruses (62,63). These findings, along with the identification of pyrrolysine and selenocysteine as natural expansions of the genetic code, suggest that the genetic code continues to evolve (64-66).

### 3.4.2 Life with mistranslation

A priori, ambiguous decoding or mistranslation should be harmful to the cell due to effects on proteome stability. We examined cells expressing suppressor tRNA\textsuperscript{Pro\_UGG} (G3:U70) to determine how mistranslation of Pro codons with alanine affects growth and response to protein stress. We found only mild reductions in the growth rates of yeast expressing one or two copies of the mistranslating tRNA\textsuperscript{Pro\_UGG} (G3:U70) alleles, and a partial induction of the heat shock response caused by SUF9\textsubscript{G3:U70} expression. These results suggest that yeast tolerate a significant amount of proline to alanine mis-incorporations. This is likely because cells have several ways to reduce the toxicity of mis-folded proteins, including the ubiquitin-proteasome system, autophagy, induction of the heat shock response and upregulation of molecular chaperones, and organization of aggregates into inclusion bodies (67,68). The synthetic slow growth upon expressing SUF9\textsubscript{G3:U70} in a strain deleted for the proteasome regulator Rpn4 suggests that the ubiquitin-proteasome system is compensating for increased mistranslation arising from Ala-tRNA\textsuperscript{Pro\_UGG} (G3:U70). The lack of a genetic interaction between SUF9\textsubscript{G3:U70} and ATG8 may indicate that low levels of Pro mistranslation does not cause larger toxic aggregates that require autophagosomes for removal.
Yeast cells overburdened by tRNA$^{\text{Ser}_{\text{CAG}}}$ mistranslation can adapt when grown for over 250 generations (69). Adaptation is mediated through large genome rearrangements that cause accelerated protein synthesis and protein degradation, and increase glucose uptake to meet energy demands (69). As a consequence of such adaptations, the extent of toxic protein aggregates within the cells is reduced. Additional studies will be required to address whether yeast compensate and adapt to Pro mistranslation by tRNA$^{\text{Pro}_{\text{UGG}}}$ (G3:U70) over multiple generations.

It is difficult to estimate the extent to which Ala-tRNA$^{\text{Pro}_{\text{UGG}}}$ (G3:U70) is incorporated into proteins, and it is likely that the rate of mis-incorporation may differ at different Pro codons or based on mRNA sequence context. As there are 10 tP(UGG) genes in the genome, the mutant tRNA may represent roughly 10% of the tRNA$^{\text{Pro}_{\text{UGG}}}$ pool. The relative amounts of charging of the mutant tRNA with alanine as compared to the wild-type with proline is more difficult to estimate because the reactions involve two different aaRSs. Measurements of initial rates actually suggest that tRNA$^{\text{Pro}_{\text{UGG}}}$ (G3:U70) is charged more efficiently with alanine than wild-type tRNA$^{\text{Pro}_{\text{UGG}}}$ with proline. In vivo comparisons are further complicated by variations in the amino acid pools and stabilities of the products, though the expression of AlaRS and ProRS is similar (70) and editing or deacylation of mis-charged Ala-tRNA$^{\text{Pro}}$ is negligible in S. cerevisiae (62). Evaluation of the concentration of the final protein products (e.g., Tti2L187A) in cells is also complicated by the potential instability of the Tti2L187P protein. Considering this, we estimated the frequency of Ala-tRNA$^{\text{Pro}_{\text{UGG}}}$ incorporation into proteins using a GFP reporter assay whereby signal intensity of a eGFP$^_{\text{D129P}}$ mutant is restored by mistranslation of the CCA Pro codon with alanine. We calculated an approximate 6% increase in eGFP$^_{\text{D129P}}$ signal intensity as a result of SUF9$_{G3:U70}$ expression. This rate of mistranslation agrees with our estimate that ~10% of the tRNA$^{\text{Pro}_{\text{UGG}}}$ pool is mischarged with alanine. The ratio of Tti2 peptides containing proline to alanine substitutions from our mass spectrometry data is also consistent with a rate of mistranslation between 5-10%.

Johansson et al. (49) found that a strain deleted for all of the tRNA$^{\text{Pro}_{\text{AGG}}}$ genes is viable and does not show slow growth. This led them to hypothesize that tRNA$^{\text{Pro}_{\text{UGG}}}$ molecules can decode all Pro codons. Our finding that SUF9$_{G3:U70}$ (tP(UGG)) does not
suppress the L50P mutation, a CCC codon, which is suppressed by $SUF2_{G3:U70}$ (tP(AGG)), suggests that Ala-tRNA$_{Pro}^{UGG}$ molecules do not efficiently decode CCC. Consistent with this, in a strain expressing $SUF9_{G3:U70}$ we detected only proline residues at both CCC codons in Tti2 by mass spectrometry, while alanine incorporations at two of the three CCT codons were identified (Table 2). The tRNA$_{Pro}^{UGG}$ species in *S. cerevisiae* contains 5-carbamoylmethyluridine (ncm$^5$U) as the only modification at U34 (71).

Uridine modifications do not have bond angles required for pairing with C in the ribosome A site (72), however, it is predicted that unmodified uridine can base pair with any nucleoside at the wobble position (72). Therefore, it is possible that uridine in the tRNA$_{Pro}^{UGG}$ anticodon undergoes frequent modification and is unable to decode CCC at the level of efficiency required for suppression of $tti2_{L50P}$.

### 3.4.3 Intron splicing in a mistranslating tRNA

Introns are found in the tRNAs of organisms across all kingdoms of life where they play a role in transcriptional regulation, tRNA modification, and prevention of viral genome integration (reviewed in (73)). In *S. cerevisiae*, 61 of the 295 tRNA genes contain introns. Removing the intron from $SUF9_{G3:U70}$ had no effect on suppression of $tti2_{L187P}$, suggesting that the intron does not play an essential role in tP(UGG) function. Interestingly, introducing an intron into the tP(AGG) allele $SUF2_{G3:U70}$ partially reduced its ability to suppress. These results are consistent with those of Winey *et al.* (74) who found that removing the intron from a frameshift suppressor allele of $SUF8$ improved its translation efficiency. In the case of both the intronless $SUF2$ and $SUF8$, the increased activity may be due to increased levels of the tRNA by removing the need of a potentially inefficient splicing step (74). In this regard, the intron may play a role in regulating the abundance of mature tRNA.

### 3.5 Conclusion

Our study prompts further evaluation of the idea that a ‘frozen’ genetic code is a prerequisite for life. Rather we demonstrate that mistranslation can arise via a single nucleotide mutation as a route to circumvent lethal mutations in the genome. The tRNAs we have identified are relatively efficient at missense suppression and these tRNAs will
continue to be useful tools for genetic analyses and synthetic biology applications (e.g. (75)). The spontaneous nature of the selection process and the limited consequences of the genetic code change on growth suggest that tRNA alleles resulting in mis-aminoacylation may become fixed in a population where they confer advantage and may be more common than previously assumed. Otherwise deleterious mutations in coding regions may become phenotypically neutral due to the cell’s ability to reinterpret the meaning of codons leading to ambiguous decoding and mistranslation.

3.6 Bibliography


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3.7 Supporting Information

3.7.1 Tables

**Table S 3-1. Yeast strains used in this study.**

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Table S 3.2. Oligonucleotides used in this study.
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<td>CATGGATCGCAAGCGCTTCCCTCCTCAAT</td>
<td>SUF2</td>
</tr>
<tr>
<td>UB0181</td>
<td>GGGAGGTCCCGGTTGAGTCCGGCTGTCCCACATTGTTT</td>
<td>SUF2G3:U70</td>
</tr>
<tr>
<td>TI5697</td>
<td>CACCACTGGGCATCGGTGATAAGCAAAAAATG</td>
<td>ALA1</td>
</tr>
<tr>
<td>TI5698</td>
<td>CACCACTGGGCATTTAAATGGAAGCGCTTCTCCTTG</td>
<td>ALA1</td>
</tr>
<tr>
<td>SUF9-1</td>
<td>CTAAGAAATACGACACTCACTATAGGGGCCTGTTG GTCTAGTGATATGGTTCTCGCTTGGTGTCGAAG GAGGCCCTGGGTCCAATTCAGCTGCCACCACCATGCATG</td>
<td>SUF9</td>
</tr>
<tr>
<td>SUF9-2</td>
<td>GATCCATGCATGGGGGGGGCCAGCTGGAATGGG AACCAGGGGCTCTCCGACCCAAAGCGAGAAATGCA CATACCACCTAGACCCACACGCCTCATATGAGTCTCGATTAT</td>
<td>SUF9</td>
</tr>
<tr>
<td>tA(AGC)D-1</td>
<td>CTAGAATAATACGACTCACTATAGGGGCCTGTTG GCGTATGGTGAGCGGCTCTCCTTGAGCATGGG GAGAGGGTCTCGGCTTCCAGATCGGACTGTCGCCACCATGCATG</td>
<td>tA(AGC)D</td>
</tr>
<tr>
<td>tA(AGC)D-2</td>
<td>GATCCATGCATGGGGGGGGCCAGCTGGAATGGG AACCAGGGGCTCTCCGACCCAAAGCGAGAAATGCA CATACCACCTAGACCCACACGCCTCATATGAGTCTCGATTAT</td>
<td>tA(AGC)D</td>
</tr>
<tr>
<td>SUF9G3:U70-1</td>
<td>CTAGAATAATACGACTCACTATAGGGGCCTGTTG GTCAGTGATATGGTTCTCGCTTGGTGTCGAAG GAGGCCCTGGGTCCAATTCAGCTGCCACCACCATGCATG</td>
<td>SUF9G3:U70</td>
</tr>
<tr>
<td>SUF9G3:U70-2</td>
<td>GATCCATGCATGGGGGGGGCCAGCTGGAATGGG AACCAGGGGCTCTCCGACCCAAAGCGAGAAATGCA CATACCACCTAGACCCACACGCCTCATATGAGTCTCGATTAT</td>
<td>SUF9G3:U70</td>
</tr>
<tr>
<td>UA9593</td>
<td>CTAGAATAATACGACTCACTATAAAA</td>
<td>SUF9</td>
</tr>
<tr>
<td>UA9594</td>
<td>TGGGGGGGCGAGCTGGGGAA</td>
<td>SUF9</td>
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<tr>
<td>UA9595</td>
<td>AGATAATACGACTCACTATAAAA</td>
<td>tA(AGC)D</td>
</tr>
<tr>
<td>UA9596</td>
<td>TGGTGGAGCGAGCTCGGAAT</td>
<td>tA(AGC)D</td>
</tr>
<tr>
<td>ID</td>
<td>Sequence</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>UA9597</td>
<td>TAGATAATACGACTCACTATAG</td>
<td>SUF9&lt;sub&gt;G3-U70&lt;/sub&gt; primer</td>
</tr>
<tr>
<td>UA9598</td>
<td>TGGGGGACGAGCTGGA</td>
<td>SUF9&lt;sub&gt;G3-U70&lt;/sub&gt; primer</td>
</tr>
<tr>
<td>UD2159</td>
<td>CGCATCGAGCTGAAGGGGATCCCATTCAAGGGAGACGG</td>
<td>eGFP-Asp129Pro</td>
</tr>
<tr>
<td>UD2160</td>
<td>CGTGCTCTTTGAATGGGATCCCCCTTCAGCTGATGCG</td>
<td>eGFP-Asp129Pro</td>
</tr>
<tr>
<td>UF4196</td>
<td>CTCTCGCAACCAAGCGAGAATCATACCACTAG</td>
<td>SUF9 intron removal</td>
</tr>
<tr>
<td>UF4197</td>
<td>CTCGCTTTGGGTGCGAGAGGCCCTGGGG</td>
<td>SUF9 intron removal</td>
</tr>
<tr>
<td>UF4198</td>
<td>TTCCTGAATAAACAGGAAGACAAGAGCATGCGGGAGGTCCCGGGGTTC</td>
<td>SUF2 intron removal</td>
</tr>
<tr>
<td>UF4199</td>
<td>TTGTCTTCTGTGTATATTCAAGGAAGTCGCCCTAAGCGAGAATCATACC</td>
<td>SUF2 intron</td>
</tr>
<tr>
<td>UF4570</td>
<td>CGCATCGAGCTGAAGGGCATCGCTTTCAAGGAGGACGG</td>
<td>eGFP-Pro129Ala</td>
</tr>
<tr>
<td>UF4571</td>
<td>CGTGCTCTTTGAAGCGATGCGCCCTTCAGCTGATGCG</td>
<td>eGFP-Pro129Ala</td>
</tr>
</tbody>
</table>
3.7.2 Figures

Figure S 3-1. tRNA aminoacylation assays.
A) tRNA$^{Ala}_{AGC}$, tRNA$^{Pro}_{UGG}$ (G3:U70), and tRNA$^{Pro}_{UGG}$ were incubated with 500 pM, 100 nM, and 1 µM AlaRS, respectively, and 10 mM alanine. Two microliters of each reaction was stopped in ice-cold sodium citrate containing nuclease P1 and incubated for one hour at room temperature. Reaction mixtures were spotted on glass TLC plates, chromatographed in 0.1 M ammonium acetate and 5% acetic acid, then stopped prior to buffer reaching the top of each plate. TLC plates were dried, exposed onto phosphor screens, and visualized using a Phosphorimager to detect free $^{32}$P-labelled AMP and $^{32}$P-labelled AMP charged with proline. B) Wild type tRNA$^{Pro}_{UGG}$ and mutant tRNA$^{Pro}_{UGG}$ (G3:U70) were incubated with 1 µM ProRS and 10 mM proline. Reactions were stopped at the indicated time points, separated and imaged using glass TLC plates as described in (A).
Figure S 3-2. Increase in Tti\textsubscript{L187P} levels by SUF9\textsubscript{G3:U70}.

Yeast strains CY6965 (TTI2 SUF9), CY7020 containing YCplac33-SUF9 (tti2\textsubscript{L187P} SUF9), and CY7020 containing YCplac33-SUF9\textsubscript{G3:U70} (tti2\textsubscript{L187P} SUF9\textsubscript{G3:U70}) were grown to stationary phase in YPD medium, diluted 1:20 and grown for 8 hours prior to protein extraction by bead lysis. The amount of protein extract loaded is indicated in each lane and was Western blotted using anti-Myc antibodies. The bottom of the gel was stained with Coomassie Brilliant Blue and shown as a loading control.
Figure S 3-3. tRNA\textsuperscript{Pro\textsubscript{UGG} (G3:U70) results in alanine incorporation into eGFP\textsubscript{D129P}.}

A yeast strain containing Sik1-RFP strain was transformed with either YCplac111-\textit{SUF9} or YCplac111-\textit{SUF9\textsubscript{G3:U70}}, and either YCplac33-\textit{eGFP\textsubscript{D129P}-SPT7} or YCplac33-\textit{eGFP\textsubscript{D129A}-SPT7}. Transformants were grown to stationary phase in medium lacking uracil and leucine, diluted 1:20 and grown for 5 hours before harvesting cells for imaging. Signal intensities were calculated from 150 cells per strain using integrated density units, background noise levels were calculated and subtracted using a Sik1-RFP strain with no eGFP. Integrated density values were divided by the nuclear area measured for each cell and plotted along the y-axis (IntDen/Area) using \textit{ggplot2} (41) on R version 3.2.0. The line within the box represents the median, the second and third quartiles are outlined by the box, and whiskers indicate 1.5 times the quartile range of the box. To better visualize the difference between samples, an outlying value in the \textit{SUF9\textsubscript{G3:U70}} strain is not shown, but was included in all calculations.
Figure S 3-4. eGFP<sub>D129P</sub>-Spt7 levels in strains containing SUF9 or SUF9<sub>G3;U70</sub>.
The Sik1-RFP yeast strain was transformed with either YCplac111-SUF9 and YCplac33-
<sup>eGFP</sup><sub>D129P</sub>-<sub>SPT7</sub> or YCplac111-SUF9<sub>G3;U70</sub> and YCplac33-<sup>eGFP</sup><sub>D129P</sub>-<sub>SPT7</sub>, grown to stationary phase in medium lacking leucine and uracil, diluted 1:20 and grown for 8
hours. Protein was extracted by bead lysis, and the indicated amounts were Western
blotted using a PVDF membrane and anti-eGFP antibodies (Sigma). The bottom of the
gel was stained with Coomassie Brilliant Blue for a loading control.
SUF9<sub>G3:U70</sub> with intron:
5’GGGCGTGTGGTCTAGTGGTATGATTCTCGCTTTGGA<sub>G</sub>c<sub>g</sub>cattctgaataaaacaggaagacaa
agcaTGCGAGAGGCGCTGGGTTCAATTCCCAGCTCG<sub>T</sub>CCC 3’

SUF9<sub>G3:U70</sub> no intron:
5’GGGCGTGTGGTCTAGTGGTATGATTCTCGCTTTGGGTGCGAGAGGCCCTGGGTTCAA TTCCCAGCTCG<sub>T</sub>CCC 3’

SUF<sub>2G3:U70</sub> with intron:
5’GGGCGTGTGGTCTAGAGGTATGATTCTCGCTTTAGG<sub>G</sub>c<sub>g</sub>cattctgaataaaacaggaagacaa
agcaTGCGGGAGGTCCCGGGTTCGAGTCCCGGCTCG<sub>C</sub>CCC 3’

SUF<sub>2G3:U70</sub> no intron:
5’GGGCGTGTGGTCTAGAGGTATGATTCTCGCTTTAGG<sub>G</sub>c<sub>g</sub>cattctgaataaaacaggaagacaa
agcaTGCGGGAGGTCCCGGGTTCGAGTCCCGGCTCG<sub>C</sub>CCC -3’

Intron sequence:
cgactttcctgaataaaacaggaagacaaagca

**Figure S 3-5. SUF9 and SUF2 intron and intron-less sequences.**
The anticodon in each tRNA is represented in bold font, underlined nucleotides indicate the C70T suppressor mutation, and the intron sequence is in lower text.
Figure S 3-6. Growth curves comparing wild type to strains containing one or two copies of tP(UGG)G3:U70.

A) Yeast strains CY7287 (SUF9 tP(UGG)N1), CY7288 (SUF9G3:U70 tP(UGG)N1) were grown to stationary phase in YPD medium, diluted 1:100 and grown for 10 hours. Optical density measurements were taken every hour across three biological replicates per strain, and the average densities were plotted. Error bars for each data point represent one standard deviation. B) Yeast strains CY7287 (SUF9 tP(UGG)N1) and CY7286 (SUF9G3:U70 tP(UGG)N1G3:U70) were grown to stationary phase in YPD medium, diluted 1:100 and grown for 13 hours. Measurements were taken and plotted as described in (A).
Figure S 3-7. Growth of cells expressing $SUF9_{G3;U70}$ and $htt103Q$ alleles.
Yeast strain BY4741 containing YCplac111-$SUF9$ or YCplac111-$SUF9_{G3;U70}$, and either YCplac33 or $CEN-GAL1-htt103Q$ were grown to stationary phase in medium lacking leucine and uracil. Cell densities were normalized and cells spotted in 10-fold serial dilutions onto minimal media plates depleted of leucine and uracil and containing either glucose or galactose.
Chapter 4

4 Discussion

Protein biosynthesis involves the translation of genetic information to a peptide chain, and the folding of peptides by chaperones into functional protein molecules (Figure 4-1 depicts the processes and components of protein biosynthesis and proteostasis pathways). Molecular chaperone systems and proteostasis pathways function in maintaining properly folded proteins, removal of toxic aggregates, resistance to stress, adapting to diverse environments, and tolerating mistranslated proteins. The PIKK family relies on Hsp90, the TTT, and R2TP complexes for expression and function in nutrient and growth signaling, transcription, and DNA damage response and repair. The PIKKs make up the only known client proteins of the TTT complex. How the TTT complex interacts with Hsp90 and whether the interaction regulates folding activity will be important for characterizing the TTT complex as an Hsp90 co-chaperone.

Protein quality control pathways involving chaperones allow cells to tolerate some errors during protein synthesis. The cellular capacity to manage proteome perturbations has enabled mistranslation as an adaptive response (1-4). The upregulation of chaperones and protein synthesis pathways occur during elevated levels of mistranslation (5), a cellular response that likely played a role in the codon reassignment of Leu to Ser in Candida albicans (6). Therefore, not only are chaperones required for the integrity of the proteome, they are involved in the evolution of the genetic code.

Defining the level of mistranslation tolerated by cells and the mechanisms that can be engineered to exploit codon ambiguity may allow for the development of novel gene therapy approaches. Moreover, manipulating ambiguously decoding tRNAs, along with their cognate synthetases, will continue to be valuable for synthetic biology applications and protein engineering.
Figure 4.1. Flow chart of protein biosynthesis and proteostasis pathways.

Genetic information stored in DNA is transcribed to RNA by RNA polymerase II using nucleoside triphosphates (NTPs). RNA is used as template by the ribosome to specify the recruitment of aminoacyl-tRNA, which are used as substrates for peptide synthesis. A tRNA is charged with a specific amino acid by its cognate aminoacyl-tRNA synthetase to produce an aminoacyl-tRNA (boxed in). Next, peptides undergo folding by chaperones and co-chaperones and a functional protein is formed. Protein misfolding or unfolding can be triggered by mutations, stress conditions, or mistranslation. Oligomerization and intermolecular interaction of misfolded proteins can form aggregates. In some cases, unfolded or misfolded proteins are re-folded back to their native state by chaperones. Irreversible misfolded or aggregated proteins are either trafficked to aggresomes for storage, or sent to the lysosome/vacuole or proteasome for degradation. Such processes involve numerous proteins and signaling pathways, such as ubiquitin ligases, chaperones, and the ERAD pathway.
4.1 The role of Tti2 and the TTT complex in protein biosynthesis and PIKK regulation

A role for the TTT complex in PIKK protein biosynthesis is indicated by the binding of Tel2 to newly synthesized PIKK peptides and the interactions of the TTT proteins with Hsp90, Hsp70, Hsp40, and the R2TP/prefoldin-like complex (7-10). In conjunction with results from Stirling et al. (11) regarding reduced Tel1 levels, our results indicate that *Saccharomyces cerevisiae* Tti2 is required to maintain the steady state levels of all PIKK proteins. The use of defective *tti2* alleles identified in a random screen, and regulating *TTI2* under the *GAL10* promoter, allowed us to investigate phenotypic and proteomic effects associated with the loss of Tti2 function.

The results presented in Chapter 2 demonstrate a specialized role for Tti2, possibly dedicated to PIKK regulation. Tti2, like Tel2 and Tti1, is essential in nature, however, severely depleting Tti2 levels does not result in lethality. The low level requirement of Tti2 may reflect the number of molecules needed for associating with a small set of client proteins. While Tti2 depletion results in Hsp42 induction, Tti2 does not function as a general heat shock protein. Moreover, the overexpression of Hsp26, Hsp42, or Hsp104 in a Tti2 depleted strain could not rescue stress sensitive phenotypes, suggesting that general protein stress is not produced in the absence of Tti2. It seems likely that Hsp42 induction could be a consequence of reduced PIKK expression. In particular, Tra1 as part of the SAGA chromatin remodeling complex was identified in the coding regions of certain *HSP* genes and is enriched at *HSP* promoters during heat shock (12). Consistent with the reduced transcription of the heat shock element seen upon Tti2 depletion, decreased Tra1 expression may somehow result in Hsp42 induction to compensate for dysregulated heat shock genes.

The role of Tti2 and the TTT complex may be more complex than simply in the recruitment of PIKK proteins for maturation by Hsp90. The cytoplasmic foci and mislocalization of Mec1 and Tra1, as well as the absence of Mec1 aggregates when Tti2 is depleted, suggests a potential role in PIKK trafficking. In a previous study, two mutations in *TTI2* were found to suppress a defective *tra1* allele containing a phenylalanine to alanine mutation in the FATC (*tra1*F3744A) domain that results in reduced
Tra1 protein levels and mislocalization (13). The rarity of gain of function mutations, and the number of mutations in *TTI2* that suppress slow growth caused by *tra1*<sub>F3744A</sub> (Brandl lab, unpublished), suggests that suppression occurs by loss of function *tti2* alleles. How loss of Tti2 function can restore protein expression and localization of *tra1*<sub>F3744A</sub> requires further investigation but demonstrates a more complex role for Tti2 in PIKK biosynthesis. A fitting model is that Tti2 targets unstable PIKK proteins for degradation, and loss of function *tti2* mutations provide more time for PIKK folding.

The stoichiometry of the TTT complex and interactions among each member is still largely unknown. The relative number of molecules per cell indicates that Tti2 is twice as abundant as Tti1 and Tel2, suggesting it could form a dimer or have functions outside the TTT complex (14). The conserved N-terminal region of Tel2 consisting of alpha-helical repeats is required for interaction with Tti1 and Tti2 (10). There lack structural data for the TTT complex, or for Tti1 or Tti2 alone, although Tti2 is predicted to consist mostly of alpha helices. Direct contact sites between the PIKK proteins and TTT components also remain unknown. However, the N-terminal region consisting of HEAT repeats of ATM and mTOR, and the C-terminal region of ATM containing the kinase and FATC domain, co-immunoprecipitate with Tel2 (9).

The possibility of independent functions of TTT proteins requires further investigation. Tel2 was initially identified for its role in telomere length regulation (15), though likely an indirect effect through regulation of Tel1 as part of the TTT complex (11). Functions described only to certain TTT proteins and not others may be a result of allele-specific effects. For example, two mutations in the N-terminal region of Tel2 result in a telomere length defect, while C-terminal mutations are associated with rapamycin sensitivity (16). This may be due to PIKK-specific binding sites of Tel2 (9).

For the most part, Tel2, Tti1, and Tti2 have mutual roles in regulating PIKK protein levels and complex assembly. It is likely that TTT members act in complex to function with Hsp90 in PIKK biosynthesis. Tel2 interaction with the R2TP complex, as well as the interaction between Tel2 and Tti1-Tti2, is required for PIKK stability, function, and complex assembly (7,10,17,18). Although some studies report only one or
two of the TTT complex members in association with PIKK proteins (19,20), the absence of other members was not investigated. In support of a discrete function for all TTT members in PIKK biosynthesis, approximately 100 overlapping genetic interactions were identified by synthetic genetic array analyses (SGA) using temperature-sensitive alleles for TEL2, TTI1, or TTI2, which mapped all three genes within the same cluster of a global network (11).

4.2 Defining an Hsp90 co-chaperone

Hsp90 co-chaperones are proteins actively involved in the function of Hsp90 but are not client proteins. Co-chaperones of Hsp90 have one or more of the following functions: recruitment of client proteins, linking Hsp90 to other chaperones, protein complex assembly, inducing conformational changes, and regulating ATP hydrolysis for client protein binding and release (21). For many of the co-chaperones, direct interaction with Hsp90 involves a TPR domain binding to the conserved MEEVD motif of the C-terminal domain (C domain) of Hsp90. Other Hsp90 co-chaperones interact with the N-terminal nucleotide binding domain (N domain), the middle domain (M domain), or associate indirectly through protein-protein interactions. Table 1 lists the functions and domain associations of yeast Hsp90 co-chaperones. The rate of Hsp90 chaperone cycles and client specificity depend on the sequential binding of co-chaperones (22,23). For example, the transfer of Hsp70 clients to Hsp90 is mediated by Sti1, which inhibits Hsp90 ATPase activity by preventing N-terminal dimerization (22,24). The binding of ATP and p23 (human homolog of yeast Sba1) displaces Sti1, promotes nucleotide binding, and induces an Hsp90 closed conformation (22). The rate of ATP hydrolysis can also be increased by Aha1 binding to the middle domain of Hsp90 (25), which accelerates the release of a client protein.
Table 4-1. List of Hsp90 co-chaperone functions, co-chaperone domains that associate with Hsp90, and Hsp90 binding sites.

<table>
<thead>
<tr>
<th>Co-chaperone</th>
<th>Function</th>
<th>Co-chaperone domain</th>
<th>Hsp90 domain of interaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cns1</td>
<td>Binds Hsp90 and Hsp70, stimulates Hsp70 ATPase activity</td>
<td>TPR domain</td>
<td>C domain</td>
<td>(26,27)</td>
</tr>
<tr>
<td>Cpr6, Cpr7</td>
<td>Peptidyl-prolyl cis-trans isomerase</td>
<td>TPR domain</td>
<td>C domain</td>
<td>(28,29)</td>
</tr>
<tr>
<td>Ppt1</td>
<td>Serine/threonine phosphatase, regulates Cdc37 phosphorylation and kinase activation by Hsp90</td>
<td>TPR domain</td>
<td>C domain</td>
<td>(30,31)</td>
</tr>
<tr>
<td>Sti1</td>
<td>Inhibits Hsp90 ATPase activity</td>
<td>TPR domain</td>
<td>M and C domains</td>
<td>(32)</td>
</tr>
<tr>
<td>Tah1</td>
<td>Recruits the R2TP complex to Hsp90, mediates protein complex assembly, inhibits ATPase activity</td>
<td>TPR domain</td>
<td>C domain</td>
<td>(33-37)</td>
</tr>
<tr>
<td>Aha1, Hch1</td>
<td>Activates ATPase activity</td>
<td>N-terminus</td>
<td>M domain</td>
<td>(25,38,39)</td>
</tr>
<tr>
<td>Cdc37</td>
<td>Inhibits ATP binding, prevents N-terminal dimerization, recruit’s protein kinases</td>
<td>C-terminus</td>
<td>N domain</td>
<td>(40,41)</td>
</tr>
<tr>
<td>Sba1</td>
<td>Stabilizes closed conformation, promotes ATP hydrolysis</td>
<td>N- and C-termini</td>
<td>N and M domains</td>
<td>(42-44)</td>
</tr>
<tr>
<td>Sgt1</td>
<td>Recruitment of Skp1 and the SCF ubiquitin ligase complexes</td>
<td>Central CS domain</td>
<td>N domain</td>
<td>(45,46)</td>
</tr>
</tbody>
</table>
Budding yeast is a valuable system for identifying and characterizing Hsp90 co-chaperones using high-throughput analyses. Tandem affinity purification of Hsp90 coupled to mass spectrometry has been successful in detecting proteins that physically interact with Hsp90, and can be performed under different conditions. Synthetic genetic array analysis (SGA) is a high-throughput technique used to screen for genetic interactions against a yeast deletion strain library (47). Zhao et al. (48) performed SGA analyses using either an Hsp90 conditional allele, or in the presence of the Hsp90 inhibitor geldanamycin, to identify and map novel genetic and chemical-genetic interactions with Hsp90. In addition to defining Hsp90 as a central hub for numerous cellular pathways, the combination of these approaches and the clustering of interaction data identified a new role for Hsp90 in chromatin remodeling. Moreover, biochemical assays supporting SGA interactions helped define two novel Hsp90-interacting proteins, Tah1 and Pih1 (48).

The TTT complex interacts indirectly with Hsp90 through the R2TP complex (8), however, direct contacts may also exist. The structure of Tel2 elucidated two phosphoserine sites that can be bound by Pih1 and mutational analysis has defined residues important for interaction with Tti1-Tti2 (8,10). In support of an interaction with Hsp90 independent of the R2TP complex, disrupting the Tel2-Pih1 interaction by phosphoserine site mutations does not result in loss of the Tel2 association with Hsp90 and PIKK proteins (7). However, the loss of Tel2-R2TP interaction causes a decrease in PIKK stability, and suggests a role for the R2TP complex in PIKK assembly (7).

The mechanism of the TTT complex in PIKK recruitment still needs to be elucidated. Whether PIKK recruitment is the only role of the TTT members remains unclear. In this respect, it may be valuable to determine whether the recruitment of PIKK proteins to Hsp90 defines the essential nature of Tel2, Tti1, and Tti2. Moreover, identifying how the TTT complex interacts with Hsp90 independent of the R2TP
complex, and investigating the influence of the interactions on Hsp90 activity, will help define the TTT complex as an Hsp90 co-chaperone.

### 4.3 Mistranslation

The universality of the genetic code is usually emphasized, stating the need for high fidelity translation to maintain the proteome. Despite this, deviations from the standard genetic code are surprisingly common (reviewed in (49)). Alternate genetic codes were first observed in the 1970s in analyses of yeast and human mitochondrial genomes (50-53). Naturally occurring changes to the standard genetic code come in the form of ambiguous decoding, codon reassignments, and genetic code expansions, such as the decoding of UAG and UGA stop codons with pyrrolysine and selenocysteine, respectively (54). Ambiguous decoding, or mistranslation, can occur in response to changing environments, nutrient availability, and in stress conditions (2,55-59). Thus, genetic code manipulations are beneficial if not essential for the organism, and cannot be viewed simply as translational errors.

Nucleotides responsible for tRNA-aaRS recognition are known as identity elements. In contrast to the aspect of the genetic code that defines mRNA decoding as rules of codon-anticodon pairing, tRNA identity elements define a ‘second genetic code’ (60). Identity elements consist of single nucleotides, nucleotide pairs, and structural motifs (61). Since the anticodon of a tRNA links an amino acid to the genetic code, it is not surprising that tRNA recognition involves identity elements within the anticodon for many aaRSs (62). However, specific nucleotides in the acceptor stem also provide discrete structural characteristics for aaRS recognition and charging (63-65).

Missense suppressor mutations within tRNAs that alter reading of the genetic code have revealed other aspects of the specificity of tRNA structure and function. The base changes that convert a tRNA into a missense suppressor map to different regions of the tRNA molecule. The most prevalent changes are to the anticodon loop, which lead to altered base pairing in the A site of the ribosome. The mutations can be base substitutions in the anticodon (66-70) or insertions or deletions that reposition the anticodon (71,72). In some cases, the indels result in altered base modification, which also affects codon-
anticodon pairing (72). Mutations to the anticodon emphasize that, even for tRNAs that contain anticodon identity elements, other sequences can at least partially direct aminoacylation.

Base changes outside the anticodon loop can also result in misacylation, an example being base changes that create a G3:U70 base pair in the acceptor stem (73,74). The G3:U70 base pair is a major identity element for aminoacylation by alanyl-tRNA synthetase (AlaRS) in many species and when present in non-alanine tRNAs can confer charging with alanine (75-77). As charging specificity often depends on multiple elements in a tRNA, additional base changes can make a suppressor tRNA more or less efficient (78). Efficiency of suppression can also be influenced by extrinsic factors. These factors include the base composition of the genome and the resulting codon bias (66), and the number and relative expression of tRNA genes for each codon. All of these will influence the frequency and extent of amino acid substitution and thus the toxicity of the suppressor tRNA. In addition, the efficiency and accuracy of tRNA charging depends on the relative abundance and affinity of different aaRSs (79).

4.4 Repercussion of mistranslation

Mistranslation destabilizes the proteome as a result of misfolded proteins and toxic aggregates (80-84). To manage harmful proteins, cells upregulate molecular chaperones, and induce the unfolded protein and the heat shock responses (81,85). Regulating protein quality control pathways provides a rapid response that decreases proteome instability caused by mistranslation. Over a longer term, selection pressure created by translation errors lead to coding sequence changes that prevent protein misfolding and increase protein stability (82,86,87). Therefore, protein quality control pathways and codon alterations that reduce the cost of translation errors are important for exploiting mistranslation as an adaptive response.

In addition to the observation of increased error rates at low abundance codons (88), high usage codons are theorized to correlate with high translational accuracy (89). Synonymous codon bias towards optimal codons may ensure translational accuracy for conserved amino acids, to minimize the cost of errors (90). Synonymous mutations to
conserved and structurally important amino acids within proteins can improve the translational accuracy of the codon (90,91). However, it is proposed that making proteins more robust to translation errors is a superior mechanism to adapt to mistranslation (86,87,92). Having translationally robust proteins maintains the function of mistranslated species, increases thermodynamic stability, and provides resistance to genetic mutations (86,92-94). These studies suggest that enhancing protein robustness, rather than improving the fidelity of translation, enables organisms to tolerate mistranslation.

A study by Kalapis et al. (55) investigated how yeast evolve in response to mistranslation levels that cause a fitness reduction. Expression of tRNA_{Ser}^{CAG} in S. cerevisiae causes ambiguous decoding of Leu as Ser. The negative effects on cellular fitness were drastically reduced after 250 generations. Kalapis et al. (55) analyzed eleven independent clones in detail. They shared chromosomal duplications containing genes involved in ribosome biogenesis, rRNA processing, and proteasome assembly. They also shared a chromosome V deletion that removed two deubiquitinating enzyme genes. In addition to chromosomal rearrangements, genes that function in amino acid biosynthesis, ribosome biogenesis, and RNA processing were upregulated. Gene expression changes and chromosomal rearrangements correlated with increased protein synthesis and turnover, reduced protein aggregation, and an increase in glucose uptake (55).

The extent to which cells tolerate mistranslation has been studied with defective aaRSs and mischarged tRNAs. Ruan et al. (95) studied auxotrophic and antibiotic resistance markers in E. coli that require missense suppression for function. Misacylated tRNAs were generated in cells by expressing error-prone or non-discriminating aaRSs. These experiments revealed that this E. coli strain grows at optimal levels even in the presence of proteome-wide mistranslation. As determined by quantifying amino acid misincorporation frequency by mass spectrometry, E. coli tolerate ~10% mistranslated protein. This represented the threshold level of mistranslation where optimal growth and a functional proteome are still maintained (95). In the same study, cellular responses were analyzed. Expression of a non-discriminating aspartyl-tRNA synthetase, which charges tRNA_{Asn} with aspartic acid, caused an induction of chaperones dnaK and mopA by 49 and
8.7-fold, respectively. Moreover, synthetic slow growth was caused by deleting major heat shock proteases in strains with defective aaRSs (95).

As described in Chapter 3, we analyzed the protein quality control pathways and cellular responses required for tolerating mistranslation of Pro as Ala (74). In yeast expressing tRNAPro\(^{G3:U70}\), which causes mis-incorporation of alanine at \(~6\%\) of Pro CCA codons, there was a partial induction of the heat shock response but only a modest effect on cell growth. When grown at increased temperatures or in the presence of tunicamycin, slow growth was exacerbated. In addition, we found synthetic slow growth of a strain expressing the mistranslating tRNA and deleted for the 26S proteasomal regulator, Rpn4. Consistent with other reports (55,81,85,95), these results demonstrate a role for the heat shock response and the unfolded protein response in mistranslation tolerance.

### 4.5 Quantifying mistranslation

Stable isotope labeling with amino acids in cell culture (SILAC) and other isobaric labeling mass spectrometry methods provide a quantitative approach to measure proteome-wide mistranslation. Using shotgun mass spectrometry, Cvetesic et al. (96) identified over 2500 spectra of peptides having substitutions of leucine with norvaline, a non-proteinogenic amino acid. The number of spectra represents an approximate 10% incorporation frequency of norvaline at Leu codons in an \(E.\ coli\) strain containing an editing-defective leucyl-tRNA synthetase. In the same study, SILAC identified the surprising absence of isoleucine mis-incorporations at Leu positions by leucyl-tRNA synthetase defective of hydrolyzing isoleucyl-tRNA\(^{\text{Leu}}\) (96).

The study by Cvetesic et al. (96) highlights the potential of mass spectrometry-based approaches to measure proteome-wide mistranslation frequencies; but measuring mistranslation by mass spectrometry does have some limitations. The accuracy in detecting single peptide change is affected by the ratio of wild-type to mistranslated species (97). Mistranslation that affects peptide ionization may result in detection variability that is not indicative of relative \(\text{in vivo}\) abundance. By spiking wild type samples with known amounts of mistranslated peptides (Ser for Asn), Wen et al. (98)
determined that their mass spectrometry analysis yielded a 15-20% greater amount of mutant peptide than expected, in part due to differences in ionization. In addition, SILAC-labeled valine can be metabolized, making certain amino acids indistinguishable (96).

Fluorescent and enzymatic reporters provide sensitive methods to calculate mistranslation frequencies (99-104). We and others have designed fluorescent reporter molecules to measure mistranslation (74,100). By mutating Asp129 to Pro, we diminished fluorescence of eGFP, which could be restored by mistranslation with alanine. Alanine at position 129 in eGFP was created to mimic complete mistranslation of the Pro codon. By comparing eGFP<sub>D129P</sub> fluorescence relative to eGFP<sub>D129A</sub>, we calculated a 6% mistranslation frequency caused by tRNA<sub>Pro</sub> (G3:U70) in yeast (74). Gomes et al. (100), designed a dual-reporter system to internally normalize fluorescence in each cell. Their reporter system has a wild type eGFP molecule fused to mCherry, which contains a Met72 to Glu (GAA or GAG) mutation and only fluoresces when Met is mis-incorporated. Fluorescence of wild type eGFP is unaffected by mistranslation and is used to normalize mCherry fluorescence.

Our reporter, and the one designed by Gomes et al., calculate mistranslation frequency at a particular codon. To detect all codon misreadings by native tRNA<sub>Lys<sub>UUU</sub></sub>, Kramer et al. (88) engineered luciferase genes with the essential Lys529 codon changed to all possible single nucleotide mutations (102). Normalizations were possible due to the in-frame expression of a second non-mutated luciferase that could be assayed under different conditions (88). With this assay the authors determined that differences in mistranslation frequencies are a consequence of decoding competition between cognate and near-cognate aminoacyl-tRNAs, with frequencies being codon-dependent and varying from $\sim 4 \times 10^{-3}$ to $\sim 3 \times 10^{-4}$ per codon in <i>E. coli</i> and $\sim 4 \times 10^{-5}$ to $\sim 6.9 \times 10^{-4}$ per codon in <i>S. cerevisiae</i> (88,102).

β-galactosidase assays have also been used to measure amino acid misincorporation. Tsai and Curran (66) used β-galactosidase activity to detect and quantitate mistranslation caused by suppressor tRNAs. Their reporter contained lacZ with the
essential Gln CAG at position 625 mutated to Arg CGA to screen mutated tRNA$^{\text{Gln}}$-expressing clones for suppression. The four suppressors identified contained C-U and U-C mutations at anticodon positions 34 and 35, respectively, allowing base pairing with CGA. The tRNA$^{\text{Gln}}$ variants increased $\beta$-galactosidase activity by 8% (66). Similarly, Gregory and Dahlberg (105) screened tRNA$^{\text{Glu}}$ mutants for suppression of missense or nonsense mutations in $\text{lacZ}$. A C-A transversion mutation at position 36 in tRNA$^{\text{Glu}}$ suppressed ochre and amber $\text{lacZ}$ mutations, while C-G transversion at position 36 suppressed a GAG to CAG missense mutation. In each case the $\beta$-galactosidase activity indicated a low level of mistranslation (105).

4.6 Benefits of mistranslation

The ability of tRNA mutations to result in new genetic codes supports the theory of genetic code evolution (77,106,107). Mistranslation and genetic code expansions represent ways to introduce proteome diversity to improve the fitness of an organism by generating novel proteins. Studies of early life forms suggest that peptides contained a subset of the 20 canonical amino acids; those that were readily available on the primordial Earth (108). Crick hypothesized that introducing new amino acids improved protein fitness (109). As described by coevolution theory, biosynthesis of additional amino acids significantly influenced the evolution of the genetic code (110). Muller et al. (111) suggest that once an enzyme reaches a “local fitness maximum”, expanding the amino acid repertoire provides the chemistry to increase protein stability and activity. Their experiment involved the evolution of a simplified chorismate mutase composed of only nine different amino acids in an attempt to resurrect the primordial enzyme. Mutations that introduced an amino acid beyond the primordial nine were selected in a growth assay favouring increased activity. Modest chemical changes brought on by mutation of Leu to Val and Ile to Thr produced a chorismate mutase variant composed of 11 different amino acids and increased activity 10-fold as the result of intricate structural changes (111). These findings emphasize that even subtle stability and functional alterations to proteins upon introducing new amino acids can drive genetic code evolution and expansion.
The same evolutionary pressures on the genetic code apply to organisms in the present day. Changing codon-amino acid designations can provide a selective advantage for viruses, prokaryotes and eukaryotes in response to environmental change. Examples of the advantage of ambiguous decoding were shown in laboratory experiments when *E. coli* and Chinese hamster ovary cells were deprived of an amino acid. Parker *et al.* (56) observed lysine mis-incorporation into bacteriophage MS2 coat protein upon starving *E. coli* host of asparagine. They speculate that mistranslation is preferred over halting translation entirely, thus killing the cell (56). Similarly, mistranslating Asn as Ser was observed in Chinese hamster ovary cells under asparagine starvation (98). Wen *et al.* (98) reasoned that Asn to Ser mistranslation was due to tRNA misacylation rather than codon misreading, because Ser codons differ from Asn in the first or second nucleotide position, and Asn misreading errors typically involve a wobble position mismatch with tRNA\textsuperscript{Lys}.

Other examples of adaptive mistranslation occur in *E. coli*, yeast, and mammalian cells in response to oxidative stress. Oxidative stress results in mischarging of non-cognate tRNAs with methionine by methionyl-tRNA synthetase (1,2,4). Methionine misincorporation is proposed to sequester reactive oxygen species as a protective mechanism (2).

An elaborate case of codon reassignment exists in *Candida* species, which have reassigned Leu (CUG) to Ser (112-114). Under standard conditions ~97% of CUG codons are decoded as Ser; however, reversion of CUG identity back to Leu is tolerated (80,115). Interestingly, engineered *C. albicans* strains with increased leucine mis-incorporations at CUG codons are phenotypically diverse (80,115). Upon infection, *C. albicans* with increased CUG ambiguity caused an upregulation in inflammatory and immune responses of mice and human cells, respectively (80). Adaptive mistranslation to evade host immunity has also been suggested for *Mycoplasma* parasites (116). Mutations in the editing domains of phenylalanyl-tRNA synthetase, leucyl-tRNA synthetase, and threonyl-tRNA synthetase cause mistranslation with near-cognate amino acids. This may increase antigen diversity and allow the *Mycoplasma* to escape host defense mechanisms (117).
Bacteriophage have evolved to infect hosts that have variations to the standard genetic code. UGA stop codon reassignment has occurred in bacterial species from SR1 and Gracilibacteria phyla (117,118). It had been predicted that bacteriophage would follow the same coding rules to utilize the host’s translation machinery (119-121). Ivanova et al. (122) demonstrated that the interaction is more complex. They identified DNA bacteriophage, of the Caudovirals order, in the human oral cavity that reassigned UAG stop codons to Gln during the infection process. The viral genomes contain a UAG-decoding tRNA$_{Gln}^{CUA}$, and expresses release factor 2 (RF2), which terminates translation at UGA and UAA. These phage were enriched in bacteria that reassigned UGA codons, but express release factor 1 (RF1), which terminates translation at UAG. Based on the observation of reassigned-UAG codon usage bias in phage genes, Ivanova et al. proposed the following antagonistic model to explain phage proliferation in a bacterial host with a different genetic code. Low UAG codon usage of early infection stage genes of phage, including RF2, prevents premature termination by bacterial RF1. Bacterial RF1 levels are reduced by phage RF2 expression, through translational inhibition of UGA-reassigned codons in RF1. The absence of bacterial RF1 allows expression of late infection stage phage genes containing UAG-reassignments that function in packaging and assembly of viral DNA (122). Therefore, rather than adapting their genetic code to that of their host, bacteriophage can exploit the differences to manipulate gene expression in UGA-reassigned bacteria.

The spontaneous appearance of suppressor tRNAs, as we found for a selected tRNA$_{Pro}^{UGG}$ (G3:U70) variant that is efficiently charged with alanine (74), raises the question of the prevalence and maintenance of tRNA variants that result in mistranslation in populations of cells and in multicellular organisms. The copy numbers of the different tRNAs alone suggests that tRNA variants may be surprisingly abundant. We estimate that in our yeast experiments, the G3:U70 mutation in tRNA$_{Pro}^{UGG}$ genes was found once per $2 \times 10^5$ cells. We found that a single copy of a tRNA$_{Pro}^{UGG}$ (G3:U70) gene resulted in modest effects on cell growth, and would likely be maintained in the population for some time, even under non-selective conditions. The tRNA$_{Pro}^{UGG}$ (G3:U70) alleles are relatively nontoxic likely because they are not ideal substrates for AlaRS. This would contrast with the toxicity of an anticodon switch of tRNA$_{Ala}$, resulting in Pro decoding.
These demonstrably more toxic anticodon swapping mutants [72] have not been identified in genetic screens as spontaneous mutants. Interestingly, tRNA\textsubscript{Pro\_UGG} (G3:U70) is not aminoacylated by prolyl-tRNA synthetase, nor is it required because of the additional wild-type copies of tRNA\textsubscript{Pro\_UGG} genes remaining in the cell. The fact that multiple copies of the tRNA isoacceptor exist in the genome, enables the cell to acquire a seemingly deleterious mutation in tRNA\textsuperscript{Pro\_UGG} (G3:U70), for at least a transient period, so that the tRNA can be repurposed by the cell. For example, an anticodon change to UGA could potentially repurpose the mutant tRNA to decode alanine UCA codons with serine.

### 4.7 Uses of mistranslation

Ambiguous decoding is a mechanism to increase proteome diversity and generate novel proteins. Natural expansions of the genetic code that add selenocysteine and pyrrolysine are prime examples of this (123-125). Decoding selenocysteine involves a unique tRNA\textsubscript{Sec} species with a UCA anticodon to recognize UGA (126). Selenocysteine incorporation is guided by a selenocysteine insertion sequence (SECIS) element that forms a stem loop in the mRNA secondary structure, and the SelB translation elongation factor for SECIS-mediated recruitment of aminoacyl-tRNA\textsubscript{Sec} (reviewed in (127)). This system directs incorporation of selenocysteine at specific recoded UGA codons so that translation termination at UGA is not disrupted. Selenocysteine-containing proteins, or selenoproteins, have increased nucleophilicity and reactivity relative to homologs with cysteine at the same position, and are required by bacterial pathogens in oxidative stress conditions (128,129). These advantages may explain why organisms invest a great deal of energy into incorporating selenocysteine into proteins.

Pyrrolysine is found in archaeal and bacterial methanogens and is decoded by a UAG reassignment (reviewed in (54)). Pyrrolysyl-tRNA synthetase aminoacylates tRNA\textsubscript{Pyl} with free pyrrolysine synthesized from two lysine molecules (reviewed in (130)). Encoding of pyrrolysine was discovered in the active site of methyltransferases (124,125,131). Pyrrolysine incorporation also allows expression of full-length tRNA\textsubscript{His\_guanylyltransferase}, strictly by nonsense suppression of UAG (132). The importance of a genetic code containing pyrrolysine was further demonstrated in \textit{Methanosarcina acetivorans}. Deleting tRNA\textsubscript{Pyl} causes differential expression of >300 proteins, including...
the down regulation of proteins involved in amino acid metabolism, translation initiation factors, and methanogenesis (133).

Natural genetic code expansions with selenocysteine and pyrrolysine signify the potential of introducing non-canonical amino acids (ncAAs) to the standard code and thus directed evolution of translation systems. Engineered translation systems are designed to site-specifically incorporate ncAAs that can function as imaging probes, affinity tags, or modified residues (134, 135). Nonsense suppression is commonly used to incorporate ncAAs in vivo for a few reasons. Aminoacylation involving ncAAs and an engineered aaRS/tRNA pair must function exclusively (orthogonally) from the host system for efficient and non-lethal translation. Since stop codons typically do not specify amino acids, engineered tRNAs that translate stop codons will avoid charging by aaRSs with recognition for anticodon identity elements (61). Furthermore, competition with release factors can be eliminated in E. coli. Lajoie et al. (120) replaced all 321 UAG codons with UAA using multiplex automated genome engineering (MAGE) and conjugative assembly genome engineering (CAGE). This permitted deletion of RF1 and genome recoding of E. coli.

The current challenges being addressed when introducing ncAAs include identifying truly orthogonal aaRS/tRNA pairs, transcription and processing of foreign tRNAs in a host system, and improving the yield of proteins containing ncAAs (reviewed in (134, 136, 137)). Genetic selections in yeast and bacteria can screen for aaRS/tRNA pairs that incorporate one or two ncAAs within the same protein (138-141). However, such selections require optimization for high yield incorporation of a ncAA by an orthogonal aaRS/tRNA pair. In this respect, coupling positive and negative growth assays in yeast that select for efficient nonsense suppression and against non-orthogonal aaRS/tRNA pairs has been successful (138, 139). Expression of a foreign tRNA in yeast has been improved when transcribed in a dicistronic tRNA cassette containing upstream A- and B- box promoter elements (142). In addition, the half-life of the targeted mRNA template can be extended when transcribed in a strain deficient of the nonsense-mediated mRNA decay pathway (143).
The pharmaceutical value of protein engineering using genetic code expansions has emerged. Incorporating ncAAs into therapeutic proteins increases protein stability, prevents immunogenicity, and generates novel antibodies (reviewed in (144)). Here we will discuss a few examples of therapeutic antibodies produced using codon expansions. Kim et al. (145) constructed Fab antibody fragments that can be specifically linked to recruit CD3+ T lymphocytes to HER-2+ breast cancer cells. An engineered aaRS/tRNA pair was used to incorporate p-acetylphenylalanine at a defined amber codon within anti-CD3 and anti-HER-2 Fab fragments. The positioning and chemistry of p-acetylphenylalanine allows for conjugation to a bifunctional ethylene glycol linker and coupling of anti-CD3 Fab and anti-HER-2 Fab fragments to form a bi-specific antibody. Formation of the bi-specific antibody leads to destruction of HER-2+ breast cancer cells (145). The same approach has since been designed to target cytotoxic T cells to leukemic stem cells with promise for treatment of acute myeloid leukemia (146).

Incorporation of ncAAs in therapeutic antibodies is an innovative strategy for improving drug targeting and antigen binding affinity. Site-specific incorporation of p-acetylphenylalanine facilitates conjugation of a microtubule toxin to an anti-HER-2 antibody by stable oxime bond formation (147). This method is advantageous over conjugation of toxins to canonical amino acids within antibodies. For example, antibody-drug conjugates made by randomly modifying cysteine or lysine residues contain heterogeneous mixtures with a range of toxicities (148,149). Moreover, ligation of toxins to cysteine residues is restricted to specific positions due to multiple disulfide bond requirements within the antibody (150,151). Exclusive chemical reactivity, or bio-orthogonality, can be accomplished for drug-antibody conjugation involving an ncAA at a defined position, and results in high yields of a homogeneous product (147). The synthesis of antibodies unrestricted to the standard 20 amino acids can drastically increase antigen binding affinity. Libraries of randomly mutated antibody fragments are expressed and displayed by phage, yeast, and bacteria, to screen for antibodies with high antigen binding affinity and specificity (152,153). Antibody library display screening in E. coli has been coupled with genetically encoded modified tyrosine residues. Dual incorporation of sulfotyrosine at nonsense and frameshift codons results in a 500-fold increase in the binding affinity of an anti-gp120 antibody, which recognizes HIV.
envelope glycoprotein (154,155). These studies demonstrate the potential for synthetic genetic code expansions in the design of therapeutic proteins and other proteins with commercial value.

It may be possible to use engineered tRNAs therapeutically. The vast number of suppressor tRNAs identified in genetic screens that restore function of defective alleles, and their limited effects on cellular fitness, suggests a potential use in gene therapy. Altered tRNA identity elements and anticodons can correct missense or nonsense mutations by mistranslation (61,73,75,77,78,156). Transfer RNAs that lack anticodon identity elements represent excellent candidates for efficient suppression. For example, if an aaRS of an engineered tRNA is anticodon agnostic, aminoacylation would be unaffected by anticodon switches, and the charged tRNA species can be directed to various codons. Each manipulation can be screened to identify tRNAs that introduce nontoxic levels of mistranslation and act as efficient suppressors (157).

In addition to genetic code expansion, mistranslation or ambiguous decoding has long been considered as a route to counteract human disease causing alleles. Treatment of disease-linked or deleterious alleles with suppressor tRNAs was first considered in the early 1980s (158), and has since been tested in mammalian cell culture and a transgenic mouse model (159-162). Temple et al. (158) mutated a tRNA\textsubscript{Lys} anticodon to CUA and demonstrated nonsense suppression of an amber mutation in human $\beta$-thalassaemia after injection of the mutant tRNA\textsubscript{Lys} gene and mRNA template into \textit{Xenopus} oocyte. Nonsense suppression was also analyzed in a transgenic mouse expressing a chloramphenicol acetyltransferase (CAT) gene containing a nonsense mutation. Suppression was examined after injection of plasmids bearing a nonsense suppressor tRNA\textsubscript{Ser} into skeletal muscle and heart tissues of live mice. Heart extracts yielded 1-2% CAT activity while 12% could be recovered from skeletal muscle cells (159). Suppression involving the selenocysteine translation components was performed in human primary fibroblasts (162). An engineered tRNA\textsubscript{Sec} species restored full-length expression of Selenoprotein N1 containing a nonsense mutation after transfection of patient samples with a plasmid expressing the tRNA\textsubscript{Sec} suppressor.
Other applications for mistranslation are likely to emerge. For example, natural variation in the sequence of an organism makes it difficult to identify a causative missense mutation in a background of numerous single nucleotide polymorphisms. It should be possible to use mistranslating tRNAs to narrow down the possible phenotype-linked mutations. If the mutation is recessive, addition of a mistranslating tRNA with an anticodon complementary to the mutated codon will at least partially restore protein function.

4.8 Conclusion

Tolerating low levels of mistranslation and maintaining proteostasis is attributed to the work of chaperones and protein quality control pathways. Variations in tRNA sequences causing mischarging, and subsequently amino acid mis-incorporation, alters what has been viewed as the standard genetic code. Genetic code variations impact the cell at the most basic, informational level. These variations provide important regulatory mechanisms, allow cells to increase genetic diversity, and reveal novel tools for synthetic biology applications. They also emphasize that the genetic code is continuing to evolve. How tRNA variation affects human biology is an exciting area for future study. Given their prevalence, one would predict that tRNA variants may alter the penetrance of neurodegenerative and a myriad of other human diseases that are driven by a loss of proteostasis.

4.9 Bibliography


deficiency in rigid spine muscular dystrophy caused by a mutation in the
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Curriculum Vitae

Name: Kyle Hoffman
Post-secondary Education and Degrees:
Wilfrid Laurier University
Waterloo, Ontario, Canada
2006-2010 B.Sc. Biology

The University of Western Ontario
London, Ontario, Canada
2010-2012 M.Sc. Biology

The University of Western Ontario
London, Ontario, Canada
2013-2016 Ph.D. Biochemistry

Honours and Awards:
Western Graduate Research Scholarship
2010-2012, 2013-2016

Related Work Experience:
Teaching Assistant
The University of Western Ontario
2010-2016

Publications:
