

Investigating the Diverse Activities of a Conserved Pseudouridine Synthase in Budding
Yeast: from RNA Modification to Prion Protein

by

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DISSERTATION ABSTRACT

Emily Meng-Meng Dennis

Doctor of Philosophy in Chemistry

Title: Investigating the Diverse Activities of a Conserved Pseudouridine Synthase in Budding

Yeast: from RNA Modification to Prion Protein

The ubiquitous yeast pseudouridine synthase Pus4 has several roles in cells. Pus4 catalyzes Ψ_{55} on nearly all tRNAs in yeast, a modification that is also highly conserved from bacteria (TruB) to mammals (TRUB1+TRUB2). This modification facilitates tRNA conformational stability and thus promotes protein synthesis. My thesis work expands our understanding of tRNA conformational dynamics resulting from Ψ_{55} and Pus4 interaction. Using biochemical and structural studies, we found that unmodified tRNAs populated three conformational states, transitioning through an intermediate structured state. The Ψ_{55} modification did not induce new conformational states, but shifted the relative abundance of these states. Biolayer interferometry permitted measurement of the timescales of Pus4:tRNA interactions. Upon Pus4 interaction, additional tRNA conformational states and dynamics were explored that were not as abundant with tRNA alone. This work overall established a new approach for exploring tRNA modification and enzyme-dependent effects on tRNA conformational dynamics.

Pus4 has also been implicated as the basis of the prion $[BIG^+]$, an altered epigenetic state which increases cell proliferation, enhances translation, and reduces lifespan. My thesis work investigated the biophysical state of Pus4 in the $[BIG^+]$ state as a possible explanation for its cellular growth phenotypes, as well as examined $[BIG^+]$ -dependent changes to the proteome. We

discovered that, unlike classical prion examples, $[BIG^+]$ is not dependent on a prion aggregate of the protein that is thought to initiate the state (Pus4). An analysis of the intrinsically disordered C-terminus of Pus4 revealed that negatively charged residues in this region modulated cell growth in the presence of the translation inhibitor, paromomycin. Pus4 and its C-terminal region appear to have a connection to translation, which could be related to its catalytic activities. Years after this work was initiated, we discovered a mutation in the gene *YAK1*, a protein kinase involved in regulating expression of genes tied to cell growth, was present in $[BIG^+]$ strains, and that this mutation contributes to at least one of the major growth phenotypes. Overall, my investigation has further clarified the molecular basis of $[BIG^+]$.

This dissertation contains previously published and unpublished co-authored material.

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Dennis, E. M. & Garcia, D. M. Biochemical Principles in Prion-Based Inheritance. *Epigenomes* **6**, 4 (2022).

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Zhang, T., Cao, L., Kumar, S., Enemchukwu, N. O., Zhang, N., Lambert, A., Zhao, X., Jones, A., Wang, S., **Dennis, E. M.**, Fnu, A., Ham, S., Rainier, J., Yau, K., Fu, Y. Dimerization of visual pigments in vivo. *PNAS* 201609018 (2016) doi:10.1073/pnas.1609018113.

Zhang, T., Enemchukwu, N. O., Jones, A., Wang, S., **Dennis, E. M.**, Watt, C. B., Pugh, E. N., Fu, Y. Genetic deletion of S-opsin prevents rapid cone degeneration in a mouse model of Leber congenital amaurosis. *Hum. Mol. Genet.* **24**, 1755–1763 (2015). doi:10.1093/hmg/ddu588

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During my time in Eugene, which began during the COVID-19 pandemic, a major source of community came from recreational soccer. What began as a single team grew into three teams that I now co-manage and benefit from every week. This sport and the people I play with have become a cornerstone in my life. I'd like to thank Bryce LaFoya for trusting me with his teams

and always pushing me to improve on the field (and even in science too). Thank you to every teammate who set me up to score goals and urged me to run harder, there are too many names to include.

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CHAPTER I

INTRODUCTION

This dissertation contains my contributions to three bodies of work, as well as published and unpublished co-authored work, spanning the space of epigenetics and the numerous activities of a conserved pseudouridine synthase, Pus4.

Chapter II is a collaborative project investigating the interplay between the binding, catalytic, and tRNA remodeling activities of the highly conserved pseudouridine synthase, Pus4. I am co-lead author of the resulting manuscript, in which I contributed major effort in all aspects of the study: project development, experimentation, analysis, writing, and manuscript preparation. My co-lead author was Nico Conoan Nieves, a former graduate student in Professor Julia Widom's lab. Other important contributions were made by Madison Kadrmas (Widom Lab), Abigail Vaaler (Garcia Lab), and Margaret Barry (Garcia Lab). Professors Julia Widom and David Garcia managed the project and conducted analyses, in addition to manuscript writing and editing. This paper is on *bioRxiv* and in revision at *RNA Journal*.

Chapter III is a review of the current biochemical understanding of prions in yeast, published in *Epigenomes* in 2022. Writing and editing was performed by both me and my advisor David Garcia.

Chapter IV is a summary of data investigating the biophysical state and molecular basis of the yeast prion [*BIG*⁺]. As project lead, I designed and implemented experiments that aimed to identify the biophysical features of the prion and its altered interactions/structures within the cell. Ethan Shaw, Abigail Vaaler, Preeti Bhattacharjee, and Julia Reinsch contributed reagents, methods, and/or supporting results. Our collaborators Neil Wood and Dr. Stephen Fried at Johns Hopkins University carried out the LiP-MS experiments and analysis, with input from me and

David Garcia. As corresponding author, David Garcia oversaw the investigation and gave feedback on the writing and editing of this data summary. This work is currently unpublished.

This introduction will provide background and context for the following chapters, with each chapter containing more specific information on its respective topic.

RNA Modifications in tRNAs:

RNA is largely composed of four nucleobases, A, G, U, and C. It forms diverse structures, from single-stranded molecules to highly structured stems and loops. It can also perform enzymatic activities, either alone or in combination with proteins, but it is most well-known for its fundamental roles in information transfer (mRNA), acting as adaptor molecules to match the appropriate codon to the appropriate amino acid (tRNAs), and facilitating protein synthesis in combination with ribosomal proteins (rRNA).

Its complexity is further enhanced by RNA modifications. There are over 170 different kinds of RNA modifications that occur in coding and non-coding regions, and in RNAs of all kinds, including tRNAs, mRNAs, rRNAs, snoRNAs, and more¹. These modifications range from simple isomerization to the addition of large bulky adducts, and everything in between. They serve diverse roles, from providing structural stability/flexibility, impacting translation speeds and regulation, and facilitating substrate recognition. Although the first RNA modification pseudouridine (Ψ) was discovered back in 1951², it was not until more recently that the field understood the prevalence and significance of these altered bases across the mRNA transcriptome. Recent method developments, such as Oxford Nanopore direct RNA sequencing, have recently transformed the field, improving the accessibility and resolution of datasets for both mRNA and tRNA modification investigations.

The average cytosolic yeast tRNA has about 13 modifications³, making them the most highly modified RNA molecules relative to their size (**Figure 1.1**). Modified bases in tRNAs serve several purposes in the cell. They determine the structural stability and/or flexibility of the molecules, facilitate proper codon-anticodon pairing when the modification lies in the anti-codon loop, and aid in recognition of specific tRNAs by aminoacyl-tRNA synthetases that charge tRNAs with the correctly paired amino acid. Finally, they can even act in “circuits”, potentially regulating the maturation of the tRNA, where the presence of one modification promotes or represses the next RNA-modifying enzyme to recognize its substrate, and deposit a sequential modification^{4,5}. This also may serve as a key control step subject to regulation based on the current growth state and resources of the cell.

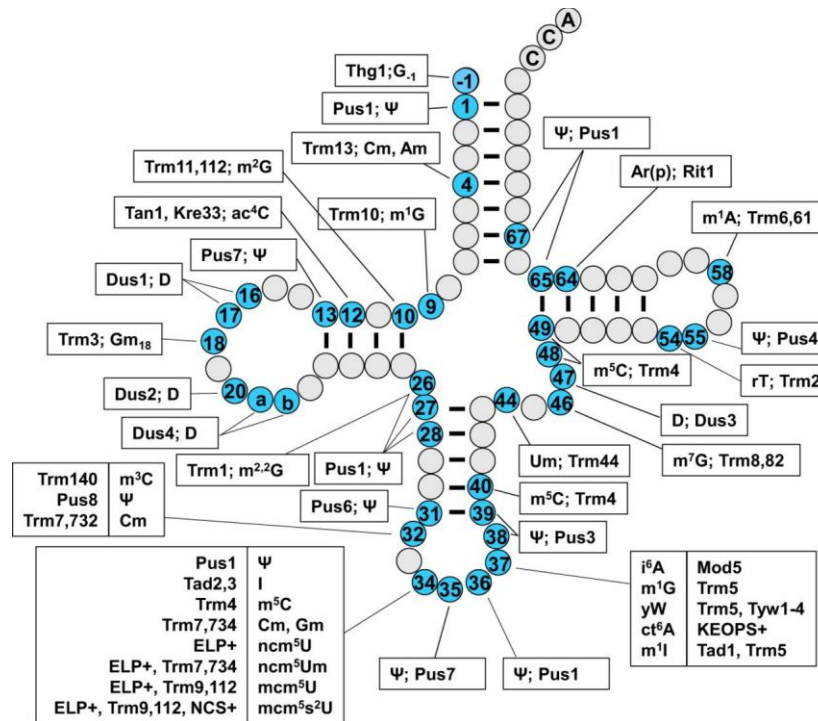


Figure 1.1: All documented yeast tRNA modifications and the enzymes that catalyze them. Figure modified from Phizicky and Hopper, 2023.

Pseudouridine is the main modification of relevance in this body of work. It is the most abundant RNA modification in cells and is a rather simple one – an isomer of uridine – meaning it is orientation specific and thus mass-silent (**Figure 1.2**). This flipped base provides an additional hydrogen bond donor, however, which can increase base-stacking and overall stability of its parent molecule, particularly in tRNA. Pseudouridine is present in tRNA, mRNA, rRNA, and various snoRNAs, and cannot be reverted to a uridine base once installed. This modification also has therapeutic implications, as it was recently used in mRNA vaccines to reduce its immunogenicity of the RNA molecule^{6,7}. Its abundance across biology and potential therapeutic uses provides strong motivation for further investigation of this modification and enzymes that deposit it.

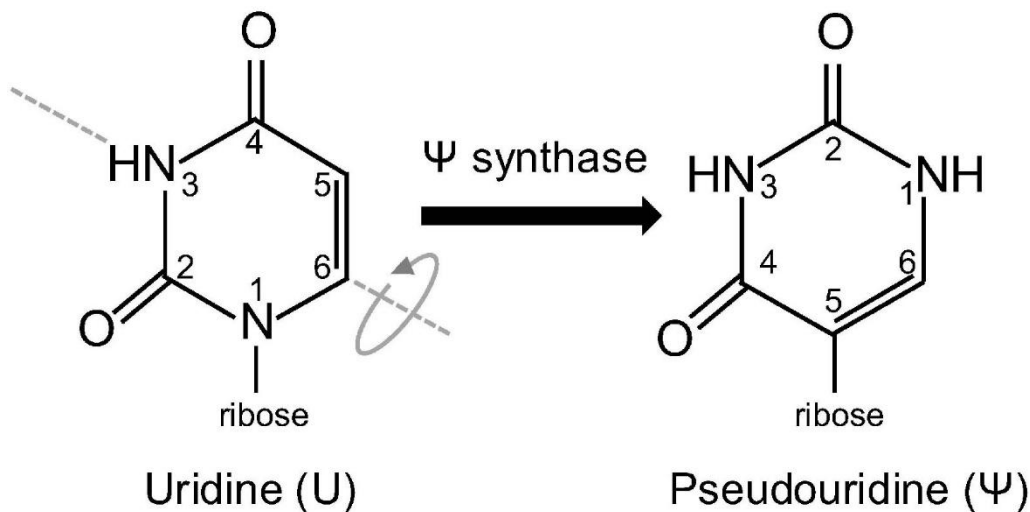


Figure 1.2: Mechanism of pseudouridine isomerization. A pseudouridine synthase cleaves the glycosidic bond, allowing the base to flip over and the bond to reform. Figure modified from Tomikawa, C. 2025.

In yeast tRNAs, pseudouridine can be installed on 15 different sites in cytosolic tRNAs and 7 sites in mitochondrial tRNAs⁸ (**Figure 1.3**). When present on stems and loops, the modification improves the structural stability of the tRNA. When pseudouridine is present in the

anti-codon region (positions 34-36), it can also affect translation fidelity. These modifications and the enzymes that catalyze them are generally highly conserved, with orthologs in bacteria and mammalian cells. Recently, Sebastian Glatt and colleagues have shown that pseudouridine incorporation near the D- and T-arm promotes folding and enhances thermal stability of most human tRNAs⁹. They found that the overall effect of pseudouridine modifications (Ψ_{13} and Ψ_{55} specifically) on tRNA structure is still tRNA-specific and sequence-context dependent. In summary, this modification is abundant in tRNA, catalyzed by numerous conserved enzymes (more below), promoting the maturation and stability of this key class of molecules.

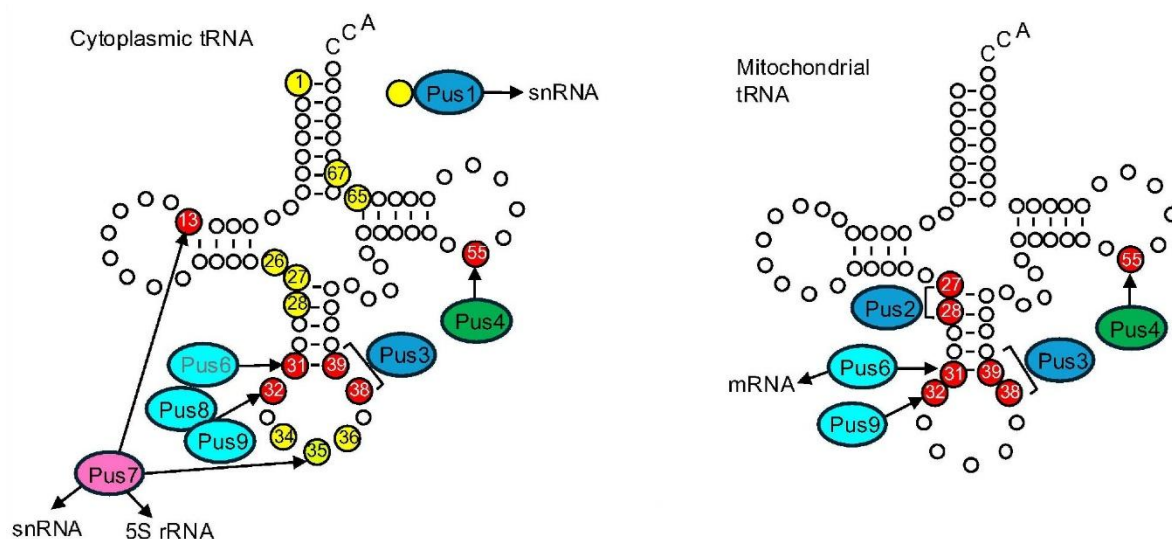


Figure 1.3: All documented pseudouridine modifications on yeast tRNA (both cytoplasmic and mitochondrial) and the Pus enzymes that catalyze them. Figure modified from Tomikawa, C. 2025.

Pseudouridine Synthase 4 (Pus4):

Pseudouridylation of tRNAs is catalyzed by five families of pseudouridine synthases: TruA, TruB, TruD, Pus10, and RluA⁸. In yeast, there are nine pseudouridine synthases that

modify tRNAs, both cytoplasmic and mitochondrial, belonging to the TruA, TruB, TruD, and RluA families. Pus10 has no ortholog in yeast. These standalone enzymes recognize their modification site based upon tRNA sequence and secondary structural context. They also pseudouridylate other RNA substrates, such as mRNAs, snoRNAs, and rRNAs, conjointly with other closely related H/ACA box enzymes (Cbf5/DKC1). These enzymes, however, are dependent on RNA guides, rather than motif sequence, distinguishing them from pseudouridine synthases.

One member of the TruB family, pseudouridine synthase 4 (Pus4), catalyzes a modification present on nearly all tRNAs in the cell. It deposits Ψ_{55} on all cytosolic tRNAs except for tRNA^{iMet}, and all mitochondrial tRNAs except for mt-tRNA^{Asp(GUC)} which does not have a U at position 55^{5,10,11}. This modification lies in the T-loop of tRNAs and promotes base-pairing with the D-loop, improving the tRNA's tertiary structure. While this enzyme is not essential, it has been shown to be a part of a modification circuit, in which Ψ_{55} promotes catalysis of m⁵U₅₄ and m¹A₅₈, by Trm2 and the Trm6/Trm61 complex, respectively⁵. This circuit suggests that since Ψ_{55} is upstream, its catalysis could regulate other modifications and therefore tRNA maturation. Pus4 also has a ~dozen documented mRNA targets¹²⁻¹⁴, and a single mitochondrial rRNA target⁴.

Pus4 was first identified because of its high homology to the enzyme TruB in *E. coli*¹⁰. Aside from its catalytic function, its ortholog TruB has an additional function as a tRNA chaperone, helping tRNAs fold for proper tRNA aminoacylation¹⁵. Multiple crystal structures of TruB have been solved, both with and without RNA bound, providing some understanding of the RNA contacts made during binding/catalysis¹⁶⁻¹⁸. While the structure of Pus4 has not yet been solved, AlphaFold3 predictions show high structural alignment to TruB, particularly in the

catalytic domain¹⁹ (**Figure 1.4**). One notable difference is the C-terminus region, in which TruB has a PUA domain, while Pus4 is predicted to have an intrinsically disordered region. Whether Pus4 also functions to remodel tRNA structure has yet to be determined.

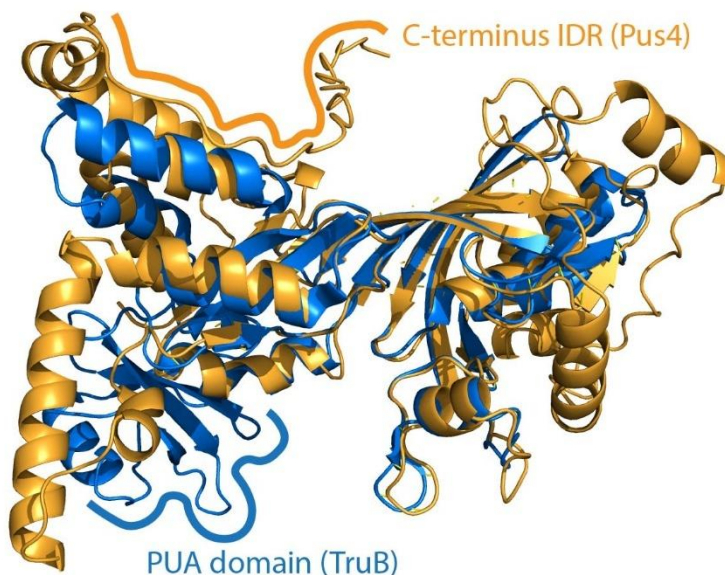


Figure 1.4: Alignment of AF3 model of Pus4 (orange) with the crystal structure of TruB (blue; 1K8W). Colored lines show the location of the C-terminus IDR of Pus4 (orange) and the PUA domain on TruB (blue).

Its catalytic mechanism of action involves residues in the RNA binding pocket, including R286, which when mutated may produce a catalytically-dead enzyme⁵. Once the tRNA is bound, the interaction with the enzyme disrupts tertiary interactions between the T-loop and D-loop, flipping out positions 55-57 on the T-loop (**Figure 1.5**). This allows the glycosidic bond of U₅₅ to be cleaved, the base flipped, and a new bond formed, resulting in the isomer pseudouridine. In the case of TruB, binding of the substrate is reportedly fast compared to catalysis that is rate-limiting²⁰. Ψ_{55} is not required for tRNA aminoacylation, however, its conservation in tRNAs throughout nature suggests it is critical for tRNA function. One study reported that while

deletion of TruB does not impact growth in optimal conditions, knockout strains are outcompeted in coculture with WT strains¹⁵. The study proposed that its function as a tRNA chaperone may be the basis for this finding, although other explanations are possible.

In part due to the lack of solved crystal structure, similar questions about Pus4's catalytic function, binding of substrates, and effects on growth remain unanswered. Specifically, the interplay between these documented functions, and perhaps still to be discovered ones, has yet to be explored. Pus4 interacts with nearly every tRNA in the yeast cell during at least one moment of their biogenesis. Generating a better understanding of this protein's functions will not only expand our understanding of Pus4, but all tRNA-modifying enzymes.

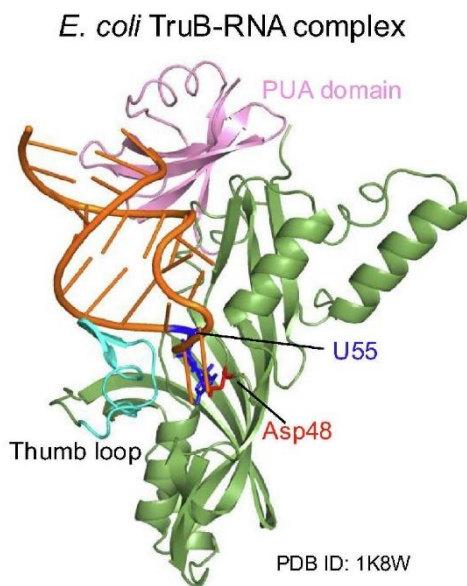


Figure 1.5: Crystal structure of TruB (1K8W) in complex with a T-loop fragment of RNA showing the flipping of U₅₅ (blue) out into the catalytic site. The catalytic residue Asp48 is shown (red), as well as the thumb loop (cyan) of TruB, which has a role in positioning the tRNA for catalysis. Figure modified from Tomikawa, C. 2025.

Mechanisms of Inheritance in Budding Yeast:

A lasting change in the function and activities of proteins like Pus4 may be inherited in the next generation of progeny. This passing on of traits, both those that are beneficial and those that are harmful, occurs through several mechanisms in budding yeast. Beyond those that follow the basic principles of biology, such as inheritance through DNA, there are others that are rare and exceptional (**Figure 1.6**).

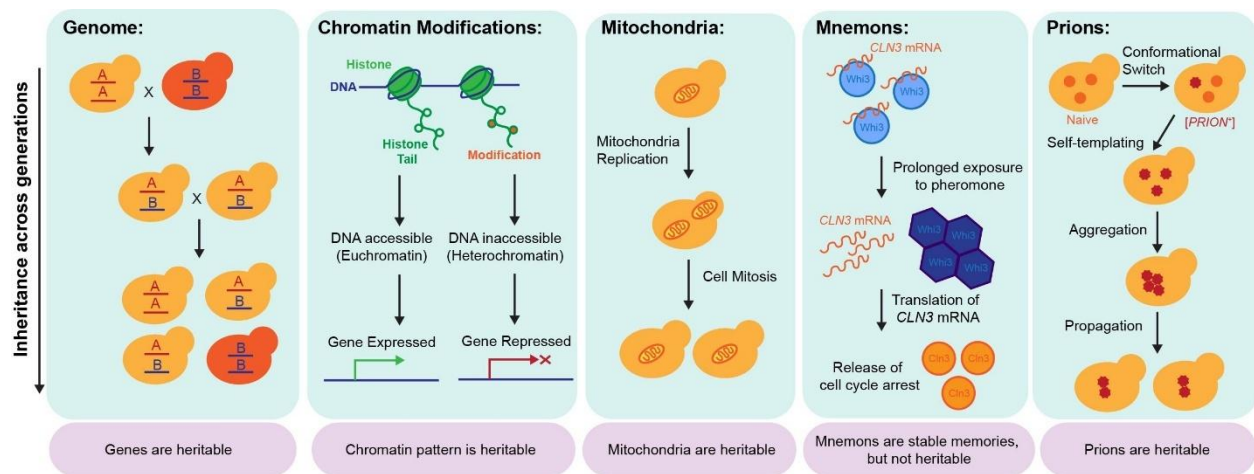


Figure 1.6: Mechanisms of inheritance in budding yeast, including the genome, chromatin modifications, mitochondria, mnemons, and prions.

Inheritance primarily works through the encoded sequence of one's DNA. The genome is sub-divided into chromosomes, which are further sub-divided into genes that encode proteins that carry out most functions in the cell. In haploid cells, which have one genome copy, yeast can reproduce through mitosis, generating identical copies of themselves that are more or less isogenic. Under nutrient-rich conditions, two haploid cells can mate to form a diploid, which will have two genome copies. This diploid can divide via mitosis, but they can also perform meiosis and sporulation, forming four distinct haploid spores which inherit new combinations of genes

sequences, and therefore traits, as compared to the parental genomes. The inheritance of alleles amongst the spores is typically Mendelian (2:2) but depending on dominant/recessive state of the phenotypes, the traits may be seen in all, half, or none of the spores. This process of meiotic inheritance generates genetic diversity and is fundamental to evolution.

Beyond genetic factors, our understanding of epigenetic inheritance has expanded greatly in the last 20 years. Chromatin modifications, and their resulting effects on transcriptional regulation, have been of particular interest, since they affect gene expression without altering the underlying genomic code. DNA is wrapped around octamers of histone proteins, which form nucleosomes. In yeast – a common model for chromatin research – this includes two molecules each of H2A, H2B, H3, and H4²¹. Each of these histone proteins have N- and C-terminus tails that extend out from the histone core and can be post-transcriptionally modified with multiple modifications, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and more. These modifications affect whether the chromatin is open and accessible (euchromatin), or closed and inaccessible (heterochromatin), which influences transcription, and dynamically controls the overall structure of the genome. Importantly, this open or closed state can be inherited across generations, demonstrating a method of information transfer independent of DNA sequence. Although several epigenetic processes such as DNA methylation, repressive histone methylation, and siRNAs are not present in yeast²², the other alterations to chromatin that are present can strongly affect traits and overall fitness, including in response to stress.

Traits can also be linked to the inheritance of organelles, specifically mitochondria. Mitochondria are important organelles in the cell for their production of ATP, amino acid and lipid metabolism, and several other metabolic pathways^{23,24}. Mitochondria have their own genome (mt-DNA), which encodes 8 proteins in budding yeast²⁵. It also contains a complete set

of mitochondrial tRNA genes and two mitochondrial rRNA genes. Thousands of other mitochondrial proteins are encoded in the host genome, synthesized in the cytosol, and transported into mitochondria. Mitochondria self-replicate and therefore must be inherited from their mother cell directly during cell division²⁶. This inheritance is temporally coordinated, since mitochondria must be shuttled to the bud site for the budding cell, while some are held back for the mother cell, and therefore, many of these traits are linked to the cytoskeleton and cell cycle checkpoints. Since they have their own small genome, mitochondria can act as a source of genetic material driving mitochondrial-linked traits, separate and independent from meiotic inheritance that is driven by the nuclear genome. Mitochondria are present in the cytoplasm and are therefore another cytoplasmic source of information that can be inherited.

Another rare yet fascinating example of molecular memory is a phenomenon called mnemons. These molecules are altered protein conformations, induced by a specific cell history²⁷. They act as molecular memories for a cell to induce altered decision-making upon re-encountering a specific stress condition. An example of this phenomenon is the cell cycle G1/S inhibitor mRNA-binding protein Whi3²⁸. Whi3 is typically bound to *CLN3* mRNA, translationally inhibiting it and promoting cell cycle arrest. Upon deceptive mating arrest – a scenario where mating cells do not find each other within a reasonable timeframe – it forms super-assemblies via its prion-like domains (PrD), releasing *CLN3* and liberating the cell from cell cycle arrest. Mnemons have much in common with prions in yeast, yet their inability to pass down this information to daughter cells distinguishes them. They remain an interesting example of how spatial-temporal control of an altered protein conformation in response to stress may allow cells to navigate their environments and achieve improved fitness outcomes.

A final important example of an epigenetic mechanism of inheritance are prions. Prions are proteins that can adopt an altered conformation that is heritably transmitted to other copies of protein, and therefore heritable across cell divisions. They are a unique biological phenomenon where protein alone can carry information, resulting in altered traits and phenotypes, upending our traditional view of the central dogma. The first yeast prion was first described in the 1960's²⁹, although the concept was only first understood to be protein-based when scrapie was identified by Stanley Prusiner in the 1980's. In budding yeast, prions allow cells to inherit traits and resistance to stress through diverse phenotypes. The first yeast prion discovered was called $[PSI^+]$, which is dependent on a protein involved in translation termination²⁹. $[PSI^+]$ causes read-through of stop codons by ribosomes, reducing translation fidelity. While some in the field view this prion as a disease of yeast³⁰, other research suggests it may provide a mechanism for genetic variation and phenotypic diversity³¹. Another prion, $[MOD^+]$, provides cells with resistance against antifungal agents and can be induced by the prolonged exposure of yeast cells to antifungal agents³². $[GAR^+]$ is a yeast prion that is resistant to glucose-associated repression of alternative carbon sources, giving its cells an advantage in fluctuating environments³³. These examples suggest that yeast prions can provide an epigenetic mechanism to confer beneficial traits, in great contrast with mammalian prion examples. Importantly, there is evidence suggesting that this prion phenomenon is conserved and present in wild yeast³⁴, demonstrating that this phenomenon is non-trivial. Many more examples exist in budding yeast, yet prions are also present in other species, including mammals, bacteria, viruses, and plants^{35–38}. The biochemical features that are inherent to different prions are discussed in more detail in Chapter II, a review published in 2022.

[*BIG*⁺]:

In 2016, a screen was performed that identified ~50 new putative prions, with behavior consistent with the definition of protein-based inheritance³⁹. Many of these prions would not have been discovered by previous methods^{40,41}, which looked for a propensity to form amyloid aggregates, low-complexity sequence bias, and Hsp104 chaperone dependence. These researchers attempted a different approach: transient overexpression of ~5300 open-reading frames. They found stable phenotypic traits many generations later, long after protein overexpression ceased and suggested that an altered protein conformation – a prion – could be responsible for many of these. Notably, hits from this screen were set apart from previous prion examples. They lacked amyloid aggregates and enrichment for glutamine and asparagine, characteristics considered hallmarks of well-characterized prions. Rather, the hits were enriched for intrinsically disordered regions. Many of these proteins were transcription factors or RNA-modifying enzymes, including the pseudouridine synthase Pus4. At the time, only a handful of prions had been identified and described in any detail, marking this a large leap forward for the field, and the first screen based on the inheritance of phenotypes as opposed to the more biochemical definition of prions: amyloid-formation.

In 2021, a follow-up study was published that better characterized Pus4 and its ability to form a prion, named [*BIG*⁺] for better-in-growth⁴². The study measured the patterns of inheritance of its phenotypes, and tested non-mendelian inheritance, cytoductions, and chaperone curing to induce loss of the prion state. It found that [*BIG*⁺] was inherited in a non-Mendelian fashion, dependent only on transmission through the cytoplasm, and the prion could only be cured by depletion of Hsp70, not Hsp104. The study also sought to better describe the phenotypes induced by [*BIG*⁺]. They found that the [*BIG*⁺] state enhanced cell proliferation, cell

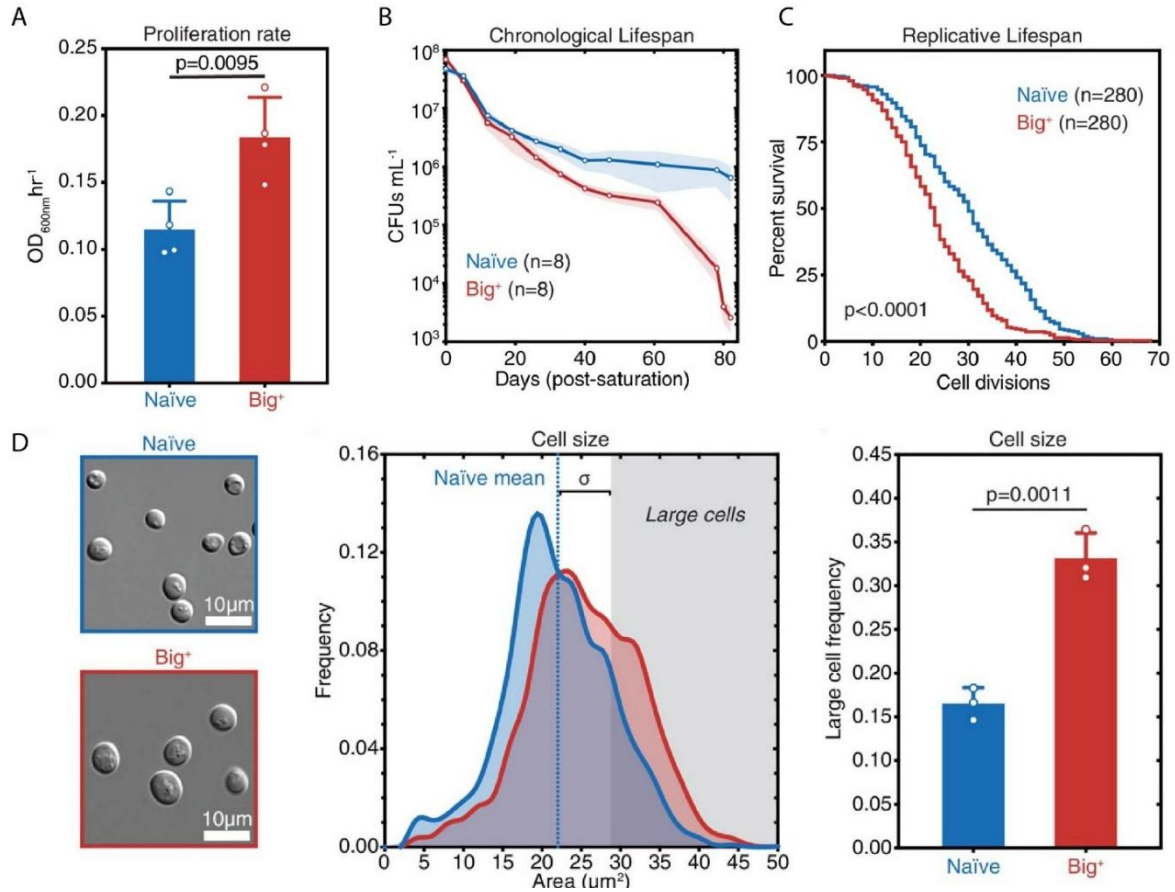


Figure 1.7: $[BIG^+]$ cells grow faster, are larger, and have reduced lifespans. **A)** $[BIG^+]$ cells proliferate faster than naïve cells. **B)** $[BIG^+]$ cells have reduced chronological lifespan compared to naïve cells. **C)** $[BIG^+]$ have reduced replicative lifespan compared to naïve cells. **D)** $[BIG^+]$ cells are on average larger than naïve cells during saturated phase. The micrograph shows a visual example, while the large cell frequency charts show the cell size distributions and quantification. Figure modified from Garcia & Campbell *et al.*, 2021.

size during saturation, altered translation, with the large trade-off that these cells have shorter lifespans (**Figure 1.7**). A closer look at its effects on protein synthesis revealed that $[BIG^+]$ cells were resistant to translation inhibitors, had increased polysomes, and an increased rate of protein synthesis by $[^{35}\text{S}]$ -Methionine pulse labeling (**Figure 1.8**). Interestingly, initial investigation indicated that $[BIG^+]$ maintained its catalytic ability on a few tRNAs and the mRNA *TEF1/2*, setting it apart from other classical prion examples. Overall, this study established Pus4 as the

causative basis of the $[BIG^+]$ growth state and suggested that this epigenetic state may give an adaptive advantage over mutation, in that it is more easily induced or reversed in response to environmental cues.

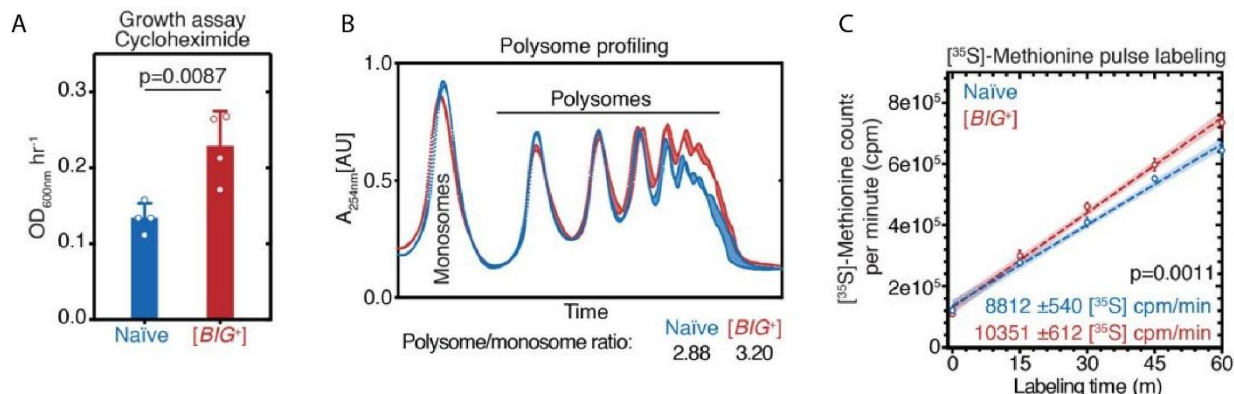


Figure 1.8: $[BIG^+]$ has altered protein synthesis. **A)** $[BIG^+]$ cells show resistance to the translation elongation inhibitor cycloheximide. **B)** $[BIG^+]$ cells have increased polysomes compared to naïve cells, measured by polysome profiling. **C)** $[BIG^+]$ cells have an increased rate of translation, as measured using $[^{35}S]$ -Methionine pulse labeling. Figure modified from Garcia & Campbell *et al.*, 2021.

This study stopped short of investigating the biophysical state of Pus4 in the $[BIG^+]$ state. Some evidence, such as its dependence on Hsp70 chaperone, the retention of its catalytic function, and GFP microscopy images, suggested that the protein state might fall into a new class of prions: non-amyloid forming. The gap that remained was an understanding of the molecular mechanism that drove this epigenetic state that caused such significant cellular phenotypes.

Bridge to Chapter II:

Chapter I provides the necessary background to contextualize the remaining chapters. I described the conventional functions of Pus4, including its catalysis of pseudouridine and its effects on RNA structure/stability and translation. I also summarized the mechanisms of

inheritance in yeast, highlighting the field of prions, with more detail surrounding the prion [*BIG*⁺]. Chapter II dives deeper into exploring canonical activities of Pus4. It investigates the interplay of the enzymatic and potential remodeling functions of Pus4, separate from its prion activities. This investigation was done in collaboration with Dr. Julia Widom's lab at the University of Oregon.

CHAPTER II

CHRONOLOGY OF TRNA STRUCTURAL DYNAMICS PRIOR TO AND DURING INTERACTION WITH A PSEUDOURIDINE SYNTHASE

Author Contributions:

This work was the result of a collaboration between Dr. Julia Widom's lab, including Nico Conoan Nieves and Madison Kadrmas, and Dr. David Garcia's lab, including Emily Dennis, Abigail Vaaler, and Margaret Barry at the University of Oregon. Emily Dennis and Nico Conoan Nieves are co-first authors. Julia Widom and David Garcia are co-corresponding authors. My contributions included establishing a recombinant purification method to generate purified Pus4 protein, performing BLI and *in vitro* modification assays, initial manuscript drafting and editing, and overall project management in combination with Julia Widom and David Garcia. This work is available on *bioRxiv* and in revision at *RNA Journal*.

Abstract:

Transfer RNA has long served as an exemplar of a thermodynamically stable, structured RNA. Yet it undergoes significant structural changes upon binding and catalysis by diverse modification enzymes. We leveraged optical binding assays and single-molecule FRET to observe the structural dynamics of two yeast tRNAs, in isolation, and upon interaction with the conserved pseudouridine synthase Pus4/TruB. We show that unmodified and pseudouridylated tRNA^{eMet(CAU)} and tRNA^{Thr(AGU)} all sample open, compact and intermediate conformations, though tRNA^{Thr(AGU)} exhibits faster dynamics. Consistent with its role in modifying RNAs with different structural properties, Pus4 binds robustly to unmodified tRNA, tRNA that was pseudouridylated prior to engaging Pus4 (“pre-modified”), and even an unrelated riboswitch RNA. Pus4 binding to tRNA^{eMet(CAU)} leads to additional tRNA conformational states. The ensemble of conformations explored by Pus4-bound pre-modified tRNA resolved within minutes back into open, compact and intermediate states. tRNA^{eMet(CAU)} that is initially unmodified more gradually approached the ensemble of structures that were attained rapidly by pre-modified tRNA. Thus, Pus4 both catalyzes a lasting chemical change on tRNA and remodels it over time after catalysis, perhaps to promote subsequent steps of tRNA maturation.

Introduction:

Pseudouridine (Ψ) is the most abundant RNA chemical modification in nature, and transfer RNAs (tRNAs) represent the most common pseudouridylated substrate. tRNAs are formed from four structural motifs that fold onto each other in three dimensions. The acceptor stem at one end of a tRNA is sparsely modified and serves as the attachment point for an amino acid. The anticodon stem loop (ASL) at the opposite end is frequently modified at multiple positions and is involved in mRNA decoding. The heavily modified D-loop and T-loop interact to form the “elbow” where they impart flexibility to the molecule to promote multiple steps of tRNA biogenesis and protein synthesis, including translocation through the ribosome. While many sites of pseudouridylation are found in tRNA, position 55 in the T-loop is pseudouridylated in nearly all tRNAs and is most commonly catalyzed by a pseudouridine synthase from the “TruB” family⁴³. Bacteria contain a single enzyme, TruB, responsible for all tRNA U₅₅ catalysis. Yeast also contain a single enzyme, Pus4, which is found in the nucleus, cytoplasm, and mitochondria. Humans encode two paralogous enzymes, TRUB1 and TRUB2, found primarily in the nucleus and mitochondria, respectively. In humans, a third enzyme belonging to a different synthase family, Pus10, is located in the cytoplasm and also modifies many tRNAs at U₅₅^{44,45}. This change from U₅₅ to Ψ_{55} creates an additional hydrogen bond donor, increasing base-stacking and helix rigidity, while promoting tertiary interactions between the T-arm and D-arm in the “elbow” region of a folded tRNA. Thus Ψ_{55} contributes to the characteristic three-dimensional structure of tRNA^{46–50}. In the budding yeast *Saccharomyces cerevisiae*, Pus4 pseudouridylates 41 out of 42 cytosolic tRNA isoacceptors⁵, and 23 out of 24 mitochondrial tRNA isoacceptors¹¹. In *E. coli*, all tRNAs are pseudouridylated by TruB⁵¹. Therefore, the majority of tRNA molecules in these species are bound and modified by

Pus4/TruB, making this enzyme a useful model for understanding the interplay between tRNA structure and dynamics and an enzyme that chemically modifies it.

Previous studies have identified various RNA sequences and/or structures important for binding and modification by various pseudouridine synthases^{13,52–67}, including Pus4/TruB^{44,68,69}. Yet binding by these enzymes can remodel RNAs in ways distinct from the lasting chemical change that they catalyze. An early study on bacterial TruB noted that growth phenotypes associated with its absence could not be explained by its catalytic activity alone⁷⁰. Subsequently, a second role was more clearly established: that it aids tRNA folding and functions as a tRNA chaperone¹⁵. Other studies have also demonstrated RNA-chaperone-like roles for other tRNA modifying enzymes^{71–74}. Thus, to exert their full function, tRNA modifying enzymes should, 1) bind tRNA, 2) catalyze tRNA chemical modification, and, in some cases, 3) remodel tRNA structure. These three components could each independently affect the dynamics of tRNA structure, or do so interdependently. We sought to clarify these activities by addressing the following questions: 1) Do structural ensembles of unmodified tRNA and pseudouridylated tRNA differ? 2) How does binding by Pus4 affect the distribution of tRNA structures over time? 3) Does the outcome depend on whether the tRNA is pseudouridylated prior to engagement with Pus4?

To address these questions, we leveraged single-molecule Förster resonance energy transfer (smFRET) to track the conformational fluctuations of tRNA and biolayer interferometry (BLI) to monitor association of Pus4 to tRNA (**Figure 2.1**). We show that Pus4 binds rapidly to both unmodified (U₅₅) and pseudouridylated (Ψ₅₅, hereafter also referred to as “pre-modified”) tRNAs, and subsequently navigates unmodified tRNA through a pathway of catalysis and remodeling lasting tens of minutes, that is partially bypassed by pre-modified tRNA. This

pathway involves a temporary population of conformations not prevalent for either U₅₅ or Ψ₅₅ tRNA in the absence of enzyme. Thus our results show that a prototypical RNA-modifying enzyme shapes the chronology of tRNA structural dynamics through both its catalysis and remodeling.

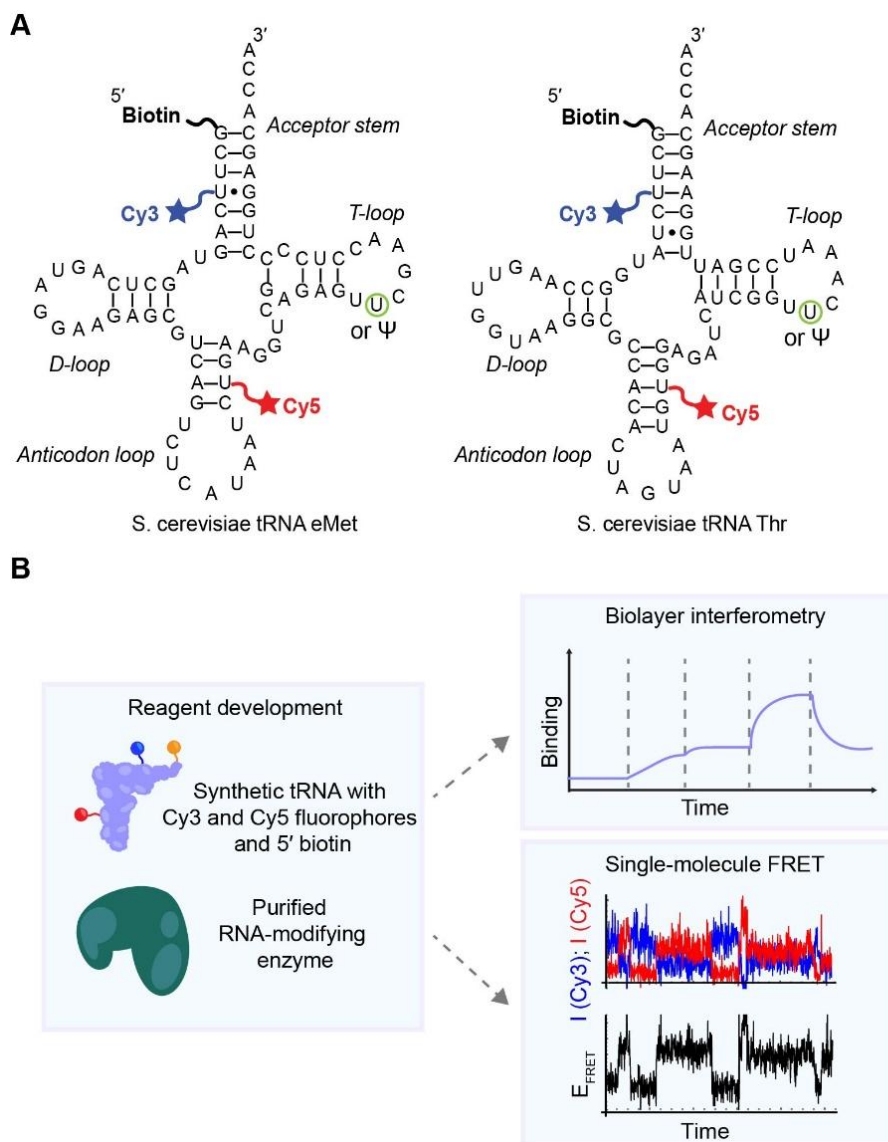


FIGURE 2.1. tRNA sequences and methodological approach to investigate how an RNA-modifying enzyme influences tRNA conformation. (A) Predicted secondary structures of the tRNA sequences used in this study, including the positions of biotin, Cy3, Cy5 and pseudouridine modification. The four structural motifs are labeled on each tRNA in italics (Acceptor Stem, T-loop, Anticodon loop, and D-loop). (B) Summary of experimental approach and workflow.

Results:

Recombinant Pus4 Pseudouridylates Yeast tRNAs *In Vitro*

We first established a protocol to express and purify *S. cerevisiae* Pus4 protein from *E. coli* cells (**Supplemental Figure S2.1A**). While we attempted to prepare untagged Pus4 protein, it showed a strong propensity to aggregate after tag cleavage, so we proceeded with a GST-tagged full-length version instead, which remained soluble throughout purification. To verify the biochemical activity of the purified protein, we tested its catalysis *in vitro* (**Figure 2.2**). Using direct RNA sequencing (DRS), a method we previously established to investigate tRNA modification *in vivo*⁵, Ψ₅₅ was newly detected on nearly all tRNAs isolated from a *pus4Δ* yeast strain following a two-minute incubation with an excess of purified Pus4. Modification levels were qualitatively comparable to those previously observed in tRNA isolated from WT strains⁵. While some differences in pseudouridine levels were observed between the *in vivo* and *in vitro* data—most notably a lack of modification of tRNAs Arg^{ACG} and Leu^{CAA} *in vitro*—the data supported near-full catalytic activity of purified Pus4 on the vast majority of these cell-derived yeast tRNAs. Robustly modified tRNAs included the two employed in this study (see below), tRNA^{eMet(CAU)} (the elongator methionine tRNA, as opposed to the initiator methionine tRNA, tRNA^{iMet(CAU)}) and tRNA^{Thr(AGU)}, hereafter called tRNA eMet and tRNA Thr, respectively.

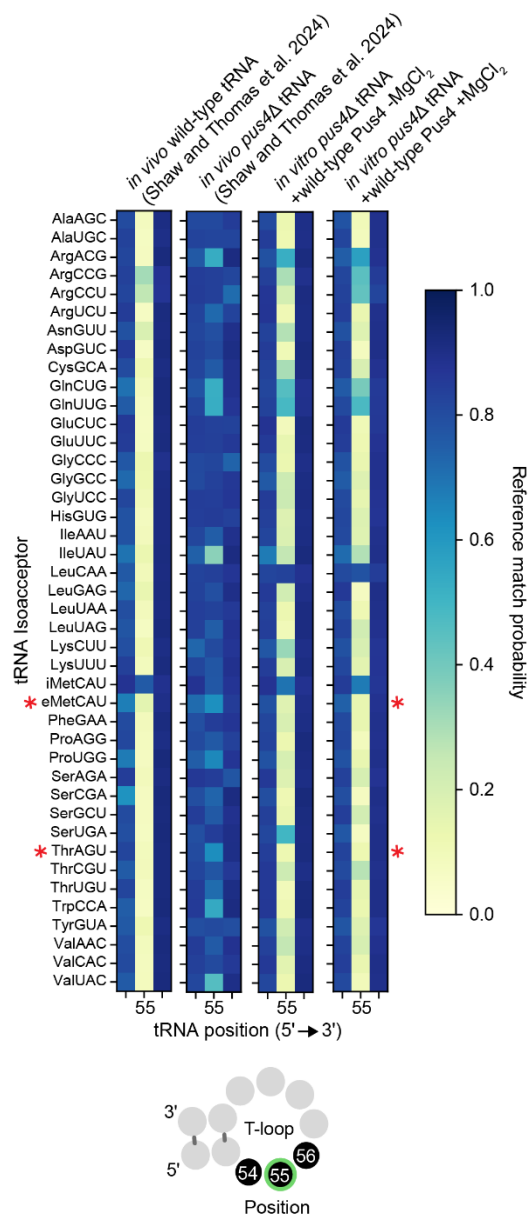


FIGURE 2.2. Nanopore direct RNA sequencing shows *in vitro* modification activity of purified Pus4. Heatmaps represent the sequenced nucleotide positions 54–56 of the 42 yeast cytosolic tRNA isoacceptors for tRNA harvested from *pus4*Δ cells and reacted *in vitro* with purified Pus4, with or without 4 mM MgCl₂. Results from *in vivo* tRNA sequencing are from Shaw *et al.* 2024. Reference match probability corresponds to the probability that a base-called nucleotide agrees with the reference unmodified nucleotide (A, G, C, or U). More frequently miscalled positions, such as pseudouridine at position 55, produce light yellow squares. When position 55 is base-called as a uridine, the square is dark blue. Red stars indicate the cellular tRNAs corresponding to synthetic tRNAs investigated in this study. At bottom is a diagram of a generic yeast tRNA T-loop with black circles denoting bases shown in heatmaps. Note that “iMet” refers to the initiator tRNA that decodes the start codon, and is different from “eMet” which is the tRNA that decodes methionine codons after the start codon.

Synthetic Modified tRNAs Enable Binding and smFRET Studies

Having established recombinant Pus4 with desired enzymatic activities on the majority of yeast isoacceptors *in vitro*, we chose two as models for our biophysical studies of RNA binding by Pus4 (using BLI) and tRNA conformational fluctuations (using smFRET). The yeast tRNA eMet and tRNA Thr were chosen for several reasons: 1) both contain uridines at positions 4 and 41, enabling us to use fluorophore-labeling sites and chemistry matching those of several prior studies that performed FRET analysis of tRNA structure^{75–78}, 2) both have a minimum free energy structure, as predicted by RNAFold^{79,80}, that closely resembles the tRNA cloverleaf secondary structure, theoretically reducing formation of non-canonical, off-pathway structures *in vitro*, and 3) prior evidence demonstrates that both tRNAs are pseudouridylated by Pus4 efficiently *in vivo*⁵, in addition to our work herein that demonstrated their efficient pseudouridylation by Pus4 *in vitro* (**Figure 2.2**). The tRNAs were labeled with the FRET donor Cy3 at the carbon-5 position of U₄ in the acceptor stem and with the FRET acceptor Cy5 at the carbon-5 position of U₄₁ in the anticodon stem. They were additionally biotinylated at the 5' end to enable immobilization of the molecules on a microscope slide, or probe, for smFRET and BLI, respectively. We prepared two versions each of tRNA eMet and tRNA Thr, containing either U or Ψ at position 55.

In previous work of ours and others, it has been observed that addition of Ψ_{55} can promote the addition of other T-loop modifications, specifically m⁵U₅₄ and m¹A₅₈⁸¹. We previously determined which yeast isoacceptors showed an influence of Ψ_{55} on promoting m¹A₅₈ *in vivo*⁵. While both tRNA eMet and tRNA Thr used here do exhibit this pattern *in vivo*, and thus would have also represented interesting models for investigating how multiple T-loop modifications influence tRNA conformation, technical considerations precluded our ability to

include m¹A₅₈ in our synthetic sequences. The m¹A modification undergoes spontaneous conversion to m⁶A via the Dimroth rearrangement under a number of different experimental conditions⁸², including polyacrylamide gels that we used here for RNA purification, which was previously documented in mass spectrometry data⁵.

Pus4 Binds Rapidly to tRNA

We utilized BLI to assess the binding specificity of Pus4 and the timescales of its association to and dissociation from both substrate and non-substrate RNAs. RNAs were immobilized on a streptavidin-coated probe that was then passivated to prevent non-specific adsorption of protein, then exposed to buffer containing Pus4 protein (the “association phase”), and finally exposed to buffer lacking protein (the “dissociation phase”) (**Figure 2.3A; Supplemental Figure S2.2A**). A BLI “shift” is observed when material accumulates on the probe, such as protein binding to immobilized tRNA during the association phase. At a concentration of 200 nM protein, the association curves show rapid binding of Pus4 to tRNA eMet and tRNA Thr, with the accumulation rate slowing after ~100 seconds (**Figure 2.3B,C; Supplemental Figure S2.2B,C**; replicate data in **Supplemental Figure S2.3**). While accumulation plateaued after ~15 minutes when Pus4 was present at our highest tested concentration (800 nM), it failed to plateau even after 75 minutes for Pus4 at 50 or 200 nM concentrations (**Figure 2.4**). The BLI shift at 200 nM Pus4 eventually converged with the data for 800 nM, suggesting that complete binding occupancy was achieved in this concentration range. We confirmed this using non-denaturing gel electrophoresis as an orthogonal tRNA binding assay, which showed that in free solution, *all* tRNA eMet molecules were bound after 30-minute incubation with Pus4 at concentrations of 200 nM or higher (**Supplemental Figure**

S2.4). Combined with the prior observation that both tRNA eMet and tRNA Thr were robustly modified within two minutes during the *in vitro* modification experiment (in which Pus4 was present at 1 μ M) (**Figure 2.2**), we presumed that the majority of the immobilized tRNAs were pseudouridylated in the early period of this association phase once engaged with Pus4. The *in vitro* modification experiment and BLI experiments were performed in the same buffer (with the exception of Tween-20 used to suppress nonspecific binding in BLI), at the same temperature, and using the same molar ratio of protein to tRNA (3.33), lending support to this assumption.

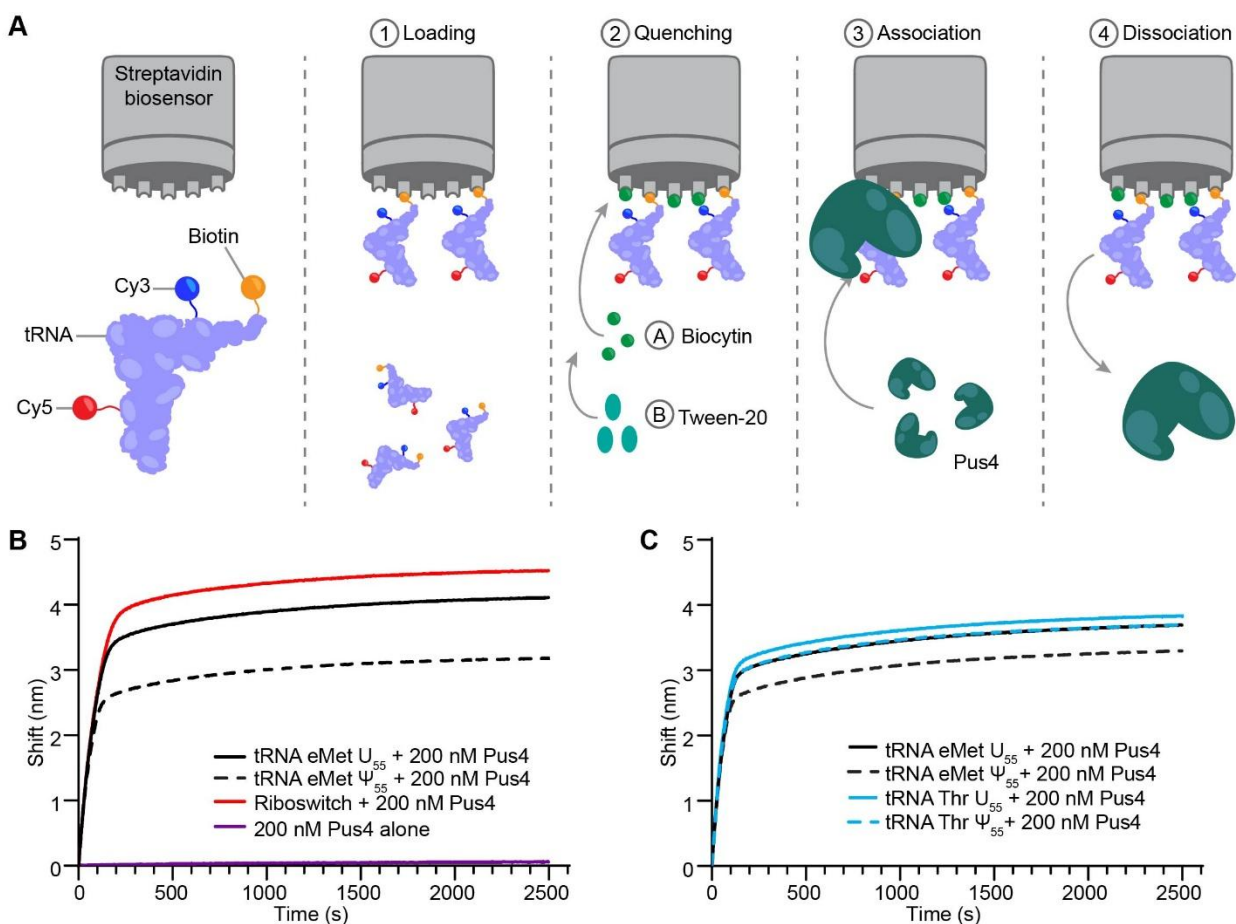


FIGURE 2.3. Pus4 binding to tRNA measured using biolayer interferometry. (A) Summary of BLI experimental design. Biotinylated and Cy3- and Cy5-labeled tRNAs are immobilized onto streptavidin-coated probes (1) that then undergo two quenching steps and baselining (2). The

probe is then transferred into buffer with purified Pus4, where association of Pus4 with immobilized tRNAs occurs (3). The probe is then transferred to a buffer without protein, permitting dissociation (4). (B) BLI traces showing the association phase of 200 nM Pus4 to U₅₅ (black solid) and Ψ₅₅ (black dashed) tRNA eMet, to an unrelated RNA (riboswitch; red), and to a passivated probe lacking RNA (purple). Traces are aligned to start at a shift of 0 nm. (C), Same as B, but with U₅₅ (black solid) and Ψ₅₅ (black dashed) tRNA eMet and U₅₅ (blue solid) and Ψ₅₅ (blue dashed) tRNA Thr. Complete traces in **Supplemental Figure S2.2**.

We analyzed the association of Pus4 to tRNA quantitatively by modeling the initial binding of Pus4 as a reversible process occurring under pseudo-first order conditions, which predicts that Pus4 should accumulate on the RNA with a profile described by an exponential (details in **Methods**). In order to obtain accurate k_{on} values, we devised and compared multiple fitting approaches to isolate the kinetics of initial binding from those of the continued slow accumulation observed by BLI and the catalysis observed by DRS (**Supplemental Figure S2.5**). In one approach, we isolated this phase of association by performing mono-exponential fits (as predicted from pseudo-first order kinetics) to the time period over which the first ~75% of the overall BLI shift occurs. In the second approach, we modeled both the initial binding and continued slow accumulation by fitting the first 1,000 seconds of the association phase with a bi-exponential function, and took the faster component to represent initial binding to the pre-existing distribution of tRNA structures. Both approaches revealed that the on-rate (k_{on}) was slightly faster for tRNA Thr ($1.36 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ from the bi-exponential fit) than for tRNA eMet ($1.00 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) (**Figure 2.4D,E**). These on-rates are comparable to ones previously observed for yeast Pus1 binding to tRNA^{Phe} and tRNA^{Val} ($4\text{--}6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$)⁸³. These fits could in principle have been used to obtain k_{off} , however the off-rate during the initial phase of Pus4 accumulation was indistinguishable from zero. When the BLI probe was transferred into a solution lacking protein, an initial fast phase of dissociation was observed, but even after 75 minutes, less than 10% of the shift observed in the association phase was reversed (**Supplemental Figure**

S2.2B,C). This suggests that Pus4 binds all RNAs investigated with high affinity and dissociates very slowly.

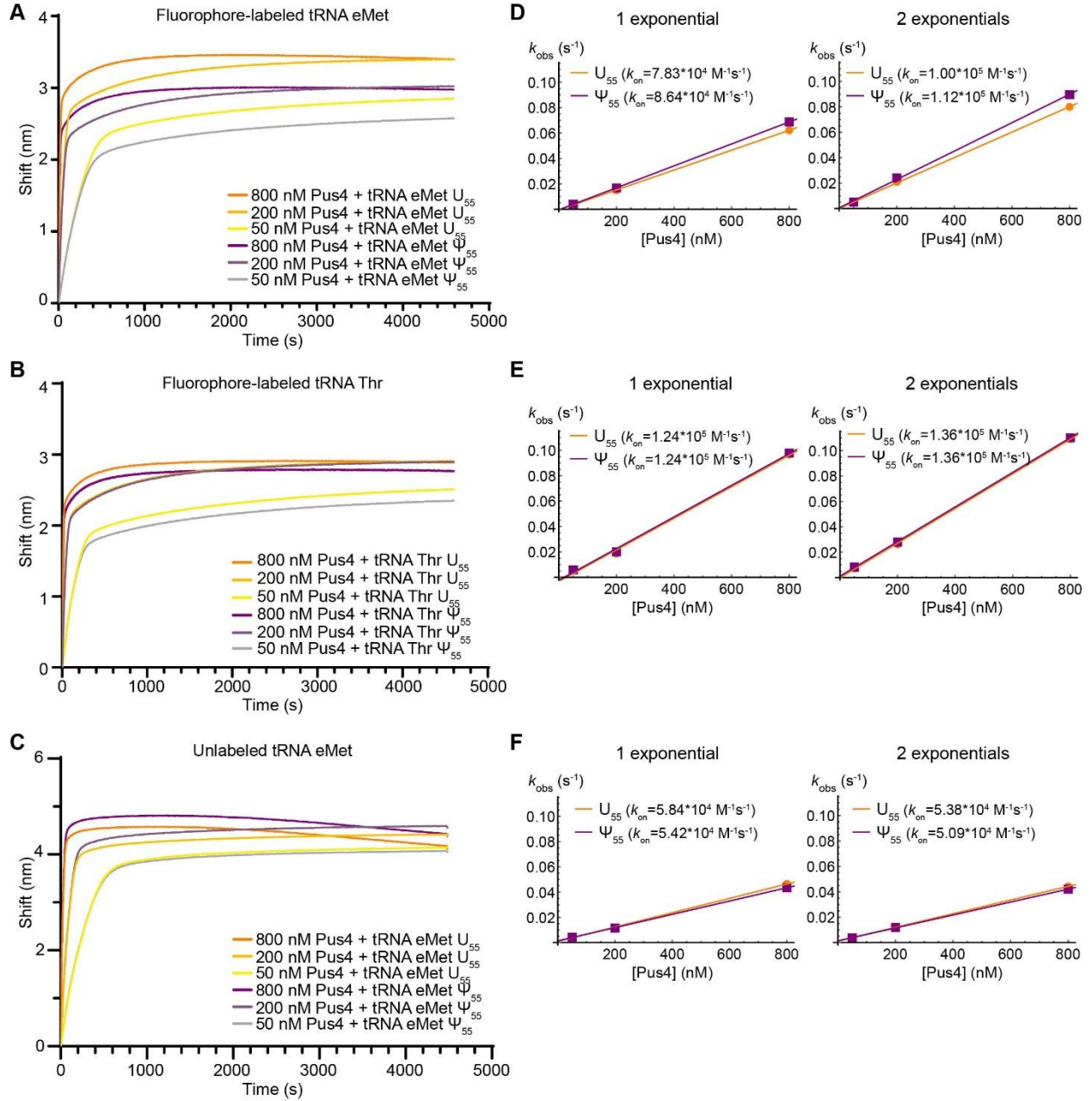


FIGURE 2.4. Pus4 binds tRNAs with a high k_{on} , but a low k_{off} , with similar affinities for unmodified and pre-modified tRNAs. (A,B,C) BLI traces showing the association phase of 50 nM, 200 nM, and 800 nM Pus4 to various tRNAs. Traces are aligned to start at a shift of 0 nm. (A) Fluorophore-labeled U₅₅ (orange) and Ψ₅₅ (purple) tRNA eMet. (B) Fluorophore-labeled U₅₅

(orange) and Ψ_{55} (purple) tRNA Thr. (C) Unlabeled U₅₅ (orange) and Ψ_{55} (purple) tRNA eMet. (D,E,F), Linear fits to the observed association rate at three Pus4 concentrations. The slope of the fit, representing k_{on} , is indicated for each plot. Left columns: k_{obs} determined from a single exponential fit to the time range of the association phase during which ~75% of the BLI shift occurs. Right columns: k_{obs} determined from a double exponential fit to the first 1000 s of the association phase for unlabeled tRNA eMet and the entire association phase for the others. (D) Fluorophore-labeled U₅₅ (orange) and Ψ_{55} (purple) tRNA eMet. (E) Fluorophore-labeled U₅₅ (orange) and Ψ_{55} (purple) tRNA Thr. (F) Unlabeled U₅₅ (orange) and Ψ_{55} (purple) tRNA eMet.

To test whether the fluorophores altered Pus4 binding affinity to tRNAs, we prepared a synthetic tRNA eMet with the same sequence as above, but without Cy3 and Cy5 fluorophores (**Figure 2.4C; Supplemental Figure S2.6**). The BLI curves showed the same overall shape with a somewhat lower k_{on} value ($5.84 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) than observed for labeled tRNA eMet, an impact comparable in size to the variation between different tRNAs (labeled tRNA eMet vs. labeled tRNA Thr). We conclude that while the labels exert a small effect on Pus4 binding, in general our strategy for investigating the conformational ensemble of tRNA using smFRET is not confounded by the effects of these fluorophores on the binding behavior of Pus4.

After the initial binding phase that was used for kinetic analysis, we noted other features of Pus4 binding behavior. In the highest concentration tested (800 nM protein), we observed that after binding plateaued, a mild dissociation occurred slowly over the remainder of the measurement (**Figure 2.4**). The effect was more distinct in the unlabeled tRNAs in comparison to the fluorophore-labeled, but was present in all tRNAs. This appeared to be a distinct phase from the binding phase; thus we did not incorporate it into our kinetic analysis. Secondly, the slow, steadily increasing second phase of the association curve observed at all concentrations of Pus4 tested may represent 1) gradual “trapping” of tRNA or Pus4 molecules that began the association phase in conformations not amenable to binding, or 2) Pus4 multimerization such that the stoichiometry of Pus4:tRNA exceeded 1:1 on some of the immobilized tRNA molecules.

Multimerization is supported by the shift of Pus4:tRNA bands to the wells for the highest Pus4 concentrations (**Supplemental Figure S2.4**), and might be facilitated by the GST tag that is known to dimerize⁸⁴. To measure the extent to which Pus4 multimerization could impact tRNA binding, we incubated total tRNA isolated from yeast cells with recombinant Pus4, and performed sucrose gradient ultracentrifugation to separate protein:RNA complexes (**Supplemental Figure S2.7**). If multimerization of Pus4 under these conditions reduced tRNA binding, we would expect no evident shift in the distribution of protein complexes upon addition of tRNA. However, addition of tRNA caused a global shift of Pus4 to fractions in the gradient containing a higher percentage of sucrose, forming complexes with an apparent molecular weight of approximately 200 kDa (implying complexes of two GST-Pus4 with two tRNAs). Additionally, our smFRET measurements (described below) were performed on sparsely surface-immobilized tRNAs, in which the average distance between tRNA molecules greatly exceeds the size of a protein dimer or even multimer. This would preclude the formation of complexes containing more than one tRNA molecule. In summary, while we can detect Pus4 multimers in our experimental conditions, we do not believe that they represent the dominant form of Pus4 that is interacting with immobilized tRNAs that are the principle substrate that we measured, and moreover our Pus4 samples demonstrated robust catalysis (**Figure 2.2**).

Pus4 Also Binds Robustly to Pre-Modified tRNA and Non-Substrate RNA

To test whether Ψ_{55} altered the binding of Pus4 to tRNA, we tested synthetic tRNAs that had pseudouridine present at position 55 using BLI. Although the BLI curve of Ψ_{55} tRNA eMet exhibited a smaller maximum shift than that of U₅₅ (**Figure 2.4A**), non-denaturing gel electrophoresis indicated that this still corresponded to complete binding occupancy at Pus4

concentrations at or above 200 nM (**Supplemental Figure S2.4**). Furthermore, the overall association and dissociation profile was similar between the unmodified and pre-modified variants of both tRNAs, and k_{on} values were unchanged or slightly higher with Ψ_{55} tRNA ($1.12 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for Ψ_{55} tRNA eMet and $1.36 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for Ψ_{55} tRNA Thr), indicating weak discrimination between binding substrate and product. We also performed experiments with an RNA that is not a natural substrate of Pus4 but is similarly sized to tRNA: the bacterial class II NAD^+ riboswitch, a 61 nt RNA that adopts a pseudoknot structural motif to regulate translation initiation^{85,86}. As we observed for tRNA, Pus4 rapidly associated with the riboswitch, followed by slow continued accumulation and slow dissociation (**Figure 2.3B, Supplemental Figure S2.2B**). It is worth noting that previous studies have shown pseudouridine synthases can be promiscuous binders of RNA^{52,54–56}, consistent with their roles of modifying multiple sequences and structures in various classes and sizes of RNA. For example, Pus1 has been found to have broad affinity for RNA, even those lacking defining tRNA structural features, as well as tight binding to catalytic products (e.g., $K_D = 14 \text{ nM}$ for pseudouridylated tRNA^{Val})⁸³. In addition to tRNA, Pus4 modifies mRNA^{12,13,87} and mt-rRNA¹⁴, *in vivo*. Our data suggest that, like Pus1, Pus4 also exhibits promiscuous binding. This may promote Pus4's ability to facilitate RNA remodeling more generally, even if its primary catalytic substrate is tRNA.

tRNA Undergoes Dynamic Fluctuations Between Three Predominant Conformational States

We utilized smFRET to investigate how tRNA conformations are affected by Pus4 binding and pseudouridylation. The biotinylated- and fluorophore-labeled tRNA constructs used for BLI were immobilized on microscope slides, and fluorescence from single tRNA molecules was recorded using wide-field prism-based total internal reflection fluorescence (TIRF)

microscopy. We recorded short movies (~2 minutes) sequentially across different regions of the slide of tRNA alone, as well as tRNA during up to 30 minutes of incubation with 200 nM Pus4 protein. Cy3 and Cy5 fluorescence intensity traces were extracted from individual molecules, and data were combined to achieve ~200 traces for each condition. In order to observe the evolution of the conformational landscape over tens of minutes, the data were combined into discrete time bins of 2–6 minutes, 7–9 minutes, 10–15 minutes and 30–34 minutes after introduction of the imaging buffer, either with or without Pus4. FRET efficiency (E_{FRET}) histograms were compiled from the first 50 frames of all traces within each condition.

To accurately recover signals reflecting the distance between the donor and acceptor fluorophores, and therefore the structure of the tRNA, FRET traces were corrected for 1) crosstalk between detection channels, and 2) gamma (γ) factor, which quantifies the relative detection efficiencies and fluorescence quantum yields of Cy3 and Cy5 (**Supplemental Figure S2.8**). We determined the γ factor utilizing changes in donor and acceptor intensity upon acceptor photobleaching⁸⁸, which yielded a distribution with a median value of 2.4 in the absence of Pus4 and 3.0 in the presence of Pus4 (**Supplemental Figure S2.8C,D**). Given the significant adjustment to the raw data imposed by a γ factor so far from 1 (**Supplemental Figure S2.8A**), we also tested whether utilizing a molecule-by-molecule γ factor correction would improve the resolution of different FRET states. However, restricting the analysis to molecules for which individual γ values could be calculated (molecules in which the acceptor photobleached first) skewed the histogram without sharpening its features (**Supplemental Figure S2.8E-G**). We thus elected to utilize the median of the distribution (with or without Pus4 as appropriate) as a global γ correction factor, permitting inclusion of all traces.

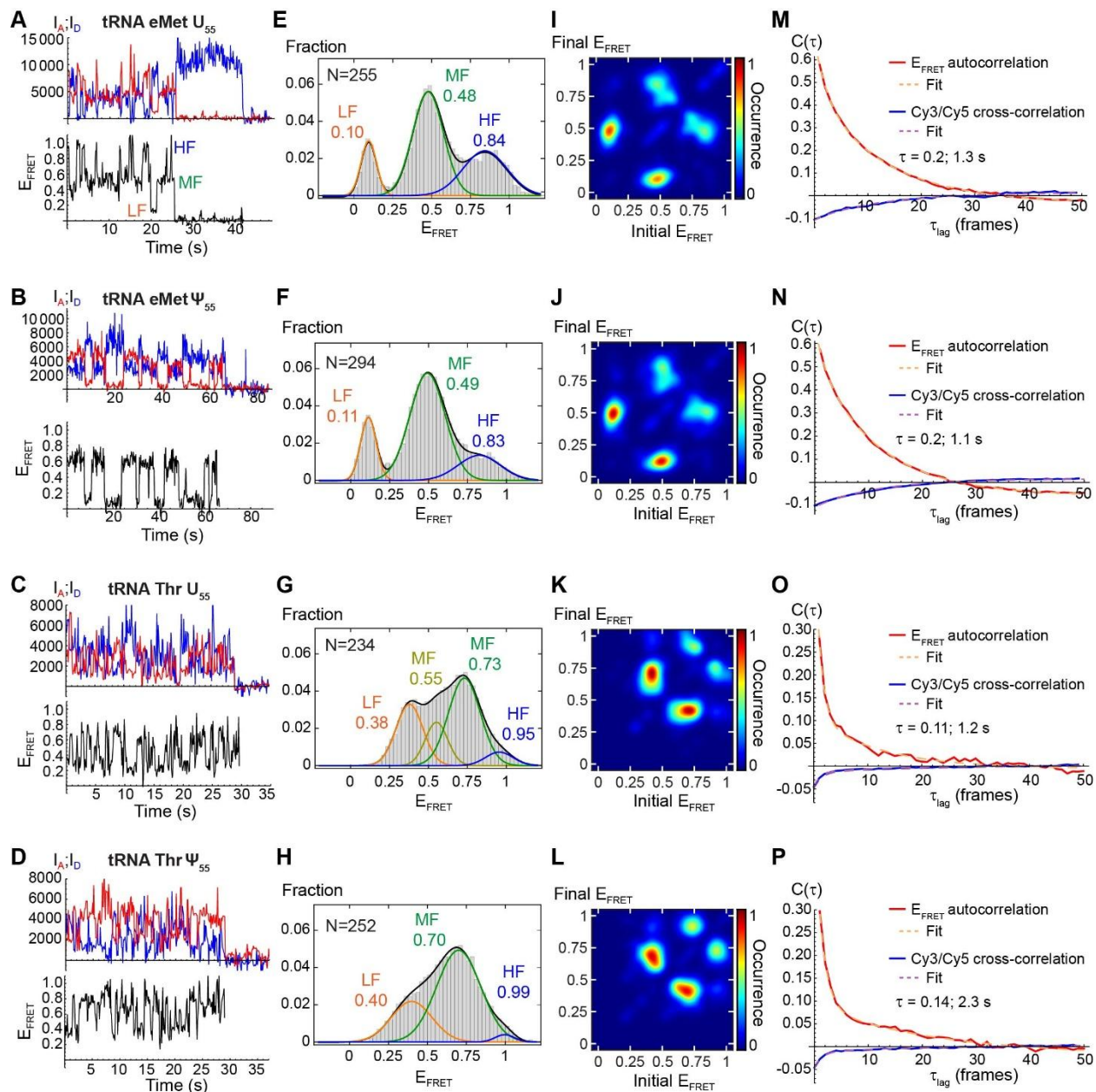


FIGURE 2.5. smFRET data on tRNAs in the absence of Pus4. (A–D) Representative smFRET traces of (A) U₅₅ tRNA eMet; (B) Ψ₅₅ tRNA eMet; (C) U₅₅ tRNA Thr; (D) Ψ₅₅ tRNA Thr. Top: Donor (blue) and acceptor (red) intensities. Bottom: FRET efficiency. Occurrences of the LF, MF and HF states are indicated in panel A. For clarity, the E_{FRET} traces are truncated after donor photobleaching. Only trace segments prior to photobleaching of either dye were subjected to further analysis. (E–H) E_{FRET} histograms compiled from the first 50 frames of the number of traces indicated by “N” for (E) U₅₅ tRNA eMet; (F) Ψ₅₅ tRNA eMet; (G) U₅₅ tRNA Thr; (H) Ψ₅₅ tRNA Thr. 3- or 4-Gaussian fits are overlaid with the peak centers labeled. (I–L) TODPs for (I) U₅₅ tRNA eMet; (J) Ψ₅₅ tRNA eMet; (K) U₅₅ tRNA Thr; (L) Ψ₅₅ tRNA Thr. The intensity of the heatmap at position (X,Y) quantifies the fraction of molecules that transition from E_{FRET} = X to E_{FRET} = Y at least once during the measurement. (M–P) FRET autocorrelation (red) and Cy3/Cy5 cross-correlation (blue) curves for the same four tRNAs.

cross-correlation (blue) functions for (*M*) U₅₅ tRNA eMet; (*N*) Ψ₅₅ tRNA eMet; (*O*) U₅₅ tRNA Thr; (*P*) Ψ₅₅ tRNA Thr. Global bi-exponential fits with the same time constants for autocorrelation and cross-correlation functions are overlaid in dashed lines, and the two time constants are indicated.

Inspection of the corrected traces revealed that in the absence of protein, all four tRNA variants investigated—U₅₅ and Ψ₅₅ tRNA eMet and U₅₅ and Ψ₅₅ tRNA Thr—dynamically sampled at least three distinct conformational states (**Figure 2.5A-D**). The shapes of the distributions and FRET efficiencies, however, differed between the eMet and Thr tRNA sequences. In the histograms of U₅₅ and Ψ₅₅ tRNA eMet, which were well-fit with three Gaussian distributions, a low-FRET efficiency feature (LF)—representing a conformation in which the fluorophores are most separated from each other relative to other states—is centered around $E_{\text{FRET}} = 0.1$. An LF feature could arise from molecules that are either less structured or that adopt a structure such as an extended helix that enforces separation of the fluorophores. Mid-FRET (MF) and high-FRET (HF) features—representing conformations in which the fluorophores are closer to each other relative to LF—were observed for both U₅₅ and Ψ₅₅ tRNA eMet and centered around $E_{\text{FRET}} = 0.48$ and 0.83 , respectively (**Figure 2.5E,F**). The HF state was moderately more populated in U₅₅ than Ψ₅₅ tRNA eMet. LF, MF and HF states were previously observed in smFRET analysis of the human mt-tRNA^{Lys} and were assigned to unfolded (U), extended hairpin (E), and cloverleaf-based-L-shape (C) conformations, respectively⁷⁶. The histograms of U₅₅ and Ψ₅₅ tRNA Thr exhibited broad, asymmetric distributions centered around $E_{\text{FRET}} = 0.6$. These were well-fit with either four (U₅₅) or three (Ψ₅₅) Gaussian distributions (**Figure 2.5G,H**) with centers ranging from $E_{\text{FRET}} = 0.38$ to 0.99 , consistent with the variety of states observed in the traces (**Figure 2.5C,D**).

We analyzed the dynamics of the four tRNAs by performing Hidden Markov Modeling (HMM)⁸⁹. For tRNA eMet, we used a 4-state model (**Supplemental Figure S2.9A**) that included the LF, MF, and HF states, in addition to a second HF state (HF2) centered at $E_{\text{FRET}} = 0.95$, which was observed in a fraction of molecules too small to yield a distinct feature in the histogram (5% in U₅₅ and 2% in Ψ_{55}). The model allowed for transitions between all pairs of the LF, MF and HF states, with the HF2 state occupied only statically, in accordance with the small number of traces that exhibited it. The isolated HF2 state was omitted for tRNA Thr based on inspection of traces, and the E_{FRET} values of the LF, MF and HF states were adjusted to match those obtained from 3-Gaussian fits to their histograms (**Supplemental Figure S2.9B**). The pathways of transitions among these states were visualized using transition occupancy density plots (TODPs), which show the prevalence of dynamic or static behavior across hundreds of molecules within each condition (**Figure 2.5I-L**). A feature on the diagonal axis of a TODP (i.e. lower left corner to upper right corner) indicates a population of static molecules that remain in one state throughout their measurement, while an off-diagonal feature at position (X,Y) indicates a population of dynamic molecules that transition from states with $E_{\text{FRET}} = X$ (initial) to $E_{\text{FRET}} = Y$ (final) at least once during the measurement. This analysis showed that the majority of molecules (>80% for U₅₅ and Ψ_{55} variants of both tRNAs) exhibited dynamic behavior involving fluctuations between conformations. This is consistent with previous bulk^{90,91} and smFRET⁷⁵ studies that showed that tRNAs can interconvert between multiple secondary and tertiary structures at physiological salt and ambient temperature. In U₅₅ and Ψ_{55} variants of both tRNAs, bright off-diagonal features in the TODPs indicate that molecules readily and reversibly interconverted between the LF and MF states and between the MF and HF states (**Figure 2.5I-L**). Significantly, almost no intensity was observed at the locations corresponding to transitions

between the LF and HF states in any of the four tRNAs investigated, indicating that interconversion between these two states requires a molecule to transition through an intermediate MF state.

TABLE 2.1. HMM-derived rate constants (weighted average from a bi-exponential fit) for transitions between each pair of interconverting FRET states. Fits shown in **Supplemental Figure S2.10**.

tRNA	$k_{LF \rightarrow MF} (s^{-1})$	$k_{MF \rightarrow LF} (s^{-1})$	$k_{MF \rightarrow HF} (s^{-1})$	$k_{HF \rightarrow MF} (s^{-1})$
U ₅₅ tRNA eMet	0.58	0.34	1.00	1.43
Ψ ₅₅ tRNA eMet	0.79	0.58	0.79	1.49
U ₅₅ tRNA Thr	2.83	1.26	0.90	1.59
Ψ ₅₅ tRNA Thr	2.64	1.22	0.91	1.70

For U₅₅ and Ψ₅₅ tRNA eMet, HMM analysis further revealed transition rates of 0.3–0.8 s⁻¹ between LF and MF states, and slightly faster transition with rates of 0.8–1.5 s⁻¹ between MF and HF states (**Table 2.1, Supplemental Figure S2.10**). The overall rates of conformational fluctuations in each tRNA were also quantified in a model-free manner using cross-correlation and auto-correlation analyses (details in **Methods**), which identify how rapidly Cy3 and Cy5 intensities become uncorrelated with one another within a collection of smFRET traces. Consistent with the HMM results, the correlation analysis confirmed that fluctuations in FRET efficiency occurred on a time-scale of ~1 s in U₅₅ and Ψ₅₅ tRNA eMet (**Figure 2.5M,N**). For U₅₅ and Ψ₅₅ tRNA Thr, fluctuations were notably faster, with HMM-derived transition rates between the LF and MF states almost 4x faster than observed for tRNA eMet (**Table 2.1**), and biphasic correlation functions dominated by fluctuations on a time-scale <0.15 s rather than the ~1 s observed for tRNA eMet (**Figure 2.5O,P**). This approaches the 100 ms time resolution of our

smFRET measurements, suggesting that the indistinct features of the histograms of tRNA Thr may have arisen in part from time averaging across transitions between states. In summary, our analysis revealed that U₅₅ and Ψ_{55} tRNA eMet and tRNA Thr all undergo frequent conformational changes involving substantial adjustments in their three-dimensional architecture, with their MF states acting as near-obligate intermediates between LF and HF states.

Pus4 Alters the Conformational States and Transition Dynamics in tRNA eMet

After Pus4 was introduced into a sample chamber containing immobilized U₅₅ or Ψ_{55} tRNA eMet, a rapid increase in brightness of both Cy3 and Cy5 indicated that the protein was binding to the RNA (**Supplemental Table S2.2**), a phenomenon known as “protein-induced fluorescence enhancement” (PIFE)⁹². This increase was largely complete by the earliest time window that we probed by smFRET, 2–6 minutes, which begins when the initial fast phase of Pus4 association observed by BLI is nearly complete, and continues into the slow accumulation phase (**Figure 2.3B,C**). Concurrently, an increase in conformational complexity was observed, indicating that the protein changed the free energy landscape of tRNA molecules. 2–6 minutes after addition of protein, the histogram of U₅₅ tRNA broadens and all three features shift to higher E_{FRET} (**Figure 2.6A, Figure 2.7A**), potentially indicating changes to specific tertiary contacts previously held. Inspection of traces suggested that the observed increase in complexity originates from interconversion between mid-FRET “microstates” with $E_{\text{FRET}} \sim 0.4$ and 0.7 (**Supplemental Figure S2.11A**). Although we used only one MF state in the HMM model to avoid overfitting and facilitate comparison to data recorded in the absence of protein (**Supplemental Figure S2.9A**), the presence of additional microstates is reflected in TODPs

(**Figure 2.8A-D**). Specifically, the features present in tRNA alone became broader in the presence of Pus4, and transitions were observed between states with a greater variety of E_{FRET} values (**Figure 2.5I,J** vs. **Figure 2.8A**). We hypothesize that these microstates involve remodeling of the T-loop in order to facilitate pseudouridylation, as observed in crystallography data of the bacterial homolog TruB with tRNA^{17,18}. The shift to higher E_{FRET} in the histogram is partially reversed in the 7–9 minute time window (**Figure 2.6**). Given that BLI (**Figure 2.3**) and PIFE (**Supplemental Table S2.2**) both indicate that protein occupancy increases monotonically throughout this time period, the non-monotonic changes in the peak E_{FRET} values and feature widths suggest that the stably-bound protein-RNA complex evolves in structure after its initial assembly. By 30–34 minutes after introduction of Pus4 (at which point our gel shift assay indicated that all tRNA molecules were bound by Pus4 in solution; **Supplemental Figure S2.4**), the conformational ensemble resolves back into distinct LF, MF and HF features, with MF and HF centered at higher E_{FRET} than before exposure to Pus4 (**Figure 2.7A**). The TODPs reflect this as well, showing a convergence of features by the 30–34 minute time window (**Figure 2.8D**). While our approach precluded real-time, simultaneous measurement of pseudouridylation of the immobilized tRNA, our *in vitro* pseudouridylation assay showed that the majority of tRNAs were pseudouridylated by Pus4 within 2 minutes (**Figure 2.2**). Therefore, results over the course of these smFRET experiments reflect the enduring influence of Pus4 binding and catalysis on the trajectory of the tRNA conformations sampled.

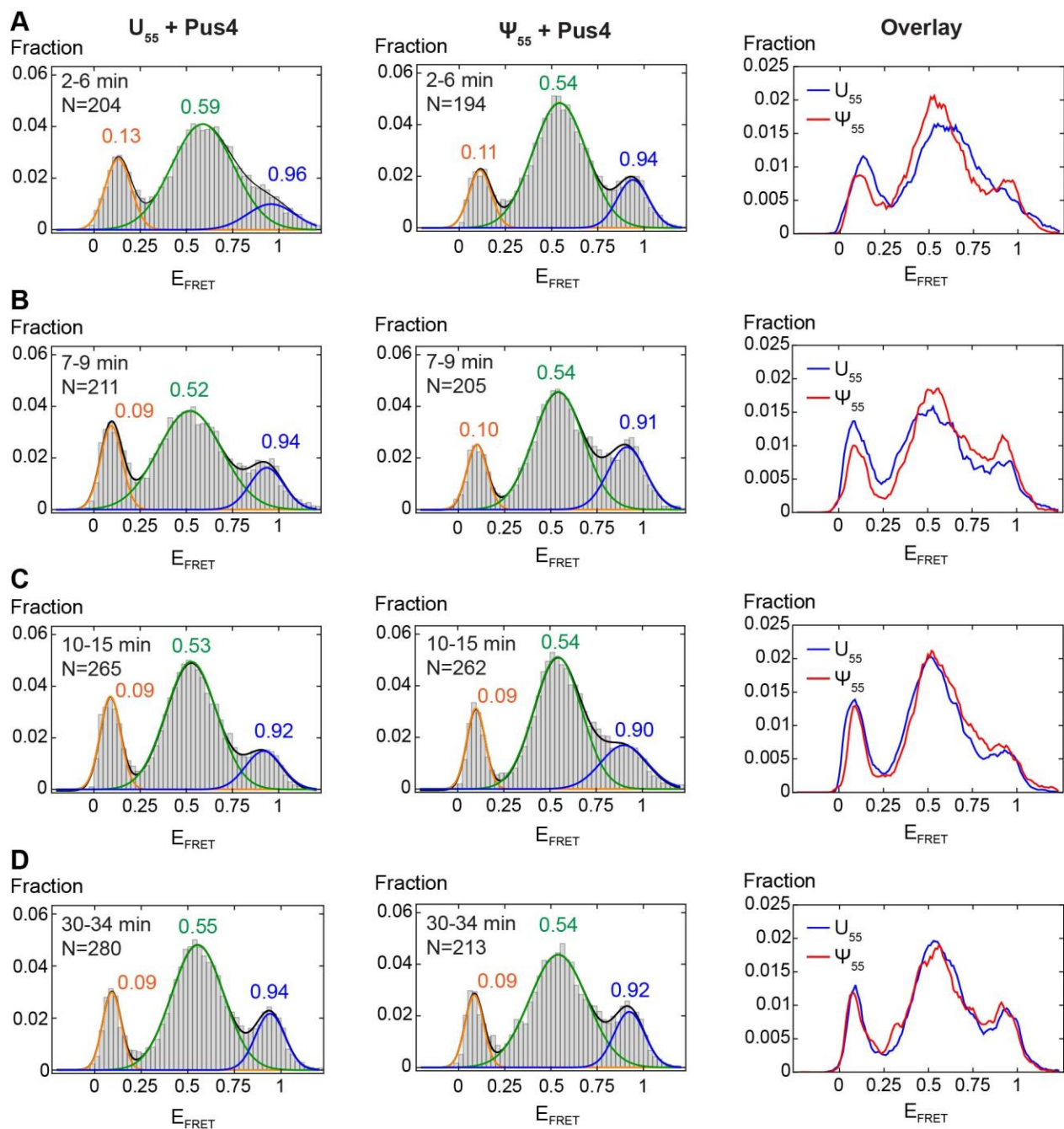


FIGURE 2.6. smFRET histograms for U_{55} and Ψ_{55} tRNA eMet in the presence of 200 nM Pus4. (A) Histograms compiled from movies recorded 2–6 minutes after injection of Pus4. Left column: U_{55} +Pus4 histogram with 3-Gaussian fit overlaid; Middle column: Ψ_{55} +Pus4 histogram with 3-Gaussian fit overlaid; Right column: Overlay of U_{55} and Ψ_{55} histograms. The E_{FRET} value of each state is indicated. (B) Same as A but compiled from movies recorded 7–9 minutes after injection of Pus4. (C) Same as (A–B) but compiled from movies recorded 10–15 minutes after injection of Pus4. (D) Same as (A–C) but compiled from movies recorded 30–34 minutes after injection of Pus4.

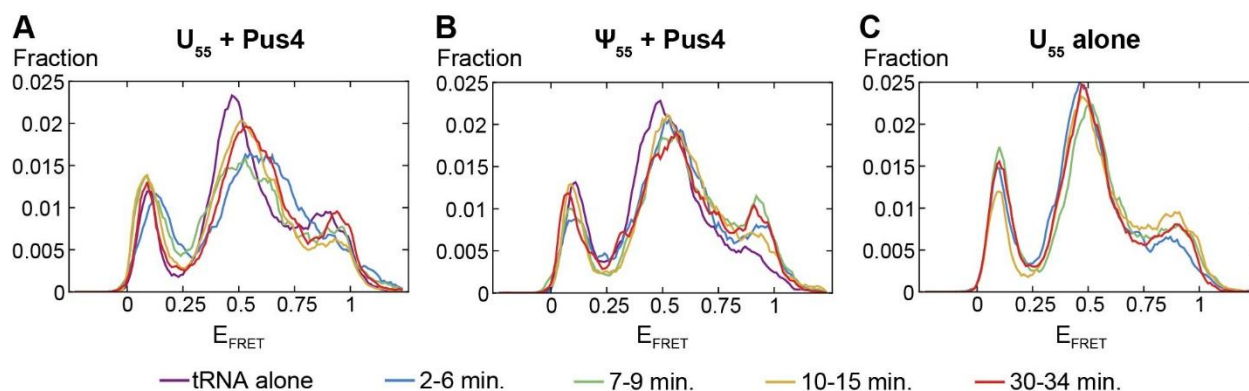


FIGURE 2.7. Overlaid smFRET histograms throughout incubation of (A) U_{55} tRNA eMet with 200 nM Pus4, (B) Ψ_{55} tRNA eMet with 200 nM Pus4, (C) U_{55} tRNA eMet with imaging buffer lacking Pus4. Blue: Histogram compiled from traces recorded 2–6 minutes after introduction of imaging buffer containing or lacking Pus4.; Green: 7–9 min.; Yellow: 10–15 min.; Red: 30–34 min. Panels A and B additionally show the histograms of the corresponding tRNA alone after 10–15 minutes of incubation with imaging buffer lacking Pus4 (purple).

As a control, we binned smFRET data recorded on U_{55} tRNA in the absence of Pus4 into the same time windows (**Figure 2.7C, Supplemental Figure S2.12**). We observed small changes in the relative amplitudes of the histogram features, particularly LF and HF. These changes were likely due to the finite number of molecules analyzed for each time window, and the gradual activation and depletion of the oxygen scavenging system used to prolong fluorophore activity. Importantly, however, U_{55} tRNA alone did not exhibit the curve broadening, shifts in E_{FRET} , or increase in overall intensity (**Supplemental Table S2.2**) that had characterized its time evolution in the presence of Pus4.

To help isolate the influence of Pus4 binding and remodeling of tRNA from its pseudouridylation activity, we performed smFRET experiments with the pre-modified Ψ_{55} tRNA eMet in the presence of Pus4. While MF microstates were observed in Ψ_{55} tRNA eMet at early times (**Supplemental Figure S2.11B**), as we also observed for U_{55} tRNA eMet, Ψ_{55} tRNA histograms and TODPs exhibited less dramatic changes. Specifically, in the 2–6 minute window

following injection of Pus4, Ψ_{55} tRNA exhibited a smaller shift of the MF state toward higher E_{FRET} than U_{55} , and an increase in the population of the HF state (**Figure 2.6A**). Ψ_{55} tRNA retained the same shape in its histogram throughout the remainder of the time course, exhibiting only minor fluctuations of the relative populations of its states (**Figure 2.6, Figure 2.7B**). These fluctuations were of comparable size to those observed in tRNA alone (**Figure 2.7C, Supplemental Figure S2.12**), so we conclude that the impacts of Pus4 on the conformational ensemble of Ψ_{55} tRNA are largely complete after 2–6 minutes of interaction. This stands in contrast to U_{55} tRNA, which continues to evolve through at least 15 minutes after injection of Pus4 despite the protein binding at a similar rate and remaining bound to both throughout the time course (as indicated by BLI and PIFE) and Pus4 presumably pseudouridylating most U_{55} tRNA molecules within 2 minutes (**Figure 2.2**). By 10–15 minutes of incubation with Pus4, U_{55} tRNA exhibited only a small skew toward lower E_{FRET} relative to Ψ_{55} , and their histograms were nearly superimposable by 30–34 minutes (**Figure 2.6C,D**). We hypothesize that Ψ_{55} tRNA thus bypassed some of the conformational changes that U_{55} tRNA transitioned through during its catalysis and subsequent remodeling by Pus4.

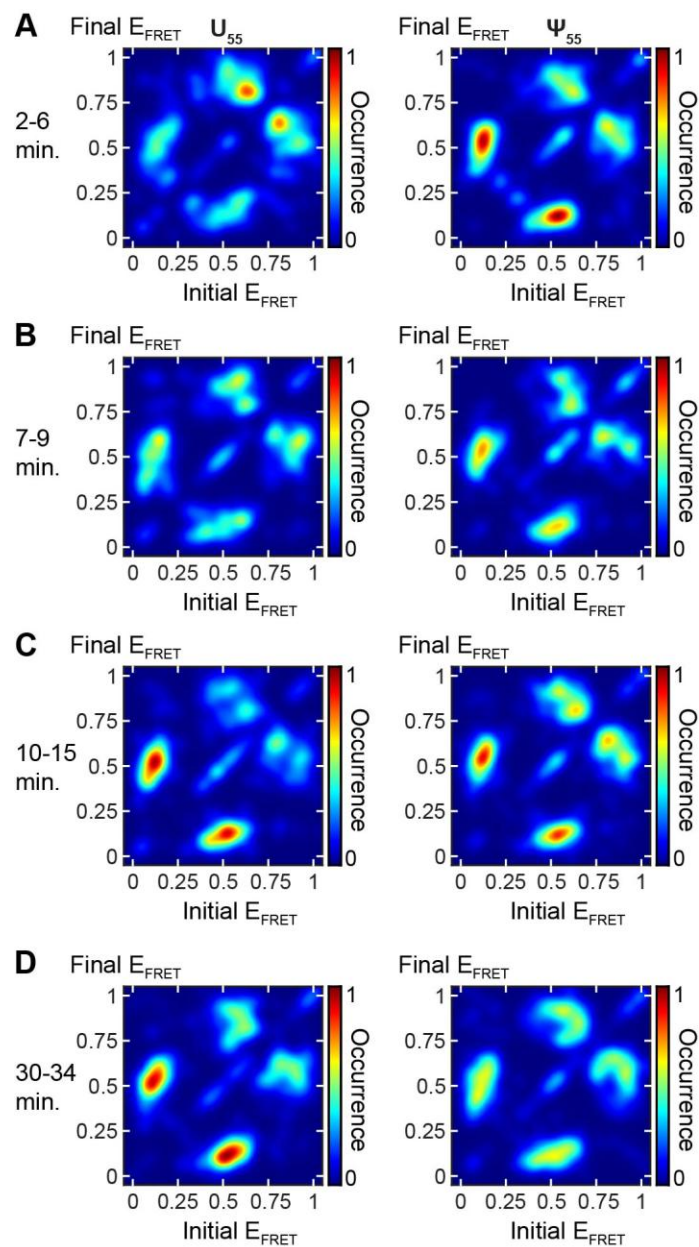


FIGURE 2.8. TODPs for U_{55} and Ψ_{55} tRNA eMet in the presence of 200 nM Pus4. TODPs for U_{55} (left column) and Ψ_{55} (right column) tRNAs compiled from traces recorded (A) 2–6 minutes after injection of protein; (B) 7–9 minutes; (C) 10–15 minutes; (D) 30–34 minutes.

Discussion:

In the decades since the three-dimensional structure of tRNA was elucidated⁹³, it has been presented as an archetype of structured non-coding RNA. Yet every tRNA undergoes many processing steps during biogenesis—including trimming, tailing and the addition of multiple chemical modifications—necessitating many conformational changes. In this study we combined biophysical and biochemical approaches to document the dynamics of conformational changes that two yeast tRNAs can undergo over time. We then measured how this chronology of tRNA structural dynamics changed upon binding by a ubiquitous pseudouridine synthase that modifies many tRNAs in nature. These data demonstrated how Pus4 impacts tRNA conformation through its binding and catalysis, leading to a more well-defined ensemble of conformations when the RNA-protein complex reached equilibrium. This outcome was reached after a period of minutes if catalysis was bypassed (i.e. Pus4 incubated with a pre-modified tRNA), rather than *tens* of minutes if catalysis occurred.

We find that tRNA eMet and tRNA Thr each sample complex free energy landscapes, with at least three conformational states resolved by smFRET. Both exhibit a hierarchical folding landscape, with their MF states as intermediates, though tRNA Thr exhibits faster dynamics. Furthermore, tRNA eMet maintains a dynamic conformational landscape of LF, MF and HF states throughout its interactions with Pus4 and independently of its modification status, with additional MF states populated upon engagement with Pus4. In the absence of protein, the E_{FRET} values of these features align reasonably closely with FRET states previously observed in human mt-tRNA^{Lys} under equivalent ionic conditions of 50 mM buffer, 100 mM monovalent cation and no divalent cation⁷⁶. However, we consistently observe a larger HF population in the yeast tRNA eMet than previously observed in mt-tRNA^{Lys}, which we propose can be reconciled by predicted

free energies. The cloverleaf secondary structure of tRNA eMet has a predicted $\Delta G_{\text{folding}} = -18.9$ kcal/mol (details of free energy calculations in **Methods**), significantly more stable than -16.1 kcal/mol for mt-tRNA^{Lys} studied in Voigts-Hoffman *et al.*, 2007 and subsequent studies^{76–78} (**Supplemental Figure S2.13A,E**).

mt-tRNA^{Lys} also substantially populated an MF state with $E_{\text{FRET}} \sim 0.4$ that interconverted with C on a time-scale of hundreds of milliseconds⁷⁵. This state was assigned to an extended hairpin secondary structure (E) that had previously been identified in this tRNA by biochemical methods⁹⁴. Interconversion between C- and E-type structures was also observed using biochemical methods in a different tRNA (mt-tRNA^{Leu})⁹¹. Consistent with these experimental observations, the E structure of mt-tRNA^{Lys} is predicted to have a free energy of -15.3 kcal/mol, within 1 kcal/mol to that of the C structure (**Supplemental Figure S2.13E,F**). To place our results into this context, we constructed a plausible E-like secondary structure of yeast tRNA eMet by identifying potential base pairs that could be formed between nucleotides in the T- and D-loops and adjacent stems (**Supplemental Figure S2.13B**). Unlike mt-tRNA^{Lys}, however, at $\Delta G = -12.9$ kcal/mol, this E-like structure of tRNA eMet is predicted to be 6 kcal/mol less stable than its C structure (**Supplemental Figure S2.13A,B**). Likewise, tRNA Thr is predicted to have a C structure that is 7 kcal/mol more stable than a plausible E structure (-24.4 and -17.4 kcal/mol, respectively) (**Supplemental Figure S2.13C,D**). In light of the substantial free energy preference for the C secondary structures of tRNA eMet and tRNA Thr, we consider it unlikely that the dominant MF states that we observe both with and without Pus4 arise from E-like secondary structures. Instead, the MF states observed in tRNA alone and the variations thereof that are populated in tRNA eMet in the presence of Pus4 may be explained by 1) relatively minor changes in secondary structure such as fraying/unpairing of the acceptor stem that contains the

donor fluorophore, and/or 2) changes in tertiary structure such as breaking of the T-loop-D-loop interaction. These structures have been observed in *E. coli* tRNAs *in vivo* under stress conditions⁹⁵. Prior steady-state and kinetic measurements also identified multiple interconvertible tertiary structures under conditions generally favoring the C secondary structure⁹⁶.

While our FRET approach reveals tRNA dynamics from the perspective of the distance between the anticodon stem and acceptor stem, complementary methods have provided insight into other degrees of freedom that characterize the accessible structural states. A recent study that used single particle cryo-EM and melting temperature assays found that tRNA folding was strongly influenced by the tertiary structure-stabilizing interaction between the D- and T-loops, and that this interaction itself was generally promoted by Ψ_{55} ⁹. Another study performed NMR on *E. coli* tRNA^{fMet}, and found that Ψ_{55} and neighboring modifications have pronounced effects on local structural dynamics⁹⁷. These dynamics were shown to facilitate interactions between the T-loop and D-loop and improve the overall stability of the tertiary fold of the tRNA. However, modification did not induce dramatic shifts in global structure, in agreement with our results showing largely similar global conformational ensembles for tRNA with and without Ψ_{55} .

Future studies of additional tRNA and Pus4 variants may improve delineation of the structures that give rise to the observed FRET states and their functional significance. For example, alternate placement of FRET pairs would permit degrees of freedom other than the anticodon stem-acceptor stem distance to be probed. Placing a third fluorophore in the D-loop of the tRNA—so as not to interfere with the T-loop pseudouridylation but enable detection of altered T-loop/D-loop interactions—may yield a more complete picture of the 3-dimensional architecture of the tRNA as it is remodeled. Alternatively, covalent attachment of a fluorophore

to Pus4 protein, with the remaining two fluorophores on tRNA, may simultaneously permit detection of Pus4 binding and tRNA structural remodeling over time by more clearly differentiating protein-bound from unbound conformations. A method to measure pseudouridylation in real-time on the microscope slide would also permit direct correlation of activity to structural remodeling, or the kinetics of binding if measured on a BLI probe. The assays developed in this study could also be applied to a catalytically impaired mutant of Pus4, such as the previously reported R286K⁵. This could further define the functional significance of the observed tRNA conformations, for example in determining if a lack of pseudouridylation activity leads to a delay in remodeling of the T-loop even in the presence of bound Pus4.

Our work herein began with the goal to characterize the conformational dynamics of tRNA as influenced by pseudouridylation and interaction with a pseudouridine synthase. Guided by our results, we propose that each instance of Pus4 engaging with tRNA follows a three-to-four-step process: 1) Initial binding of Pus4 occurs, 2) Pus4 “tests” whether Ψ_{55} is already present, 3) If Ψ_{55} is absent, Pus4 may convert U_{55} to Ψ_{55} , 4) Pus4 remodels tRNA into a new distribution of structures. Pus4 effectively discriminates U from Ψ , remodeling pre-modified tRNA toward the final structural ensemble within minutes. Upon initial engagement with unmodified or pre-modified tRNA, Pus4 induces an additional population of intermediate (MF) states. Such structural states may play a role in discrimination between substrate and product.

Overall our results demonstrate two important layers of influence on tRNA conformational dynamics: 1) chemical catalysis, and 2) remodeling resulting from extended and recurrent interactions with modification enzymes. Why Pus4 would promote such malleability in tRNA conformational dynamics on already pseudouridylated tRNAs remains an important question. Since Pus4 catalysis can strongly influence the addition of subsequent modifications *in*

*vivo*⁵, and some are added over many hours after pseudouridylation⁸¹, we propose that part of the role of Pus4 is to make tRNA structure amenable to binding and modification by subsequent modification enzymes that are critical for tRNA function.

Materials and Methods:

Ligation of synthetic tRNA sequences

Full-length tRNA constructs were made by ligation of two RNA oligonucleotides (Dharmacon, HPLC-purified by the manufacturer) using a DNA splint (Integrated DNA Technologies, PAGE-purified by the manufacturer) (**Supplemental Table S2.3**). Oligonucleotide concentrations were quantified using A260 measurements on a Nanodrop spectrophotometer and extinction coefficients provided by the manufacturer. Ligation was performed using 2 nmol of each of the 5' and 3' RNA segments and splint in a combined volume of 60 μ l to which 7.5 μ L hybridization buffer (150 mM HEPES pH 7.5 and 500 mM KOAc) was added. This solution was heated for 2 minutes at 90°C and allowed to cool at room temperature for 30 seconds before 10X T4 RNA ligase 2 buffer (NEB) was added to a final concentration of 1X. The mixture was then cooled at room temperature for an additional 10 minutes, at which time additional ligase buffer, ATP, water, and T4 RNA ligase 2 (MC Labs) were added to achieve a reaction volume of 100 μ L containing 0.4 mM ATP, 1X RNA ligase 2 buffer, and 100 units T4 RNA ligase 2. The ligation mixture was incubated at room temperature for 3 hours. Following this, 16 units DNase I (NEB) was added along with 10X DNase I buffer to bring the final concentration of that buffer to 1X. This mixture was incubated at 37°C for 15 minutes to digest the DNA splint.

A 2X FALB denaturing loading buffer consisting of 90% Formamide (TCI) in 1X TBE was added to a final concentration of 1X and the ligation mixture was heated for 2 minutes at 90°C to inactivate the enzyme and unfold the RNA. The resulting RNA mixture was run on a 12% polyacrylamide, 8 M urea denaturing gel and imaged using an Amersham Typhoon (GE). The gel was then stained with Sybr gold in 1X TBE for 15 minutes and the 76 nt band corresponding

to the full length tRNA was excised while visualizing the gel on a Dark Reader transilluminator (Clare Chemical Research). The RNA was extracted using an electro-elution system (BioRad) run at 10 mA per fritted tube for 15 minutes in 0.5X TBE. After elution, the polarity was reversed for 1 minute to release the RNA from the membrane and the product was recovered from the apparatus. 1/10 volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of -20°C 100% EtOH were added and the solution was mixed and stored at -20°C overnight. Tubes were centrifuged at 4°C, 10,000 rcf for 40 minutes. The supernatant was removed, 500 µL of -20°C 70% EtOH was added, the tube was vortexed for 30 sec, and then centrifuged at 4°C, for 10 minutes at the maximum RCF rating of the tube. The supernatant was again removed and pellets were dried in a vacuum centrifuge at room temperature for 15 minutes until dry. The pellets were dissolved in 50 µL DEPC-treated H₂O and quantified using a NanoDrop.

Protein expression and purification

A pGEX-5X-3-ScPus4 backbone plasmid encoding GST-Pus4 with a Factor Xa cleavage site between the GST tag and Pus4 protein sequence (PDG322) was used (**Supplemental Table S2.1, S2.3**). The GST-tagged Pus4 vector was transformed into *E. coli* BL21(DE3) competent cells (Thermo Fisher Scientific). A single transformant of each was inoculated to make a 50 mL overnight starter culture, which was then diluted into a 4 L culture grown until reaching OD₆₀₀=0.80. Cultures were grown at 37°C in TB medium containing 100 µg/mL ampicillin and shaken at 225 rpm. Upon reaching the desired OD₆₀₀, the 4 L culture was shifted to 16°C for 30 minutes. Protein expression was induced by adding IPTG (Gold Bio) to a final concentration of 1 mM, and cells were grown overnight for ~16 hours at 16°C with continuous shaking at 225 rpm. Cells were harvested by centrifugation, and the cell pellets were resuspended in 30 mL of Buffer

A (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 10 mM MgCl₂, 10% Glycerol, 0.5 mM EDTA, 5 mM BME, 1 tablet of Pierce EDTA-free Protease Inhibitor (Thermo Fisher Scientific)), divided into two 50 mL conical tubes, and then flash-frozen in liquid nitrogen before storage at -80°C. Each tube contained enough material for a single protein purification. All subsequent procedures were performed at 4°C unless otherwise stated.

Conical tubes were thawed on ice. The following reagents were added to achieve their final concentrations, respectively: lysozyme (Sigma) to 1 mg/mL, DNase (GoldBio) to 10 µg/mL, MgCl₂ (Fisher) to 0.5 mM, and RNase A (Fisher) to 30 µg/mL. This cell suspension was incubated for 30 minutes. Cells were lysed via sonication using a sonicator (Branson Sonifier 450) with 30-seconds-on/30-seconds-off cycles (50% duty cycle, power level of 4), repeated three times. Insoluble material was removed by centrifugation for 30 minutes at 30,000 x g in a JA-20 rotor. After clarification, the remaining soluble material was incubated with 2 mL of a 50% slurry of Glutathione-Sepharose beads (Cytiva) that had been pre-equilibrated with Buffer A for 1 hour with end-over-end rotation at room temperature. After incubation, the protein-bound beads were isolated using centrifugation (5 minutes at 500 x g) and then washed with 20 mL of Buffer A. This wash step was repeated for a total of five times. Protein was then eluted from the beads with 1 mL of freshly prepared Elution buffer (50 mM Tris-HCl pH 7.6, 10 mM reduced glutathione) after incubating for 5 minutes at room temperature with end-over-end rotation. This elution step was repeated five times and the elutions were pooled together (~5 mL).

The eluate was concentrated to ~1 mL final volume using a 30 kDa Vivaspin concentrator (GE Healthcare). Using the same concentrator, the sample underwent exchange into Buffer B (50 mM Tris-HCl pH 7.5, 100 mM KCl) by adding 10 mL of Buffer B, and performing centrifugation to concentrate to ~1 mL final volume. This was repeated two more times. These protein samples were aliquoted into Eppendorf tubes and flash frozen in liquid nitrogen before storage at -80°C. The yield was quantified using A₂₈₀ measurements on a Nanodrop spectrophotometer. Protein purity was determined using SDS-PAGE with samples collected across steps of the protein purification process (**Supplemental Figure S2.1A**).

Testing purified protein for RNase contamination

Approximately 1 µg of tRNA isolated from yeast was incubated with 0.4 µM of Pus4 in a 10 µL total volume for one hour at room temperature. Control samples of tRNA without protein and tRNA plus protein without incubation were also prepared. Samples were combined with 10 µL of 2X RNA loading dye (NEB), heated at 70°C for 8 minutes on a heat block, and then loaded onto a fresh pre-run PAGE gel, along with RNA ladder, using an Apogee V16 gel system. After running the gel at 60 mA for about 1.5 hours, the gel was stained with SYBR Gold for 10 minutes at room temperature, and imaged using an Amersham Typhoon (GE) to assess tRNA degradation (**Supplemental Figure S2.1B**).

Yeast growth conditions

*pus4*Δ (YDG127) cells were streaked out onto YPD agar media plates from a frozen -80°C stock and grown at 30°C for 2 days (**Supplemental Table S2.1**). Colonies were inoculated into 5 mL YPD media in culture tubes and grown at 30°C in a rolling drum overnight. In the morning, 400

μL of each culture was transferred to culture tubes with 5.2 mL liquid YPD and grown at 30°C in a rolling drum to an OD₆₀₀ of ~0.8. Cultures were transferred to 15 mL conical tubes and centrifuged at 4°C, 3000 x g for 2 minutes. Media was decanted, and the cell pellets were resuspended in 1 mL cold 1X PBS and transferred to microcentrifuge tubes. Tubes were centrifuged at 4°C, 9500 rpm for 1 minute to pellet. Supernatant was decanted and cell pellets flash frozen by placing microcentrifuge tubes in liquid nitrogen. Cell pellets were stored at -80°C until used for total RNA isolation.

tRNA isolation from yeast

pus4Δ (YDG127) yeast pellets were thawed on ice (**Supplemental Table S2.1**). For cell lysis and RNA preservation, pellets were resuspended in 400 μL TES buffer (10 mM Tris, 10 mM EDTA, 20% SDS). 400 μL acidic phenol was added to each tube. Tubes were vortexed for 10 seconds and incubated in a 65°C water bath for one hour, with 10 seconds of vortexing every 15 minutes. Tubes were put on ice for 5 minutes then centrifuged at 4°C, 13,000 rpm for 10 minutes. The top aqueous layer of each sample was transferred to a new tube. 400 μL of chloroform was added to each of the new tubes. Tubes were vortexed for 10 seconds then centrifuged at 4°C, 13,000 rpm for 10 minutes. The top aqueous layer of each sample was transferred to a new tube and the addition of chloroform, vortexing, and centrifuging repeated. The top aqueous layer of each sample was transferred to a new tube. To precipitate RNA, 1/10 volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of ice cold 100% EtOH were added to each sample and mixed. Tubes were stored at -80°C for one hour. Tubes were centrifuged at 4°C, 13,000 rpm for 10 minutes. The supernatant was removed and 500 μL cold 70% EtOH added to each tube to wash pellets of salt. Tubes were centrifuged at 4°C, 13,000 rpm for 10 minutes.

Ethanol was removed and tubes left open for 5–10 minutes to allow residual ethanol to evaporate. Dried pellets were resuspended in 25 μ L water, and their concentrations were quantified using a Nanodrop.

An 8% urea acrylamide gel was made using the National Diagnostics UreaGel 19:1 Denaturing Gel System. The gel was pre-run in 1X TBE running buffer at 45 mA for 30 minutes. Total RNA samples were aliquoted to 10 μ L volumes and 10 μ L 2X RNA loading dye (NEB) added to each sample. 3 μ L Low Range ssRNA Ladder (NEB) was mixed with 7 μ L water and 10 μ L 2X RNA loading dye. Samples were incubated at 70°C for 8 minutes. Samples were loaded on the gel and empty wells loaded with 10 μ L water + 10 μ L 2X RNA loading dye. The gel was run at 20 mA for 15 minutes and then at 60 mA for ~1 hour. The gel was stained with SYBR gold (Invitrogen) in 1X TBE running buffer for 10 minutes. The gel was imaged with an Amersham Typhoon (GE). Using UV shadowing, tRNA bands were cut from the gel. Gel fragments were placed in tubes with 450 μ L 0.3 M NaCl. Tubes were put on a tube inverter overnight at 4°C. Liquid was transferred to new tubes and 1.05 mL 100% EtOH was added to each sample and mixed. Samples were stored at -80°C for one hour. Samples were centrifuged at 4°C, 13,000 rpm for 35 minutes. The supernatant was removed and tubes left open for 5–10 minutes to allow residual ethanol to evaporate. Dried pellets were resuspended in 10–20 μ L water and stored at -80°C.

***in vitro* modification**

To perform the *in vitro* modification, 1 μ M Pus4 protein was mixed with 300 nM total tRNA harvested from *pus4* Δ cells and incubated at 30°C for 2 minutes in a 200 μ L reaction volume in Buffer B (50 mM Tris-HCl pH 7.5, 100 mM KCl). Buffer B supplemented with 4 mM MgCl₂

was also tested to observe whether magnesium affected catalysis. Since it did not have a strong effect (**Figure 2.2**), MgCl_2 was excluded from the buffer used for all smFRET and BLI assays. These reactions were halted with the addition of 200 μL of saturated phenol (pH 6.6). Samples were mixed and then stored at -80°C .

To purify *in vitro* modified tRNA, samples were thawed on ice, then vortexed for 10 seconds and centrifuged at 4°C , 13,000 rpm for 10 minutes. tRNA was re-purified as done in the tRNA isolation (described above), beginning with the addition of 400 μL chloroform. Samples were resuspended in 10 μL water and concentrations quantified using a Nanodrop. Samples were stored at -80°C .

Direct sequencing of *in vitro* modified tRNA

To perform library preparation for sequencing using Oxford Nanopore Technology (ONT), samples were first thawed on ice. Samples were incubated at 95°C for 2 minutes, room temperature for 2 minutes, then on ice for 2 minutes. In a new tube, 7.25 μL tRNA sample was mixed with 2 μL 10X RNA ligase 2 buffer (NEB), 0.8 μL each of: 10 μM ACCA, 10 μM GCCA, 10 μM UCCA, 5 μM CCCA, and 5 μM CCA specific adapters⁵, 2 μL 50% PEG, 2 μL 250 mM MgCl_2 , 1.25 μL 100 mM DTT, 2 μL 20 mM ATP, and 1 μL RNA ligase 2 (NEB), for a total volume of 20 μL . The reaction was incubated at room temperature for 45 minutes. 20 μL water was added to the reaction. tRNA was purified with 1.8X volume RNAClean XP Beads (Beckman Coulter). Samples were washed twice with 500 μL 80% EtOH, eluted with 23 μL water. Samples were mixed with 8 μL 5X quick ligase buffer (NEB), 6 μL RMX adapter (ONT), and 3 μL T4 DNA ligase (2,000,000 U/mL; NEB) for a total volume of 40 μL . The reaction was

incubated at room temperature for 30 minutes. tRNA was purified with 1.5X volume RNAClean XP Beads. Samples were washed twice with 150 μ L WSB (ONT), then eluted with 15 μ L ELB (ONT). Water was added to bring the total volume to 37.5 μ L. Samples were sequenced with MinION devices following the ONT SQK-RNA002 protocol.

Pseudouridylation of U₅₅ in tRNA leads to a base miscall, due to an alteration in the ionic current when a pseudouridine transitions through the nanopore, as compared to the different disruption in ionic current caused by the unmodified uridine base. This causes a “mismatch” between the sequenced base and the unmodified reference base. The “reference match probability” refers to the probability that a given base will match the reference base. Therefore, a low reference match probability at position 55 is indicative of a pseudouridylated base. For more details see Shaw and Thomas *et al.* 2024.

Sequencing results were basecalled using Guppy v3.0.3, aligned with BWA-MEM (-W 13 -k 6 -xont2d), and quality controlled with SAMtools (Q1). marginAlign was used to create alignment and error models, and marginCaller was used to calculate mismatch probabilities. Python was used to calculate the reference match probability—equal to a value of 1.0 minus the mismatch probability value—and generate heatmaps. DRS data files are listed in **Supplemental Table S2.4**.

Preparation of sucrose gradients

A 6X Buffer B solution was prepared, then mixed with a 60% sucrose solution dissolved in water at the appropriate ratios to achieve five sucrose solutions of 10%, 15%, 20%, 25%, and

30% — each achieving a final concentration of 1X Buffer B. Once prepared, 980 µl of the 30% sucrose solution was pipetted into Ultra-Clear centrifuge tubes (½ inch x 2 inch, Beckman Coulter catalog #344057), then allowed to freeze at -80°C for 20 minutes. The 25% solution was layered on top, allowed to freeze, and this was repeated for the remaining solutions. Any samples to be compared were run with the same batch of sucrose gradients to control for batch variation. Sucrose gradients were prepared ahead of time and stored at -80°C.

Sucrose gradient ultracentrifugation

Gradients were thawed overnight at 4°C prior to use the next day. A 100 µL solution with 10 µM purified GST-Pus4 with and without 10 µM tRNA derived from YDG1 (**Supplemental Table S2.1**) was allowed to sit at room temperature for 30 minutes prior to loading onto a gradient. Gradients were balanced, and then spun in an ultracentrifuge (Beckman Coulter Optima XPN-100) in a SW 55 Ti swinging bucket rotor (Beckman Coulter) for 4 hours at 55,000 rpm. Following ultracentrifugation, gradients were fractionated by hand, serially removing 200 µl from the top of the gradient and aliquoting into 24 tubes. (Note that the final fraction had a volume in the range of 200–400 µl, due to subtle differences in the total gradient volume and aspiration efficiencies of the dense sucrose mixture after spinning). Fractions were flash frozen in liquid nitrogen prior to storage at -80°C.

Five nanomoles of each gel filtration chromatography-purified standard (beta-amylase and carbonic anhydrase, Millipore Sigma) were combined and run together in a separate gradient.

SDS-PAGE of sucrose gradients

Sucrose gradient fractions were mixed 1:1 with 2X Laemmli buffer (Bio-Rad) containing 5% v/v 2-mercaptoethanol, vortexed for several seconds, boiled on a heat block at 95°C for 10 minutes, then vortexed again and briefly spun down to condense liquid. Equal volumes from each fraction were loaded across three 4–15% PAGE gels (Bio-Rad). After electrophoresis in 1X SDS running buffer, gels were stained with GelCode Blue stain reagent (Thermo Fisher Scientific) to visualize protein abundance across the fractions.

Biolayer Interferometry (BLI)

BLI was performed using the biotinylated and fluorophore-labeled RNA substrates used for smFRET as well as versions of tRNA eMet lacking fluorophores. After the ligation steps described above, the RNA was diluted in Buffer B to a concentration of 60 nM. The solution was heated to 90°C on a heat block for 2 minutes, then allowed to cool for 10 minutes at room temperature before placing it on ice. BLI measurements were performed at 30 °C on a GatorPlus Biolayer Interferometer (GatorBio, Palo Alto, CA) utilizing proprietary GatorOne software for data collection. GatorBio streptavidin-coated glass probes were presoaked in Buffer B for at least 20 minutes before collecting baseline signal for 300 seconds. The probes were immersed in 60 nM biotinylated tRNA until an adequate shift (~1.2 nm) in signal was observed (~60 seconds). The probes were then submerged in 10 nM biocytin (Cayman Chemical) in Buffer B to quench the remaining streptavidin sites. This was followed by a second quenching in Buffer B supplemented with 0.2% Tween-20, which further aided in reducing non-specific protein binding. For this reason, Buffer B supplemented with 0.02% Tween-20 (“Buffer C”) was used for all following steps. Loaded probes were then incubated in Buffer C until baseline was achieved (~300 seconds). To measure association of the protein to the tRNA, the loaded probes

were then incubated in 200 nM of GST-Pus4 in Buffer C for ~2500 seconds or more. To measure dissociation, the probes were then submerged in Buffer C for the same amount of time as the association step. Reference probes were included in some runs to assess non-specific protein binding and RNA dissociation from the probe.

Analysis of BLI data

To estimate the kinetics of Pus4 binding to tRNA, we attempted to isolate the initial rate of association before pseudouridylation (in the case of tRNA that was not premodified) or refolding occurred. In isolation, this early phase of binding represents a reversible second order reaction ($\text{RNA} + \text{Pus4} \rightleftharpoons \text{RNA} \cdot \text{Pus4}$). We approximated its kinetics as pseudo-first order in Pus4, as the protein was present in bulk solution while the quantity of tRNA was limited by the number of binding sites on the probe. This is expected to yield an exponential shape to the association curve (**Eq. 1**):

$$(1) \quad \frac{[\text{RNA} \cdot \text{Pus4}]_t}{[\text{RNA} \cdot \text{Pus4}]_{\text{final}}} = 1 - e^{-k_{\text{obs}} t}$$

where the observed rate of the reaction is:

$$k_{\text{obs}} = k_{\text{on}}[\text{Pus4}] - k_{\text{off}}$$

Considering that at each timepoint t , $[\text{RNA}]_0 = [\text{RNA} \cdot \text{Pus4}]_t + [\text{RNA}]_t$, and that at the final timepoint $[\text{RNA}]_{\text{final}} = [\text{RNA}]_0 - [\text{RNA} \cdot \text{Pus4}]_{\text{final}}$, **Eq. 1** becomes:

$$(2) \quad \frac{[\text{RNA} \cdot \text{Pus4}]_{\text{final}} - [\text{RNA} \cdot \text{Pus4}]_t}{[\text{RNA} \cdot \text{Pus4}]_{\text{final}}} = e^{-k_{\text{obs}} t}$$

To estimate k_{on} and k_{off} , the association phases of the BLI curves were converted to fractional saturation (FS) using **Eq. 3**, where $s(t)$ is the shift at time t and s_{final} is the shift at the final timepoint of the association phase:

$$(3) \quad FS(t) = 1 - \frac{s_{final} - s(t)}{s_{final}}$$

Two methods were then used to fit the fractional saturation curve: 1) The early segment of the association phase over which ~75% of the overall shift occurred was fitted with a single exponential (**Eq. 4**), or 2) a longer period of the association phase was fitted with the sum of 2 exponentials (**Eq. 5**). The first 1000 s were used in the bi-exponential fits for tRNA without fluorophores due to the prominent downturn in the curves at 800 nM, while the entire association phase was fitted in all other cases. In **Eq. 5**, the faster of the two rates was taken to be k_{obs} .

$$(4) \quad FS(t) = y - a \cdot e^{-k_{obs}t}$$

$$(5) \quad FS(t) = y - a_1 \cdot e^{-k_1t} - a_2 \cdot e^{-k_2t}$$

These fits were performed for BLI curves obtained at 50 nM, 200 nM and 800 nM Pus4. k_{obs} was then plotted against [Pus4] and the slope of a linear fit yielded an estimate for k_{on} . In principle, the y-intercept of the fit yields k_{off} . However, determining it accurately would require measurements at lower [Pus4], conditions that reduce both the accuracy of the pseudo-first order kinetics approximation and the separation of the timescales on which Pus4 accumulates on the probe and on which it modifies and/or remodels the tRNA it has already bound to. Because k_{obs} was less than 0.01 s^{-1} at 50 nM Pus4 (the lowest concentration tested) for all RNAs and fitting approaches, we can reasonably assume that k_{off} is less than that value. Given the values obtained for k_{on} (**Supplemental Figure S2.5**), this constrains the dissociation constant $K_D = k_{off}/k_{on}$ to a value less than ~130 nM.

Electrophoretic Mobility Shift Assay

The RNA was annealed as described above for BLI at a concentration of 300 nM. The RNA was then further diluted to 30 nM in Buffer B and incubated with varying concentrations (0 nM, 15 nM, 30 nM, 60 nM, 120 nM, 200 nM, 400 nM, 600 nM, 800 nM) of Pus4 in a total volume of 10 μ L for 30 minutes at room temperature. Each 10 μ L solution was mixed with 10 μ L of loading buffer (2X TBE, 40% glycerol). The mixtures were run on an 8% Polyacrylamide native/non-denaturing gel at 300 V for 2.5 hours at 4°C. The gel was prepared from 40% 19:1 Acrylamide/Bisacrylamide (Bio-Rad), TEMED (Thermo Fisher Scientific), and Ammonium Persulfate (Thermo Fisher Scientific) and pre-run in 1X TBE running buffer at 300 V for 90 minutes. The gel was imaged using an Amersham Typhoon (GE).

Single molecule FRET (smFRET)

RNA was annealed as described above at a concentration of 10 nM, then diluted to 10 pM in Buffer B supplemented with 5 mM D-glucose (Sigma Aldrich), 165 U/mL glucose oxidase from *Aspergillus niger* (Sigma Aldrich), 2150 U/mL catalase from *Corynebacterium glutamicum* (Sigma Aldrich) and 5 mM Trolox (Sigma Aldrich) as an oxygen scavenging system (OSS). This OSS was used to visualize the RNA during immobilization due to its fast acting capability. This mixture was flowed onto a slide that had been passivated with DDS (Sigma Aldrich) as previously described⁹⁸, incubated with 0.2 mg/mL BSA (Thermo Fisher Scientific) for 5 minutes, 0.2% v/v Tween-20 for 10 minutes, and 0.2 mg/mL streptavidin (Thermo Fisher Scientific) for 5 minutes. Imaging was performed with Buffer C containing 5 mM 3,4-dihydroxybenzoic acid (Sigma Aldrich), 5 mM Trolox (Sigma Aldrich), and 50 nM Protocatechuate 3,4-dioxygenase from *Pseudomonas sp.* (Sigma Aldrich or MP Biomedicals) as an OSS and, for experiments with protein, 200 nM Pus4. This imaging buffer was incubated in a

tube with minimal headspace for 10 minutes to allow the OSS to begin depleting oxygen before injection into the sample chamber. Data were collected at room temperature on a home-built prism-type TIRF microscope (Leica DMI8 inverted microscope, 63X oil immersion objective). Cy3 and Cy5 were excited by a 532 nm laser (Coherent OBIS 532 LS) at 140 mW and a 637 nm laser (Coherent OBIS 637 LX) at 60 mW, respectively. The slide was illuminated at 532 nm continuously and at 637 nm at the beginning and end of each movie to confirm that Cy5 was active. 2-minute-long movies were recorded on an Andor iXon 888 EMCCD with an exposure time of 100 ms. An EM gain setting of 300 was used for movies recorded in the presence of Pus4. tRNA-only movies used for intensity comparison (**Supplemental Table S2.2**) were recorded at the same EM gain, whereas tRNA-only movies used for FRET analysis (**Figure 2.5**, **Supplemental Figure S2.12**) were recorded at an EM gain of 450 due to lower fluorophore brightness in the absence of protein. Movies were collected back to back for the first 15 minutes following injection of imaging buffer, and additional movies were collected 30–34 minutes after injection. Unless otherwise noted, data reported for tRNA in the absence of Pus4 was recorded for 10–15 minutes following injection of imaging buffer.

Coordinates for each molecule were extracted from raw video files using FIJI and fluorophore intensity traces were extracted using custom MATLAB code. Traces that exhibited single-step photobleaching of Cy3 and Cy5 and anticorrelation between donor and acceptor signals when transitions occurred were selected manually and analyzed using custom MATLAB codes. FRET efficiency was calculated from the measured donor and acceptor emission intensities (I_D and I_A) using **Eq. 6**:

$$(6) \quad E_{FRET} = \frac{I_A - x \cdot I_D}{\gamma I_D + (I_A - x \cdot I_D)}$$

Variable x corrects the acceptor signal for bleedthrough of donor emission into the acceptor channel and was determined to be 0.129 through analysis of tRNA molecules that lacked an active Cy5. γ was determined through analysis of traces in which Cy5 photobleached before Cy3, as previously described⁸⁸, and was found to be 2.42 in the absence of protein and 3.01 in the presence of protein (**Supplemental Figure S2.8C,D**). Noise in the corrected donor and acceptor traces was suppressed using a bilateral filter with a spatial spread of 2 frames and a signal value spread of approximately 1/4 of the mean signal intensity across all traces in a dataset for Cy3 and approximately 1/5 of the mean signal intensity for Cy5. This resulted in signal value spreads of 900–1000 for Cy3 and 800–1100 for Cy5 in the absence of protein, and spreads of 2200–3100 for Cy3 and 1900–2500 for Cy5 in the presence of protein, which preserved rapid excursions in the resulting E_{FRET} traces.

FRET histograms were compiled from the first 50 frames of ~200 traces and fitted with a sum of three or four Gaussian functions in Mathematica (Wolfram Alpha). Hidden Markov Modeling was performed in QuB (Quantifying unknown Biophysics) Online^{99–101} and TODPs were generated from the resulting idealized traces using custom MATLAB codes. Correlation analysis was performed in Mathematica by computing the cross-correlation $C(\tau)$ between Cy3 and Cy5 intensities using **Eq. 7**¹⁰²,

$$(7) \quad C(\tau) = \frac{\langle \Delta Cy3(t) \Delta Cy5(t + \tau) \rangle}{\langle Cy3(t) \rangle \langle Cy5(t + \tau) \rangle}$$

where $\Delta X(t) = X(t) - \langle X \rangle$ and $\langle X \rangle$ is the average value of X across all frames t within the trace. For autocorrelation analysis, Cy3 and Cy5 intensities in the above equation were both replaced by the E_{FRET} value. Traces used for correlation analysis were corrected for bleedthrough and γ factor but not subjected to the bilateral filter. Autocorrelation and cross-correlation curves were fitted simultaneously with a two-exponential model (**Eq. 8 and 9**, respectively) with the time constants t_1 and t_2 fixed across the two curves.

$$(8) \quad \text{Fit}(\tau) = a + b_1 * e^{-\tau/t_1} + b_2 * e^{-\tau/t_2}$$

$$(9) \quad \text{Fit}(\tau) = a_2 + b_3 * (1 - e^{-\tau/t_1}) + b_4 * (1 - e^{-\tau/t_2})$$

HMM-derived rate constants for transitions between FRET states were determined by extracting the durations of dwells in each state and fitting their cumulative distribution functions with **Eq. 9**, where $k_i = 1/t_i$.

Bulk emission spectroscopy

Emission spectra were recorded on an Edinburgh Instruments FS5 spectrofluorometer using a 4 mm square cuvette. RNA was annealed as described above at a concentration of 150 nM in Buffer B and used without further dilution. Cy3 was excited at 532 nm with a bandwidth of 2 nm and emission was scanned from 500 to 800 nm in 1 nm steps with a bandwidth of 2 nm and a 1 s dwell time.

Free energy calculations

The free energies of different secondary structures were predicted using the RNAfold web server¹⁰³. The sequence used for mt-tRNA^{Lys} was the one utilized in Voigts-Hoffman *et al.*, 2007 with U instead of dT at its fluorophore labeling sites, and the sequence used for tRNA eMet was the one utilized in this work. Mild base-pairing constraints were applied as needed to achieve the C and E secondary structures of mt-tRNA^{Lys} as depicted in Figure 1 of Voigts-Hoffman *et al.*, 2007, the canonical tRNA eMet and tRNA Thr structures as depicted in **Figure 2.1A** of this work, and plausible E-like structures of tRNA eMet and tRNA Thr as depicted in **Supplemental Figure S2.13 B and D**. The secondary structures were optimized under these constraints with energy parameters re-scaled to 25°C and 0.1 M salt and isolated base-pairs allowed. The resulting minimum free energy structures were re-rendered using R2DT¹⁰⁴ to generate the visualizations shown in **Supplemental Figure S2.13**.

Bridge to Chapter III:

In Chapter II, I described our investigation into the activities of Pus4, including its catalysis and binding of tRNA, and how this enzyme and its modification affect the structural conformation of tRNA. We found that binding of Pus4 and the application of its modification has a measurable effect on tRNA structure, giving better clarity into the specific way that tRNA structure is affected. Chapter III also looks at structure, but rather of a class of proteins known as prions. It summarizes how traditional prions, or amyloid-forming prions, are distinct and different from a newer class of prions that do not form amyloids and presents the current knowledge about our biochemical understanding of these phenomena.

CHAPTER III

BIOCHEMICAL PRINCIPLES IN PRION-BASED INHERITANCE

Author Contributions:

Emily M. Dennis and David M. Garcia both contributed to the ideas, writing, and editing of this review, published in *Epigenomes* in 2022.

Abstract:

Prions are proteins that can stably fold into alternative structures that frequently alter their activities. They can self-template their alternate structures and are inherited across cell divisions and generations. While they have been studied for more than four decades, their enigmatic nature has limited their discovery. In the last decade, we have learned just how widespread they are in nature, the many beneficial phenotypes that they confer, while also learning more about their structures and modes of inheritance. Here, we provide a brief review of the biochemical principles of prion proteins, including their sequences, characteristics and structures, and what is known about how they self-template, citing examples from multiple organisms. Prion-based inheritance is the most understudied segment of epigenetics. Here, we lay a biochemical foundation and share a framework for how to define these molecules, as new examples are unearthed throughout nature.

Introduction:

Prion proteins are a type of “protein-based inheritance” in which a conformational change in protein structure alters protein function and becomes self-templating without any requisite

changes to DNA sequence. Prions are therefore an epigenetic phenomenon, albeit one not as well-studied as chromatin modifications, DNA chemical modifications, and certain classes of non-coding RNAs. Phenotypes that were later understood to be prion-based were studied as early as the 1960s²⁹, although it was not until the work of Stanley Prusiner and others that a more rigorous definition of their protein-only based nature was established. Prusiner's work helped cement a protein expressed in the brain of mammals, known as PrP, as the defining example of a prion³⁸. When this protein converts into a prionogenic conformation, however, it invariably leads to death, in the form of a variety of spongiform encephalopathies, including Creutzfeldt-Jakob Disease, Mad Cow Disease, Scrapie, and Chronic Wasting Disease. These diseases are highly infectious, and they can also occur spontaneously in very rare cases.

Today, our understanding of prion proteins is significantly more expansive—in rare cases they can cause disease, but there are even more examples of how they can be beneficial to organisms, permitting adaptation to environmental change⁴⁰, altered metabolism³³, increased cellular growth⁴², and resistance to chemical stressors³². Prions also fundamentally alter our view of inheritance, as their ability to phenotypically alter an organism is based on heritable differences in protein structure and not any change to DNA sequence.

In this minireview, we focus our attention on the biochemical principles in prion-based inheritance. We outline sequence features of prions, their structures, and their aggregation/assembly properties. We hope this provides a useful introduction to non-experts, while supplying a framework for profiling features of newly discovered examples that we anticipate will further expand our understanding of this fascinating class of transgenerational molecules of inheritance.

Structural Motifs and Amino Acid Sequence Features Observed in Prion Proteins:

A major shortcoming to understanding the general biochemical features of prion proteins is that only a handful of examples have been studied in detail. Nevertheless, some key features, sometimes predictive, are associated with prions and informative of the types of properties one could look for as new examples are discovered.

Prions can be grouped into two major classes based on the structural motifs that they can adopt: amyloid-forming prions and non-amyloid-forming prions³⁹. Amyloid-forming prions are proteins that contain regions that can form highly ordered, β -sheet-rich protein aggregates^{105–108}. Many proteins in nature can in fact form amyloids, many of them also with functional roles, although this feature does not make all of them prions that are capable of self-templating their protein conformation to other protein copies, which are in turn inherited across cell division or generations. Well-studied examples of amyloid-forming proteins in nature include Curli proteins in bacteria that are involved in biofilm formation¹⁰⁹. Understanding amyloid structure or its mechanism of assembly into aggregates is underscored by the fact that dozens of human diseases are also associated with amyloid-forming proteins¹¹⁰, including A β in Alzheimer's disease¹¹¹, and α -synuclein in Parkinson's disease¹¹². It has been suggested that some of these amyloid-forming proteins may be bonafide prions, since they undergo a conformational change and self-assemble into structured protein aggregates that can spread across brain tissue, for example. These aggregates can even form proteinaceous seeds to propagate and contribute to the progression of disease¹¹³. It remains unclear, however, whether these mammalian amyloids meet other definitions of prions in the classical sense, such as being infectious to other organisms, as is the case with PrP in mammals, or for fungal prions. Indeed, this remains a fascinating proposition, while in our view, further investigation is needed to lend support to this prion

hypothesis of neurodegenerative diseases, particularly with respect to their natural infectiousness from one organism or generation to another. From a molecular or evolutionary standpoint, the differences in how these proteins self-template their structures onto other copies, the reasons, and the outcomes, may indeed begin to blur with further study, perhaps encouraging reconsideration of what it means to be a “prion”.

The first prion protein identified, PrP^{Sc} (Sc = “scrapie”), forms amyloids and causes fatal brain spongiform encephalopathies in mammals¹¹⁴. After the discovery of PrP^{Sc}, all prions discovered during approximately the next two decades were also found to form amyloid aggregates, in fungi, bacteria, and even viruses^{35,37,115}. While the formation of amyloid structures universally reduced the activity of their encoded proteins, through sequestration of the protein into structured fibrils, the formation of these aggregates was not itself fatal to their host organisms, in contrast to the mammalian prion, PrP^{Sc}. Moreover, altering the activity of these proteins often produced altered growth phenotypes that could be physiologically beneficial under a range of conditions^{31,32,116}. More recently, through a different discovery pipeline, prion proteins lacking evidence of forming amyloids have been found in budding yeast³⁹. When cells contain these proteins in their prion conformation, activity is often maintained, and sometimes is even increased^{117,118}. The physical structures formed by these non-amyloid-forming prions are not well-understood, since only low-resolution methods have been used thus far. It also cannot be ruled out completely that these “non-amyloid” prions never form amyloid structures, even if only transiently. (A more extensive comparison of amyloid and non-amyloid prions is provided below.) A list of all known prions is provided in **Table 2.1**, separated by those that are amyloid-forming and those not known to form amyloids.

Table 3.1. A summary of all known amyloid-forming prions and non-amyloid-forming prions. All are from budding yeast (*Saccharomyces cerevisiae*), except: ^a = mammals, ^b = *Podospora anserina*, ^c = *Clostridium botulinum*. Brackets denote the non-Mendelian inheritance of this element in genetic crosses, and capital letters denote its dominance.

Amyloid-Forming Prions	Non-Amyloid-Forming Prions
PrP ^{Sc} ^{38 a}	[<i>GAR</i> ⁺] ³³
[<i>URE3</i>] ^{119,120}	[<i>SMAUG</i> ⁺] ¹¹⁷
[<i>PSI</i> ⁺] ^{29,120}	[<i>ESI</i> ⁺] ¹¹⁸
[<i>RNQ</i> ⁺]/[<i>PIN</i> ⁺] ^{41,121}	[<i>BIG</i> ⁺] ⁴²
[<i>MOT3</i> ⁺] ⁴⁰	Others ³⁹
[<i>MOD</i> ⁺] ³²	
[Het-s] ^{122 b}	
[<i>SWT</i> ⁺] ¹²³	
[<i>OCT</i> ⁺] ¹²⁴	
[<i>ISP</i> ⁺] ^{125,126}	
[<i>NSI</i> ⁺] ¹²⁷	
Cb-Rho ^{35 c}	

One common but not ubiquitous feature present in several prion protein sequences—including [*URE3*], [*PSI*⁺], [*RNQ*⁺]/[*PIN*⁺], [*MOT3*⁺], [*SWT*⁺], [*OCT*⁺] and Cb-Rho—is the enrichment of glutamine (Q) and/or asparagine (N). These Q/N-rich regions are most strongly associated with amyloid-forming prions in budding yeast, such that they have been predictive for identifying other potential prion candidates⁴⁰. Alberti *et al.* performed a hybrid computational–experimental prion screen, in which Q/N-rich regions served as one principle selection criteria, and identified 24 proteins with prion-like qualities out of an initial candidate list of 200 genes computationally screened from the proteome. Five of these were already known to be prions from prior work, validating the approach while producing an additional 19 new prion candidates. While informative about the prevalence of these types of prion sequences across the proteome, one caveat of this approach was that the first discovered and perhaps most well-characterized prion protein, PrP, does not contain Q/N-rich regions, and therefore would not have emerged

from such a screen. Other prions since identified—such as [*ISP*⁺], [*MOD*⁺], [*GAR*⁺], [*ESI*⁺], [*BIG*⁺] (**Table 2.1**), and others³⁹—would also not have been “hits” in this screen, because their proteins also do not contain Q/N-rich regions.

Given their recent discovery, less is known about the sequence characteristics of non-amyloid-forming prions. Chakrabortee and colleagues reported in 2016 an experimental prion-discovery screen, in which there was no computational pre-filter for Q/N-rich sequences or other features typical of previously discovered fungal prions³⁹. Instead, cells were screened for the capacity of nearly every protein in the proteome to individually elicit a long-lasting, epigenetic growth phenotype in response to transient overproduction of the protein. Among the dozens of “hits” emerging from this screen, the most consistent feature among the protein sequences were regions with high predicted disorder. Regions predicted to be structurally disordered, also known as “intrinsically disordered regions” (IDRs), do not resemble sequences found in structured portions of proteins. They are often depleted in hydrophobic amino acids that mediate cooperative folding. Additionally, they typically contain a higher proportion of polar or charged amino acids¹²⁸. Notably these regions were frequently poorly conserved at the level of linear amino acid sequence, even while the IDR itself was maintained over vast evolutionary timescales.

For some prion proteins, specific regions, sometimes referred to as “prion domains” (PrDs), have been characterized. These regions can be both necessary and sufficient to impart a protein with prionogenic behavior, including when added exogenously to other proteins, even with GFP^{40,129}. This modular nature—the regions are often separable from segments of the protein that carry out canonical activities—suggests the importance of structurally independent PrDs, and their role in prion conformational switching. Not all prions, however, contain such

separable regions. For example, for the budding yeast protein Rnq1, which forms $[RNQ^+]/[PIN^+]$, the regions involved in prion formation are not concentrated in one continuous segment of amino acid sequence, but instead are spread across multiple, distant segments in the protein¹³⁰. Many PrDs are also predicted to be IDRs, and sometimes these two terms are used interchangeably. It is not conclusively known whether a high degree of structural disorder is a prerequisite for a PrD to be able to undergo conformational changes that endow it with prion behavior. It is also unlikely that all proteins that contain relatively large IDRs are prions. Disordered regions have been shown to play many other important roles, such as in the regulation of cell signaling^{131,132}, where these functions would not necessarily require them to be prions.

High-Resolution Structures of Prions or Prion-like Proteins:

Few high-resolution structures of prions or prion-like proteins currently exist. Only three, all amyloid-forming, are known; one of these is a protein from fruit flies that has prion-like features¹³³ (**Table 2.2**). Their structural features vary significantly, and thus further investigations are needed before generalizable features of prion protein structure may be elucidated (**Figure 2.1**). The diversity of structural features may portend a larger number of potential prion proteins in proteomes. Investigating proteins with similar structural features to these is likely to be informative about the features that distinguish prions from non-prion proteins.

The first high-resolution structure of an infectious prion particle was the PrD of [Het-s], determined using solid state-NMR. [Het-s] is responsible for heterokaryon incompatibility in the filamentous fungus *Podospora anserina*. Its amyloid fibrils form a left-handed β -solenoid with a compact triangular hydrophobic core (residues 218–289)^{134,135}. An unusual structural

feature is the presence of pseudo-repeats of the β helix along the fibril axis, allowing a structure in which one molecule forms two turns of the solenoid. This early example of a functional amyloid was the first look into the structural features that govern prion amyloids.

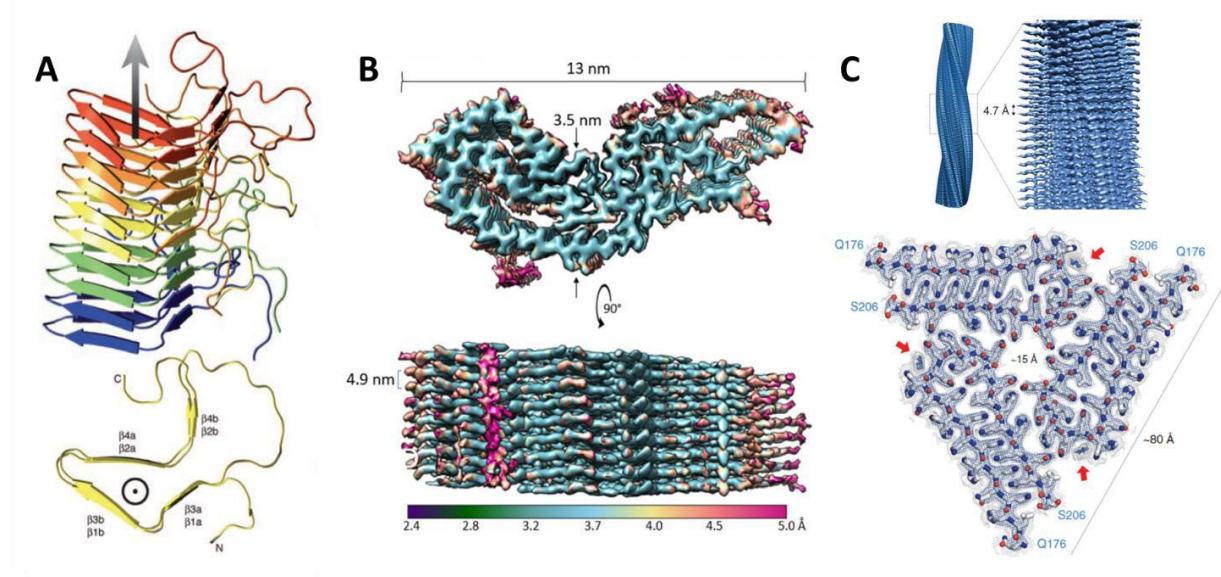


Figure 3.1. High-resolution structures of prion/prion-like amyloids. (A) [Het-s], reprinted from ¹³⁵ with permission from AAAS. (B) PrP^{Sc}, reprinted from ¹³⁶ with permission from Elsevier. (C) Orb2, reprinted from ¹³³ with permission from AAAS. As amyloids, these proteins show a similar packing of monomers along a fibril axis, however their core patterns vary considerably.

Table 3.2. A summary of the features of the high-resolution prion/prion-like protein structures^{133,135,136}.

Prion/Protein	[Het-s]	Prp ^{Sc}	Orb2B
Gene name	<i>het-s</i>	<i>PRNP</i>	<i>orb2</i>
Core residues (aa)	218-289 (72)	95-227 (133)	176-206 (31)
Structure/Symmetry	Left-handed solenoid	Parallel in-register β -sheets	Threefold triangular symmetry
Method to interpret structure	ss-NMR	Cryo-EM	Cryo-EM
Core	Hydrophobic	Hydrophobic	Hydrophilic
Stabilizing features	23 hydrogen bonds, three salt bridges, two asparagine ladders	GPI anchor, “Greek Key” motif, β -arches	Interdigitated cross- β structure, protonation of histidine

Post-translational modifications	Unknown	N-linked glycosylation	Unknown
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The culprit of all known mammalian prion diseases, such as Creutzfeldt-Jakob Disease (CJD) in humans and Mad Cow Disease in bovines, PrP is perhaps the best characterized prion protein. A 3.1 Å structure of an infectious, hamster brain-derived PrP core protein was recently solved by Kraus and colleagues using cryo-electron microscopy, marking an important advancement in the study of how prion structure relates to its function¹³⁶. PrP forms amyloid fibrils comprised of monomers with parallel, in-register, intermolecular β -sheets and connecting chains. This new structure differs from previous predictions from lower-resolution structures that PrP would contain β -solenoids or independent protofilaments, like other prion proteins^{137–141}. In a point of particular interest, this high-resolution structure revealed more about the positioning of asparagine (N)-linked glycosylation and the C-terminal GPI anchor, indicating that they lie toward the lateral edges of the fibrils. The GPI anchor, which will be explored in more detail in the next section, localizes PrP to the membrane and glycosylation stabilizes the stacking of monomers to form amyloid fibrils, allowing for precise self-templating of monomers and faithful propagation of the prionogenic form of PrP. This high-resolution structure represents a huge leap in understanding of how the structure of the protein monomer may promote its self-templating function.

A high-resolution structure was also recently solved of the putative prion protein Orb2, a conserved RNA-binding protein, also known as CPEB in other animals, that plays a role in long-term potentiation/memory formation in *Drosophila melanogaster*^{133,142}. The 2.6 Å cryo-EM structure shows that Orb2 forms 75 nm long, threefold-symmetric amyloid filaments. Of the two

isoforms of Orb2 in the fly brain, Orb2A is less abundant, but seeds aggregation of the more abundant Orb2B. The protofilament core of Orb2B, which extends from residues 176 to 206, forms a hairpin fold made up of two β -strands with a wide turn between them. The conformational change induced by this structure converts the protein's activity from a translational repressor into a translational activator. It was hypothesized that the hydrophilic, glutamine/histidine-rich fold could influence amyloid stability when the histidine residues are protonated, suggesting a mechanism for regulation. This structure provides a fascinating example of how an amyloid can serve as a stable, yet dynamic substrate of memory.

Conformational Switching, Self-Templating, and Factors Involved in Prion Propagation:

Perhaps the most important step in prion formation is the conformational switching of a protein from its non-prion, or “naive” state, into a prionogenic, self-templating state. The human PrP protein has long been known to be able to switch from its native alpha conformation, characteristic of PrP^C (c = “cellular”), into a more compact, highly soluble, β -rich monomer, a precursor to larger multi-protein, insoluble fibrillar forms¹⁴³. In the [*PSI*⁺] yeast prion, composed of the aggregates of the Sup35 translation termination factor, the intrinsically disordered and Q/N-rich “NM” domain at the N-terminus of the protein is necessary and sufficient for nucleation of prion “seeds”, or small multi-protein aggregates or assemblies that are capable of mediating transgenerational inheritance and growing into larger amyloid fibers. Assembly of these aggregates proceeds by monomer addition^{144–146}. Within the NM-domain, also known as the PrD of Sup35, relatively short, specific sequences are required to nucleate the conversion of Sup35 from a soluble monomer into aggregates¹⁴⁷. One recent study demonstrated how the size of the nucleation seed itself is also responsible for persistence of amyloid aggregation,

highlighting how this biochemical feature influences transgenerational stability of the prion-based phenotypes¹⁴⁸.

While prions are commonly thought to self-template conformational changes in other copies of the same protein, there is also evidence for one prion protein seeding the formation of a second prion protein. In fact, formation of the $[PSI^+]$ prion in vivo requires pre-existing $[RNQ^+]/[PIN^+]$, and there are particular sequences in both Sup35 and Rnq1 proteins that facilitate this cross-seeding¹⁴⁹. $[RNQ^+]/[PIN^+]$ can even promote amyloid formation of exogenously expressed proteins, such as those involved in some human neurological diseases¹⁵⁰.

There are also cases where cellular structures, such as membranes, are also key for promoting self-association of monomers that could increase the likelihood of conformational changes or self-templating behavior. PrP protein is anchored to the membrane via a GPI anchor in its C-terminus. Its conformational switching involves the peeling of the C-terminal β 1-helix 1- β 2 loop away from the helices 2 and 3, and the unspiraling of each of the helices to form the extended strands of the middle and disulfide β arches that are central to the structure of the prionogenic form, PrP^{Sc}¹³⁶. PrP is not the only prion protein known to reside in membranes. $[GAR^+]$ is a prionogenic form of the highly abundant yeast plasma membrane protein Pma1, however it is not known to what extent its cellular localization influences its propensity for prion conversion or self-assembly³³.

Another important feature of prion propagation and inheritance, as established from many studies in budding yeast, is the requirement for protein chaperones. Amyloid-forming prions depend on the heat shock protein and disaggregase, Hsp104^{32,40,151}, while non-amyloid-forming prions depend on Hsp70 and Hsp90^{33,39}. Thus, while prions are believed to undergo autonomous conformational switching and self-templating, their transgenerational inheritance depends on

these important co-factors. For Hsp104 clients, the need arises for the amyloid aggregates to be divided sufficiently to facilitate passage during cell division^{152–154}. For Hsp70 clients—i.e., non-amyloid-forming prions—the specific physical interactions that the protein chaperones have with prion proteins that enable their propagation are not known.

There is extensive evidence that amyloid-forming prion proteins can assume more than one conformational state in their self-templating form, known as the “strain” phenomenon. For example, the $[PSI^+]$ prion can undergo stable propagation of different strains, reflected in both the cellular phenotypes as well as their physical structures^{31,130,155–157}. Similarly, the [URE3] prion has also been shown to contain different strains^{155,158,159}, as has $[RNQ^+]/[PIN^+]$ ¹⁵⁵. The concept of prion strains is not well explored for the non-amyloid-forming prions (**Table 2.1**). This remains an important question for future studies, especially when coupled with structural studies of these more recently discovered prions. Prion conformations are also reversible. While measurements of the frequencies of loss of the prion state are not widely known, some measurements suggest that they are relatively infrequent¹⁶⁰.

Contrasting Amyloid-Forming vs. Non-Amyloid Prions, and Methods to Identify Them:

While high-resolution detail of prion protein structures from biophysical studies may be limited, there is evidence that they can form a variety of macromolecular structures in vivo. When taking into consideration proteins with prion-like domains, we can observe structures that vary from rigid amyloid fibrils, to gel-like condensates, to liquid–liquid assemblies^{117,136,161,162}. As described above, prions can be generally categorized into amyloid-forming and non-amyloid-forming. Below, we discuss what is known about how these two classes of prions aggregate in vitro and in vivo and how they differ.

More is known about amyloid-forming prions, as they were amongst the first prions discovered^{32,40,116,151,163}. Amyloid-forming prions can produce highly ordered β -sheets that lead to structured and thermodynamically stable fibrils^{106,133,136}. As naïve monomeric protein is sequestered into filaments, this generally leads to loss of or inactivation of protein function. The amyloid structures formed are also resistant to certain physical and chemical perturbations, including temperature and detergents^{164,165}. Indeed, an established property to test the formation of amyloids is testing a protein's resistance to SDS detergent^{32,117,164}.

Less is known about non-amyloid-forming prions. They are sensitive to denaturants such as SDS, a property which can be used to distinguish them from amyloid-forming prions^{39,40,117}. Genetic evidence also supports this distinct class of prions as not forming an amyloid intermediate that is essential for propagation of the prion structure. Curing of amyloid-forming prions can be accomplished through transient inhibition of the protein disaggregase chaperone, Hsp104. In contrast, the non-amyloid-forming prions can be cured by transient Hsp70 inhibition, but not Hsp104 inhibition^{33,39,42}. IDRs are another feature of non-amyloid-forming prions that have been identified as drivers of prion phenotypes³⁹ (as noted earlier, these may also be found in amyloid-forming prions). For example, the IDR region in the [*SMAUG*⁺] prion drives its self-assembly properties, allowing gel-like condensates to form¹¹⁷. It is possible that the more dynamic structure of non-amyloid assemblies allows manipulation of phenotypic states without elimination of protein activity^{39,42,117,118}, permitting a regulatory paradigm of prion induction and loss in response to environmental cues. More characterization is needed before such conclusions can be established.

Biochemical techniques have been key to characterizing prion protein assemblies.

Dyes that specifically bind to amyloids have been used to analyze the kinetic and structural properties of amyloid-forming prions. Compounds such as Thioflavin T (ThT)¹⁶⁶ and Congo Red (CR)¹⁶⁷ produce fluorescence when bound to amyloid β -sheets, making them an excellent method to detect the presence of amyloids. Another method to detect amyloid-forming prions is semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)^{168,169}. This technique exploits the fact that amyloid-forming prions are resistant to 2% SDS at room temperature, permitting visualization of the distribution of large biopolymer assemblies and monomeric protein. Additionally, it allows detection of amyloid fibers from cell lysate rather than from purified protein and has been used to characterize [*PSI*⁺], [*RNQ*⁺]/[*PIN*⁺], and [*MOT3*⁺]³⁴, among others.

Microscopy is another approach for characterizing prion aggregation, both in vivo and from purified protein^{39,40}. GFP-fusion proteins allow visualization of prion aggregation in vivo, a useful tool for observing protein localization, aggregation formation, and induction of the prion state¹⁷⁰. This has been used in characterizing [*PSI*⁺], [*MOT3*⁺], [*BIG*⁺], [*SMAUG*⁺] and more^{40,42,117,171}. Fluorescent-reporter systems have also been developed for studying the kinetics of prion assembly formation, such as DAmFRET¹⁷² and yTRAP¹⁷³, using [*PSI*⁺] to demonstrate their effectiveness. Several prions have been characterized using centrifugation, such as [*PSI*⁺]¹⁷¹. Combined with native separation techniques using sucrose gradients, the distribution of protein assemblies can be observed and quantified¹⁷⁴. Altogether, these techniques have proven useful over the years to investigate the biochemical properties of prions.

Conclusions and Challenges:

An inherent difficulty in the field of prion research is that, despite this phenomenon being described in the literature as early as the 1960s²⁹ and studied in fine detail in the case of PrP,

insight into the basic biochemical properties has been somewhat shallow. Limitations in prion discovery have yielded too few examples from which to extract general principles. As prions cannot be unambiguously identified from DNA or protein sequences, from co-immunoprecipitations, from proteomics, from standard genetic screens, or from microscopy and other standard cell biological methods, new examples can be challenging to determine. We do not know of a discrete property of prions that could be used to identify them easily. Indeed, most descriptions of new examples involve a battery of tests that help exclude other possibilities that are consistent with the observed phenotypes, while also establishing known behavior associated with other prions.

Yet, we know that they are conserved across domains of life and several existing examples show that they exert a strong influence over biological processes. Without identifying and understanding more of them, we are overlooking a critical determinant of transgenerational inheritance and organismal function in nature.

In the early 1990s, when knowledge of the “histone code” and the general influence of chromatin modifications on the structure and function of genomes came to light, it was unlikely many could have foreseen the importance of this epigenetic code on both basic cellular functions and a myriad of human diseases. After decades of study, prions are a well-established biological phenomenon. Yet, the challenge of discovering them and measuring them remains, and we believe they must be explored more deeply to assess the extent of their broader impact in nature. Grounding our understanding of their inheritance and regulation in biochemical terms is an excellent starting point and should accelerate further discovery. It will be necessary to revisit these terms periodically, as new examples mature our understanding of this important but still underappreciated epigenetic phenomenon.

Bridge to Chapter III:

Chapter III summarizes our biochemical understanding of prions, and how their structure/biophysical state informs their behaviors and functions in the cell. This was the starting point for my work in Chapter IV, in which I investigated the biochemical state of $[BIG^+]$ with more rigor, aiming to find evidence for an altered protein conformation, perhaps one distinct from amyloids. This avenue of investigation also explores several possible explanations for the mechanism resulting in its growth phenotypes, including 1) catalysis, 2) biophysical state, and 3) new associations with other factors.

CHAPTER IV

THE MOLECULAR BASIS OF THE PRION [*BIG*⁺]

Author Contributions:

This work was led by me with guidance from my advisor Dr. David Garcia. Several other members of the Garcia lab contributed reagents, strains, and supporting results, including Abigail Vaaler, Ethan Shaw, Julia Reinsch, and Preeti Bhattacharjee. The LiP-MS experiment was performed in collaboration with Neil Wood and Dr. Stephen Fried at Johns Hopkins University. [*PSI*⁺] strains were provided by the Halfmann lab at the Stowers Institute. Quantitative mass spectrometry was performed by the IDeA National Resource for Quantitative Proteomics. Currently, none of these materials are published.

Introduction:

Prions are a class of proteins that undergo a conformational switch in folding, potentially altering the activity of the protein. Through self-templating, these conformational changes can be inherited across generations, thereby altering cellular activities epigenetically based on protein conformation, rather than genetic code. Until recently, scientists exclusively viewed prions as deadly agents of disease, such as in Mad Cow disease and Creutzfeldt-Jakob disease^{30,38}. However, recent research has highlighted the adaptive role of prions in response to environmental signals, across many domains of life^{32,33,42}.

The budding yeast prion [*BIG*⁺] (better-in-growth), was first reported in depth in 2021⁴². This study established its phenotypes as prion-based through classical prion inheritance and propagation experiments and identified the protein responsible for this inheritance as Pus4, an RNA-modifying enzyme. The study went on to characterize [*BIG*⁺] phenotypes, including its notable effects on cell growth and lifespan, and began investigations into its effects on translation, the proteome, and how its canonical cellular function might be altered to produce these phenotypes.

At the time of this investigation⁴², the evidence for the [*BIG*⁺] prion met the genetic definition of the state. Its phenotypes were inherited in a non-mendelian fashion, were inherited through the cytoplasm, and its curing was chaperone-dependent. The propagation of the state was shown to be dependent on the presence of the gene *PUS4*. The primary phenotypes for which this state was tracked were that of large cell size and increased translation of a rare codon-enriched luciferase reporter gene. While this study provided evidence for these and other phenotypes being driven by an epigenetic factor – presumably a prion – it lacked a description of its biophysical state, nor did it establish a mechanism by which an altered protein conformation

could drive the inheritance of these traits. The goal of my thesis work was to 1) measure the biophysical state of Pus4 involved in propagating these traits, and 2) investigate the molecular mechanism of this epigenetic phenomenon.

Results:

Pus4 does not form large oligomers or aggregates in the $[BIG^+]$ state

To investigate the biophysical state of Pus4, I performed a pellet-supernatant sedimentation assay. This method has previously been used when detecting aggregates of classical prions, such as $[PSI^+]$, where nearly all Sup35 is sequestered into the pellet fraction^{171,175}. When performing this assay on a naïve and $[BIG^+]$ strain, we found no increased sedimentation of Pus4 into the pellet in the $[BIG^+]$ compared to the naïve (**Figure 4.1A**). Thus, if Pus4 is physically altered in the $[BIG^+]$, it is not due to the formation of large aggregates or oligomers that sediment under these conditions. While large oligomers were not detected, we wanted to better resolve the size distribution of potential Pus4 assemblies. We used a native (non-denaturing) lysis protocol paired with sucrose gradient density ultracentrifugation, followed by SDS-PAGE and western blot, to detect an endogenously expressed HA-tagged Pus4 in naïve and $[BIG^+]$ strains (**Figure 4.1B**). This technique relies on sedimentation through a sucrose gradient to separate native assemblies and without disruption of inter-molecular interactions. In this assay, we observed no shifts in the distribution of assemblies that would suggest that a new Pus4-based structure is formed. A recombinantly purified Pus4 sample was also run on these gradients, showing the same distribution as Pus4 from cell lysate, suggesting that the signal observed for Pus4 alone represented monomers and/or dimers, and not larger multimers or interactions with other large complexes.

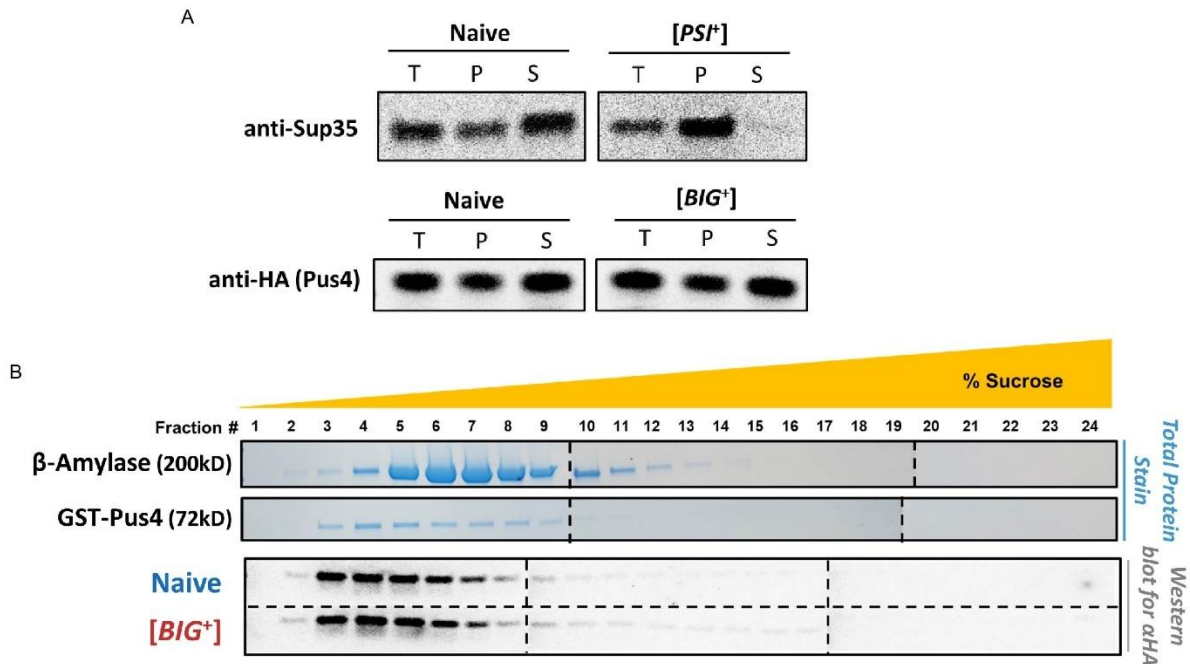


Figure 4.1: Pus4 does not form aggregates or higher order oligomers in the $[BIG^+]$ state. **A)** Western analysis of whole cell lysate sedimentation shows the separation of the total (T) input into the pellet (P) and supernatant (S) fractions. **B)** Sucrose gradient density ultracentrifugation shows the distribution of protein assemblies from native lysate of naïve and $[BIG^+]$. Recombinantly purified GST-tagged Pus4 shows a similar profile. A loading control showing the sedimentation of β -amylase is included for reference.

Some possible explanations that could explain the lack of prion-like aggregates are that

- 1) These methods are not sufficient to detect a non-amyloid biophysical state, which may be distinct from previous examples in the literature, 2) The prion aggregate is unstable in the conditions tested here, or 3) The epigenetic mechanism for $[BIG^+]$ does not involve a prion aggregate. To discriminate further between these explanations requires a different approach, but the exclusion of a prion aggregate, that is detectable under these conditions in the case of other classical prions, remains striking.

Pus4 has no detectable altered structural features in the $[BIG^+]$ state

To detect a Pus4 structural difference in $[BIG^+]$ that might be localized to a specific region of the protein, we collaborated with the Fried lab at Johns Hopkins University. We first performed whole cell lysate limited proteolysis-mass spectrometry (LiP-MS) comparing naïve strains to $[BIG^+]$ strains. A short list of proteins was implicated with altered peptides/structure (**Table 4.1**). Of these, the most interesting was Sui1/eIF1, which has a key role in translation initiation. Sui1 is essential for survival and mutants show alternative start codon initiation, such as at near cognate codons or AUGs in poor sequence context¹⁷⁶. Altered structure of this protein could be related to the large translation phenotypes seen in $[BIG^+]$ cells. Pus4 itself, however, was not detected in this experiment, likely because its level of protein expression remained below detection levels.

Table 4.1: Significantly altered peptides from whole cell lysate LiP-MS of naïve vs. $[BIG^+]$ cells. The proteins listed have a significant difference in at least two peptides in $[BIG^+]$ cells compared to the naïve cells.

Gene	Protein Description	No. of Valid Peptides	No. of Significant Peptides (P-value)	No. of Significant Peptide (Adj. P-value)
TDH3	Glyceraldehyde-3-phosphate dehydrogenase 3	188	9	2
CPA2	Carbamoyl-phosphate synthase arginine-specific large chain	81	2	2
SUI1	Eukaryotic translation initiation factor eIF-1	12	2	2
PGM1	Phosphoglucomutase 1	25	2	2
CPR5	Peptidyl-prolyl cis-trans isomerase D	14	2	2

To address the problem of Pus4 detection, I established a method to pull down HA-tagged Pus4 using HA magnetic affinity beads (**Supplemental Figure 4.1**). These samples were then analyzed via LiP-MS. This experiment allowed us to achieve two goals: 1) enrich for peptides of Pus4 to allow us to detect Pus4-specific structural differences, and 2) look for any differences in the association of other proteins to Pus4 in both the naïve and [*BIG*⁺] strains. While we were able to enrich Pus4 to detectable levels, we found no significant differences in the structure of Pus4 in [*BIG*⁺] cells. Ultimately, this result agrees with the pellet-supernatant sedimentation and sucrose gradient sedimentation results. Additionally, there were no significant differences in the list of associated proteins — both strains pulled out an identical short list of primarily ribosomal proteins (**Supplemental Table S4.1**). Thus, it appears that no stable altered protein interaction or structural difference in Pus4 could explain the basis for the phenotypes in [*BIG*⁺] cells.

WCL-MS reveals Yak1 and its targets have reduced expression in [*BIG*⁺]

To look more broadly for proteins whose expression is altered in the [*BIG*⁺] state, I prepared whole cell lysates of naïve and [*BIG*⁺] strains. These samples were sent to the IDeA National Resource for Quantitative Proteomics core for quantitative mass spectrometry analysis. Twelve proteins were found to be downregulated in [*BIG*⁺] cells (**Table 4.2**). The protein with the largest difference in expression was Yak1, a serine-threonine protein kinase at the center of glucose sensing and stress response signaling (**Figure 4.2A**). We also verified this downregulation via western blot analysis (**Figure 4.2B**). No proteins were found to be upregulated in [*BIG*⁺] cells.

Table 4.2: Significantly downregulated proteins from the whole cell lysate IDeA proteomics quantitative dataset. All proteins are downregulated in [*BIG*⁺]. Hits from the RNA-seq dataset from Garcia and Campbell *et al.*, 2021 that overlap with this dataset are marked with an asterisk. Protein descriptions are sourced from SGD.

Downregulated in [<i>BIG</i> ⁺]	Protein description
ALD3*	Aldehyde dehydrogenase [NAD(P)+] 2, Cytoplasmic aldehyde dehydrogenase; involved in beta-alanine synthesis; uses NAD ⁺ as the preferred coenzyme; very similar to Ald2p; expression is induced by stress and repressed by glucose
CTT1*	Catalase T, protects from reactive oxygen species
FMP45*	SUR7 family protein FMP45, Integral membrane protein localized to mitochondria; required for sporulation and maintaining sphingolipid content;
FRE2	Ferric/cupric reductase transmembrane component 2, Ferric-chelate reductase involved in copper ion import and iron ion transport
GND2*	6-phosphogluconate dehydrogenase, decarboxylating 2; catalyzes an NADPH regenerating reaction in the pentose phosphate pathway;
HBT1	Protein HBT1, Shmoo tip protein
HSP26	Heat shock protein 26, Small heat shock protein (sHSP) with chaperone activity
HXT5	Probable glucose transporter HXT5, Hexose transporter with moderate affinity for glucose; induced in the presence of non-fermentable carbon sources, induced by a decrease in growth rate.
MSC1	Meiotic sister chromatid recombination protein 1, Nuclear envelope protein involved in DSB repair during late mitosis; forms nuclear envelope patches associated with Rad52p-containing DNA repair foci, and accelerates homologous recombination downstream of DNA end resection;
RTN2	Reticulon-like protein 2; involved in nuclear pore assembly and maintenance of tubular ER morphology; promotes membrane curvature; regulates the ER asymmetry-induced inheritance block during ER stress;
SSA3	Heat shock protein SSA3, Cytosolic ATPase involved in protein folding and the response to stress; member of the heat shock protein 70 (HSP70) family;
YAK1	Serine-threonine protein kinase; component of a glucose-sensing system that inhibits growth in response to glucose availability;

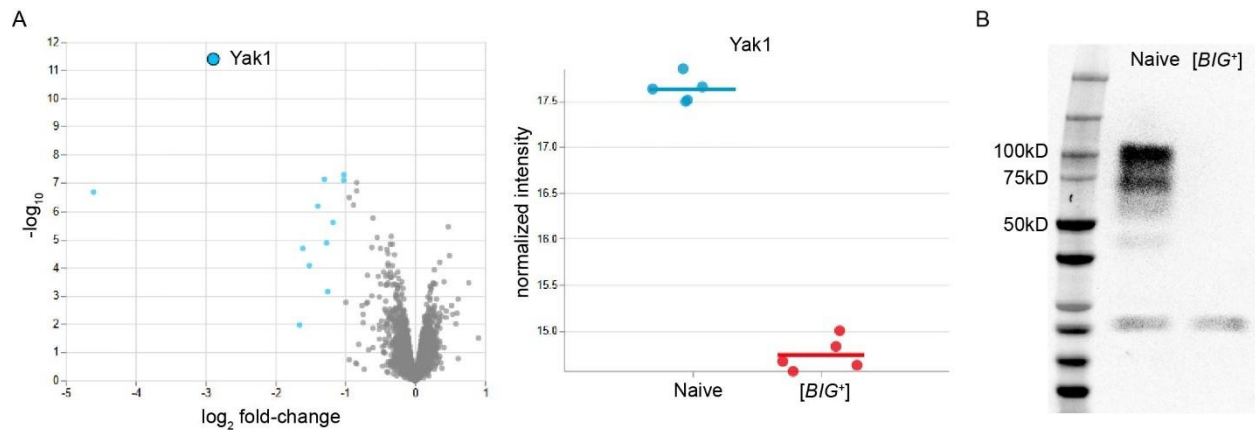


Figure 4.2: Yak1 has reduced protein expression in $[BIG^+]$ cells. **A)** Volcano plot showing the differential expression of naïve vs. $[BIG^+]$. The blue dots are the twelve proteins listed in **Table 4.2**, with Yak1 labeled at the large blue dot. The plot to the right shows the normalized intensity of Yak1 signal in naïve and $[BIG^+]$ lysate. **B)** Anti-HA western blot of a C-terminal 3xHA-tagged Yak1 (98 kDa) strain in a naïve and $[BIG^+]$ background (40 mg of total protein loaded).

Yak1 is a protein kinase that regulates cell growth and the general stress response (**Figure 4.3**). High glucose activates PKA (Protein Kinase A) and the TOR kinase (target of rapamycin), which both negatively regulate the kinase activity of Yak1, ultimately promoting cell growth¹⁷⁷. During periods of low stress/glucose abundance, PKA can phosphorylate Yak1, which restricts its localization to the cytoplasm. During heat shock stress or glucose scarcity, PKA activity is reduced, freeing an unphosphorylated form of Yak1 to enter the nucleus, where it phosphorylates the transcription factors Hsf1 and Msn2/4, in turn activating them^{178–180}. Hsf1 and Msn2/4 increase the expression of many heat shock element genes and stress response element genes. Several of these genes are also proteins that were downregulated in the IDeA proteomics dataset (**Table 4.2**). Therefore, we reasoned that this gene expression profile could explain the basis of several $[BIG^+]$ phenotypes, since lower Yak1 availability would enable $[BIG^+]$ cells to have increased proliferation during glucose scarcity. Essentially, $[BIG^+]$ cells are relatively insensitive to glucose levels and thus grow beyond the normal limits of the cell even

when nutrients are low. While this finding suggested a potential mechanism for $[BIG^+]$, linking metabolism with its growth phenotypes, it remained unclear how Pus4 might be connected to reduced abundance of Yak1 in $[BIG^+]$ cells.

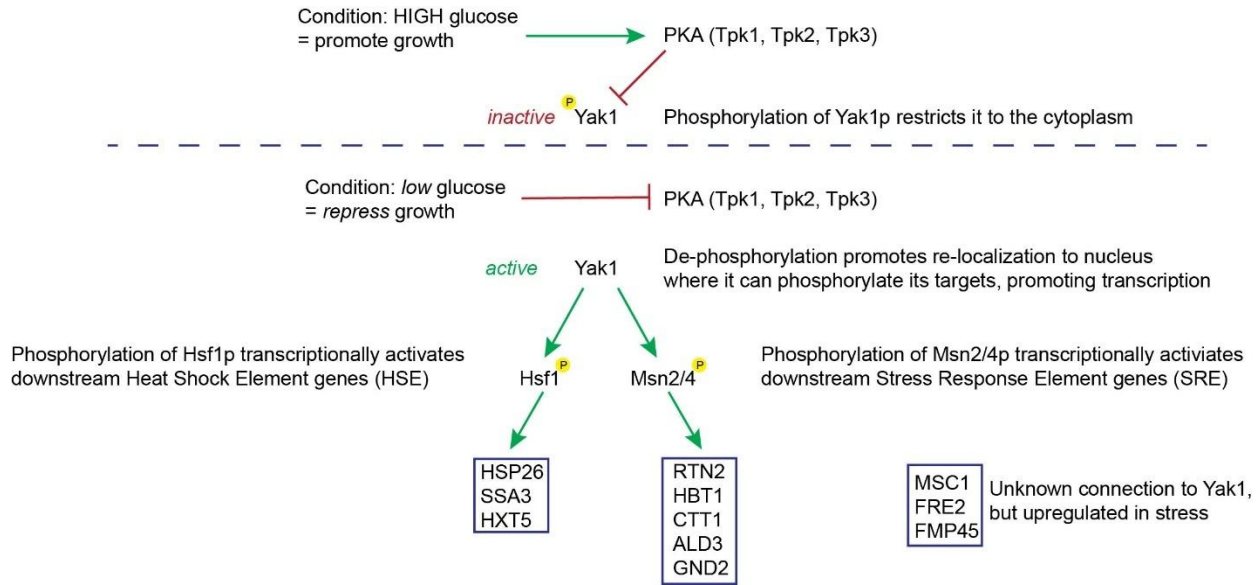


Figure 4.3: Summary of Yak1 activity, as related to hits in the WCL-MS IDeA dataset in **Table 4.2**. When glucose is abundant, PKA phosphorylates Yak1, keeping it in the cytoplasm. When glucose levels are low, PKA activity is diminished, allowing an unphosphorylated Yak1 to enter the nucleus and phosphorylate Hsf1 and Msn2/4, along with other targets that are not shown here in this diagram. Hsf1 and Msn2/4 have been shown to target the following hits in the WCL-MS dataset from IDeA, and the hits without connection are in the blue box to the side.

Whole genome sequencing reveals an unexpected nonsense mutation in *YAK1* in $[BIG^+]$ strains

While many of the earlier observations were consistent with an epigenetic phenomenon, we had not sequenced the genome of $[BIG^+]$ strains to rule out any possible mutations that may have been involved with the phenotypes. We performed whole genome sequencing of our naïve and $[BIG^+]$ strains to look for any mutations that could explain $[BIG^+]$ phenotypes. While we generally uncovered very few mutations present in one strain, but not the other, one change stood

out and was a shock for us to discover — a nonsense mutation, Q578*, that leads to an early stop codon at position 578 in the *YAK1* open reading frame which was present in all the [*BIG*⁺] strains, but not our naïve strain. To determine if this mutation was involved in these phenotypes, we tested growth of these strains in paromomycin, a translation inhibitor. We had previously determined that [*BIG*⁺] cells were sensitive to paromomycin, so we prepared [*BIG*⁺] strains that had the *YAK1* Q578* mutation reverted, and naïve strains that had the *YAK1* Q578* mutation inserted. The growth assay results show that the *YAK1* Q578* mutation is responsible for the paromomycin sensitivity in [*BIG*⁺] strains (**Figure 4.4**). While we do not yet know if other [*BIG*⁺] phenotypes are dependent on this mutation of Yak1, this discovery prompted us to reconsider our earlier data, both published and unpublished. Other [*BIG*⁺] phenotypes will need to be investigated before more definitive conclusions can be made.

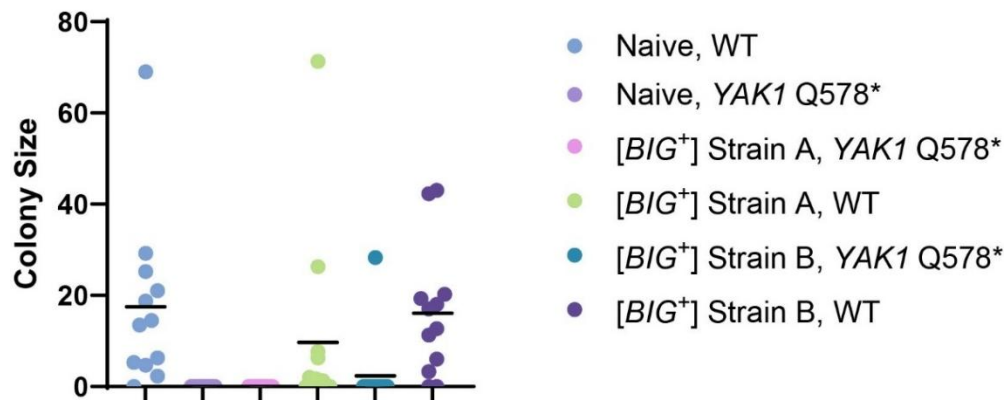


Figure 4.4: The *YAK1* Q578* mutation causes a sensitivity to paromomycin. 72-hour growth assays in CSM with 10 mg/mL paromomycin. The black line marks the average colony size.

A closer look into the literature revealed that this *YAK1* Q578* variant was previously reported as present in other yeast laboratory strains with the same lineage as the [*BIG*⁺] strains have (BY4741). This mutation was found in many strains from the GFP- and TAP-tagged

libraries¹⁸¹. These researchers looked at the RNA and protein levels resulting from this variant, as compared to strains with a WT *YAK1* locus, and changes they observed showed some agreement with our WCL-MS dataset (**Supplemental Table S4.2**). They also performed growth assays and found that both *YAK1* Q578* strain and *yak1*Δ strain grew better in high salt compared to WT strains, suggesting that the *YAK1* Q578* variant likely phenocopies a deletion of the *YAK1* gene¹⁸¹. These yeast collections or a close relative of their ancestor may have been the original source of the mutation back when the prion screen was performed in the early 2010's³⁹. A look at the AlphaFold3 predicted structure of the mutant suggests that with the last third of the protein missing, it's likely that the whole protein is nonfunctional as it is missing a large, folded domain¹⁹ (**Figure 4.5**). Unfortunately, the western blot evidence for a reduction in Yak1 expression cannot show us the presence of truncated products, since the 3xHA tag is on the C-terminal end of the protein (**Figure 4.2**). This could be tested by generating an N-terminally tagged strain of Yak1 in the [*BIG*⁺] background and repeating the western. Overall, it seems unlikely that there are stable, abundant protein truncations in the cell, and the downregulation in the WCL MS dataset likely reflects a complete knockdown of Yak1 protein levels.

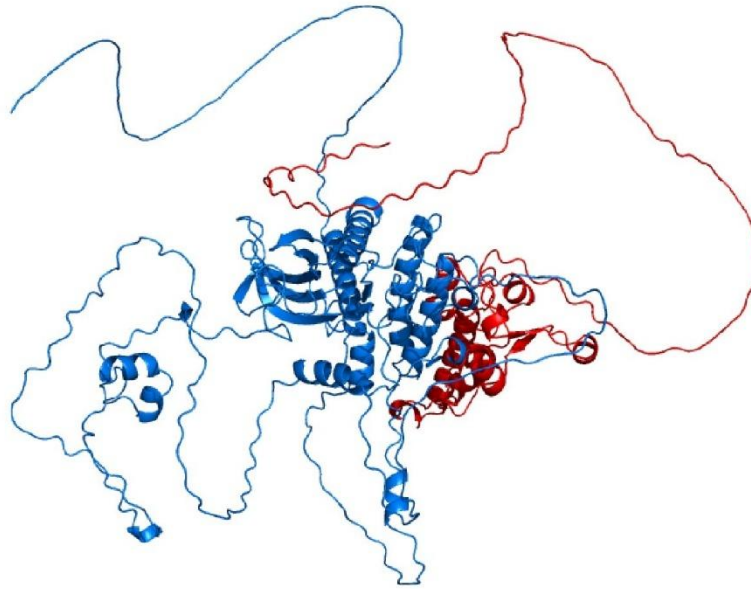


Figure 4.5: AlphaFold3 predicted structure of wild-type yeast Yak1 (blue). The red portion of the structure represents the region deleted in the nonsense mutant Q578*.

***pus4*Δ strains are sensitive to paromomycin**

It has been previously reported that *pus4*Δ also has reduced growth in paromomycin¹⁸². This suggests that Pus4 could have an undescribed role in affecting growth or translation, perhaps through a modification of *TEF1* mRNA or a Ψ_{55} -dependent effect on tRNA aminoacylation. This phenotype was initially challenging to confirm, since we found that the drug-resistance marker used for replacement in the standard deletion collection strain itself conferred resistance to paromomycin (**Figure 4.6**). Therefore, we generated a nonsense mutant of Pus4 to inactivate it without the need to replace it with another gene that might impact growth in paromomycin. In summary, caution should be exercised when designing experiments using yeast collections or examining proteins involved in translation. Regardless, sensitivity to this translation inhibitor when cells are missing Pus4 does link this enzyme to translation, and in the

future, we hope to investigate this further to determine whether this is due solely to its modification of tRNA, or whether it also involves other roles of the protein.

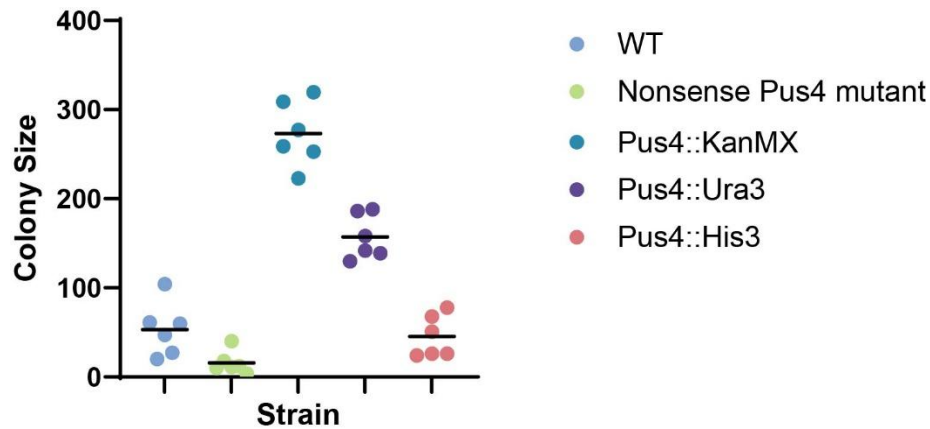


Figure 4.6: Growth of Pus4 deletion strains in 10 mg/mL paromomycin on CSM agar after 72 hours. Strains include a wild-type strain, a Pus4 strain with an early nonsense mutation, and three replacements of the Pus4 gene with an auxotrophic marker.

The C-terminus of Pus4 influences paromomycin sensitivity

A specific role for the C-terminus of Pus4 has yet to be identified. Its specific sequence is poorly conserved, in contrast to other regions of the protein, yet the presence of a C-terminal extension is highly conserved. One possible hypothesis is that it generally assists in tRNA binding via electrostatic interactions, like TruB's PUA domain, in helping to position the tRNA in the catalytic site. Alternatively, it could be involved in binding to a different unidentified protein or RNA. To test its possible role in the $[BIG^+]$ state, mutants of Pus4 with the charged features of the intrinsically disordered C-terminus removed or enhanced were generated in naïve and $[BIG^+]$ backgrounds. (This investigation was performed prior to our discovery of the *YAK1* mutation, and thus all strains involved here did contain the mutation.)

These Pus4 mutant strains were grown in paromomycin sulfate to observe C-terminus-dependent changes in their sensitivity to this translation inhibitor (**Figure 4.7**). In the naïve strains, these mutants did not consistently impact sensitivity/resistance. But in the [*BIG*⁺] strains, a mutant with all of the charged residues of Pus4's C-terminus mutated to alanine ("No Charge") rescued the paromomycin sensitivity. Another mutant in which all the charged residues of Pus4's C-terminus were mutated to Aspartic Acid ("All Positive"), showed a similar trend. This suggests that the loss of negative residues in this region (and in the presence of the *YAK1* Q579* mutation) can modulate this sensitivity to paromomycin.

Since the deletion of Pus4 also results in a sensitivity to paromomycin (**Figure 4.6**), how could altering the sequence of its C-terminus in the context of the *YAK1* Q579* mutation provide resistance? While we do not currently understand the exact mechanism to produce this result, it does suggest a few possibilities: 1) Pus4 has a role in translation, that can be tempered based on the sequence of its C-terminus, suggesting the C-terminus has a role in this function. 2) The *YAK1* Q578* mutant appears to mask this effect in Pus4, suggesting a genetic suppression of Pus4 phenotypes. Whether this occurs through direct interaction or parallel pathways remains unclear.

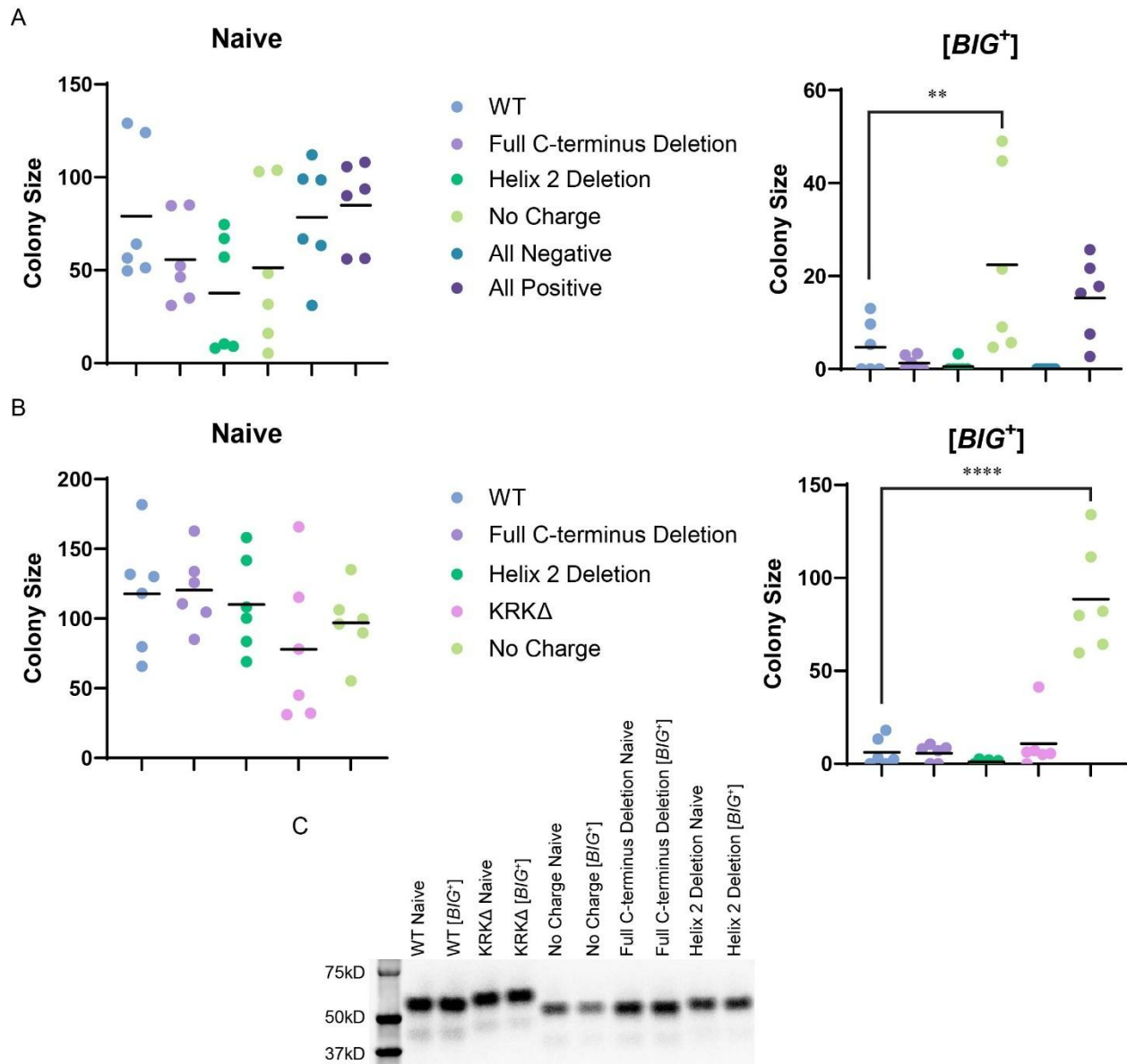


Figure 4.7: The C-terminus of Pus4 modulates growth in the translation inhibitor paromomycin. 72-hour growth assays of Pus4 C-terminus mutants in 10 mg/mL paromomycin in CSM agar. Two separate experiments are shown (**A**, **B**). The average of each dataset is shown by the black line. Significance determined by student's T-test. **C**) Western blot showing protein expression of 3xHA-tagged Pus4 C-terminus mutants. 10 mg of total protein was loaded into each lane.

Discussion:

The discovery of the *YAK1* nonsense mutation Q578* in $[BIG^+]$ strains has introduced many questions about its definition as a prion and the premise of my thesis work. I have clearly shown that the sensitivity of $[BIG^+]$ strains to paromomycin is due to this mutation, yet other experiments must be repeated to directly assess the effect of this mutation on those phenotypes, such as large cell size, increased proliferation, enhanced translation, and reduced lifespan. While it remains possible that those phenotypes may be, in part, independent of *YAK1* Q578*, a putative knockdown of this protein kinase involved in cell growth regulation and glucose signaling could contribute to such phenotypes. Additionally, my other line of investigation in examining the biophysical state of $[BIG^+]$ revealed no aggregates or altered features, using several approaches. Therefore, my thesis work has developed our understanding of genetic context of $[BIG^+]$, tied this context to $[BIG^+]$ phenotypes, and found no evidence of a prion aggregate driving this epigenetic state.

While my thesis work finds limited evidence for a prion definition based on the formation of a classical prion aggregate, there remains convincing evidence for this epigenetic state in the previous publication⁴². In particular, the non-mendelian inheritance of $[BIG^+]$ across four spores derived from a diploid suggests a Pus4-dependent prion state. The curability of the $[BIG^+]$ state by Hsp70 also supports a prion mechanism, and it's hard to imagine other explanations for this. Other prions have been shown to be Hsp70-dependent in their curing, such as $[GAR^+]$ and $[SMAUG^+]$ ^{33,117}. Additionally, the cytoductions demonstrate that the state can be inherited through the cytoplasm, which would not be consistent with the genomic *YAK1* mutation as the sole cause of these phenotypes. Studies that have examined the chronological lifespan of *yak1Δ* strains do not demonstrate the same magnitude of defect in lifespan¹⁸³, suggesting an additional

causative source in $[BIG^+]$ cells. Overall, it seems challenging to explain all of these observations with a *YAK1* mutation alone.

One possible explanation is that there is another protein or RNA factor involved in the $[BIG^+]$ state. While the HA-IP LiP-MS experiment ruled out a stable altered interaction of Pus4 with a protein factor, it was unable to detect RNAs or transient interactions with proteins. Another pulldown strategy could assess this, such as RIP-seq and a mass spectrometry approach where the protein products of the pulldown are stabilized via cross-linking. $[GAR^+]$ is dependent on two protein factors, giving weight to this possibility, although in this case the interaction is highly abundant/stable and detectable via a simple pulldown strategy and protein stain³³. Another factor aside from or in combination with Pus4 may be driving the original epigenetic inheritance of phenotypes.

To more definitively determine if $[BIG^+]$ is a prion, several other experiments should be performed testing other phenotypes of $[BIG^+]$. This includes re-testing the large cell size phenotype, the luciferase reporter composed of rare codons, and lifespan experiments that were the focus of the previous publication⁴². Ultimately, it will require reverting the *YAK1* Q578* mutation in these strains or inducing a new $[BIG^+]$ strain entirely through transient overexpression of *PUS4* and repeating the inheritance experiments. Repeating this work in $[BIG^+]$ strains with the *YAK1* mutation reverted will allow clear delineation of whether an epigenetic factor, prion or not, is driving $[BIG^+]$ phenotypes.

Beyond expanding our fundamental understanding of $[BIG^+]$, my thesis research supports a newly discovered role for Pus4 in translation. While it was previously reported that *pus4Δ* had a sensitivity to the translation inhibitor paromomycin, my work provided the first examination of the C-terminus of Pus4 and found evidence for its involvement in translation. Future work may

continue to test these mutants to better understand this connection to translation. Mistranslation reporters would directly follow up on *pus4Δ*'s sensitivity to paromomycin. Quantifying the resulting pseudouridine levels on tRNA or mRNA substrates in these mutants would clarify if the C-terminus played a role in the catalytic function of Pus4. Altered pseudouridine modification levels are a possible explanation for these effects on translation. Identifying the specific role of the C-terminus of Pus4 remains an exciting future direction. Additionally, my work highlights a *YAK1* mutant's phenotypes and its ties to translation. This presents several motivating questions: 1) How could this mutation have arisen? 2) Is it more likely to occur through selection/evolution, perhaps in combination with an epigenetic state such as [*BIG*⁺]? We do not know if this mutation arose by complete random chance or was selected for in laboratory conditions, but latter would be interesting and motivate more investigation into this mutation and its phenotypes.

Methods:

Yeast growth conditions for protein extracts

From the -80°C freezer, strains were streaked to single colonies on YPD agar plates and grown for 2 days at 30°C. Single colonies were inoculated in 10 mL of YPD liquid media and grown to saturation overnight. These cultures were then added to 140 mL of YPD media in flasks and grown for 4–6 hours at 30°C at 225 rpm until $OD_{600} = 0.6-0.8$. Cultures were spun down at 4200 rpm for 5 minutes, the media was removed, and pellets were flash frozen by dipping the tubes into liquid nitrogen, after which they were stored at -80°C.

Protein extraction

Frozen cell pellets were thawed on ice, then resuspended in 500 µl of ice-cold freshly prepared Lysis buffer (20 mM Tris pH 8.0, 140 mM KCl, 1.5 mM $MgCl_2$, 0.5% Triton X-100, 1 tablet of mini protease inhibitor (Thermo Fisher Scientific catalog# PIA32955)). This volume was flash-frozen into “dippin’ dots” by pipetting slowly into a small dewar containing liquid nitrogen. The yeast dippin’ dots were collected from the liquid nitrogen using a stainless steel wire strainer and then stored in 50 mL conical tubes at -80°C. Dippin’ dots were lysed in a cryomill (SPEX 6870) in 12 mL canisters under the following settings (pre-cool for 3 minutes, 3 minutes of cooling between cycles, 12 cycles of 3 minutes at 15 Hz). Samples were then thawed on ice, and pre-cleared at 4200 rpm for 5 minutes. In the case of preparation for sucrose gradients, this supernatant was transferred to a clean microcentrifuge tube and precleared again at 12000 rpm for 20 minutes. This sample was then snap-frozen in liquid nitrogen and stored at -80°C. Total protein concentration was estimated using a BCA assay/standard curve.

Pellet-supernatant sedimentation

Starting with lysates described above, 500 μ l of the original sample was set aside as the “Total”. 1000 μ l of lysate was spun down for 30 minutes at 100,000 x g at 4°C in a benchtop ultracentrifuge (Beckman Coulter Optima MAX-XP) using a TLA120.2 rotor. The supernatant was removed and added to a new 1.5 mL Eppendorf tube, while the pellet was resuspended in 1000 μ l of Lysis buffer (above). Sample concentrations were estimated using a BCA assay and assessed via Western blot.

Preparation of sucrose gradients

Sucrose gradients were prepared as previously described but with 6X Lysis buffer¹⁸⁴.

Sucrose gradient ultracentrifugation

Gradients were thawed overnight at 4°C. Equal amounts of total protein were loaded onto each gradient. Gradients were balanced and then spun in an ultracentrifuge (Beckman Coulter Optima XPN-100) in a SW 55 Ti swinging bucket rotor (Beckman Coulter). Samples were run for 4 hours at 55,000 rpm. Following ultracentrifugation, gradients were hand-fractionated, serially removing 200 μ l from the top of the gradient and aliquoting into 24 tubes. (Note that the final fraction had a volume in the range of 200–400 μ l, due to subtle differences in pouring the gradient and pipetting/aliquoting after spinning). Fractions were flash frozen in liquid nitrogen—as described above—prior to storage at -80°C.

Pus4 protein purification was performed as previously described¹⁸⁴. 100 µg of purified GST-Pus4 protein was loaded onto the gradients and run as described above. Gel-filtered purified standards were run in parallel (Millipore Sigma).

Western blot

Protein samples were mixed 1:1 with 2X Laemmli buffer (Bio-Rad catalog# 1610737) with 2-mercaptoethanol added, vortexed for several seconds, boiled on a heat block at 95°C for 10 minutes, then vortexed again and briefly spun down to condense liquid. For the whole-cell lysates, 10 µg of total protein was loaded onto 4-15% PAGE gel (Bio-Rad catalog# 4561084) with 1X SDS running buffer on a mini-electrophoresis gel system (Bio-Rad). For the sucrose gradient fractions, equal volumes were loaded across three 4-15% PAGE gels. Gels were then transferred to nitrocellulose membrane sandwiches (Bio-Rad catalog#1704158) using the “mid-weight” transfer setting on a Transblot Turbo Transfer system (Bio-Rad). Blots were stained with Ponceau to verify relatively even lane loading and transfer, then blocked with 5% milk in 1X PBST for one hour at room temperature. To detect HA-tagged Pus4, blots were incubated with 1:1000 anti-HA polyclonal antibody (Invitrogen catalog# 26183) in 5% milk in 1X PBST overnight at 4°C. Blots were washed 3 times for 5 minutes with 1X PBST, then incubated with 1:1000 goat anti-rabbit secondary HRP conjugated (Sigma-Aldrich catalog# A4914) in 5% milk in 1X PBST for one hour at room temperature. The blot was again washed 3 times for 5 minutes with 1X PBST, then developed using ECL (Bio-Rad catalog# 1705061) and imaged on an Amersham Typhoon. To detect Sup35, blots were incubated with 1:1000 Sup35 primary antibody (Mouse) from the Halfmann lab, then 1:1000 rabbit anti-mouse secondary antibody (Millipore Sigma catalog# A9044), with the same workflow as anti-HA detection.

Yeast cell culture for LiP-MS

Strains for LiP-MS experiment were streaked from glycerol stocks stored at -80°C onto YPD-agar plates and grown for 2 days at 30°C. Biological replicates were picked from single colonies and grown in 5 mL overnight culture in YPD media at 30°C and 700 rpm. Saturated overnight cultures were seeded into 4 x 150 mL (1 L for immunoprecipitation experiment) YPD media to a starting OD₆₀₀= 0.1 and harvested in mid log-phase at OD₆₀₀= ~0.6. Pellets were flash-frozen in liquid nitrogen and stored at -80°C until lysis.

Preparation of yeast lysates and limited proteolysis of whole yeast lysates

Cell pellets for LiP-MS analysis were prepared as described previously¹⁸⁵. Briefly, naïve and [*BIG*⁺] cell pellets (N = 4 for both strains) were resuspended in native buffer (100 mM NaCl, 2 mM MgCl₂, 20 mM Tris pH 8.0) with freshly added protease inhibitors (500 µM PMSF, 15 µM E64, 50 µM bestatin final concentration, added from 100X stock in DMSO) and 100 µg/mL DNase I. Suspensions were dripped slowly over liquid nitrogen to freeze into dippin' dots, and lysed by cryogenic pulverization with a freezer mill (SPEX Sample Prep) with 8 cycles consisting of 1-minute grinding/1-minute cooling. Lysates were allowed to thaw at room temperature and were then clarified at 16,000 x g for 15 min to remove insoluble cell debris. Supernatants were then moved to new microcentrifuge tubes and incubated for 2 hours at room temperature to allow PMSF to quench. Lysates were then normalized to a concentration of 700 µg/mL using protein concentration values measured by bicinchonic acid (BCA) assay (Pierce BCA Protein Assay Kit).

Normalized lysates were each divided into one trypsin-only and one limited proteolysis sample per replicate. Each limited proteolysis sample was added to a microcentrifuge tube containing 0.7 µg proteinase K (Thermo Fisher Scientific) in 2 µL suspension. Immediately upon addition to the tube, each sample was briefly mixed by vortex and incubated for 1 minute at room temperature with proteinase K, followed by submersion of the sample tube in a 105°C oil bath for 5 minutes to terminate proteolysis. Trypsin-only samples were also incubated for 5 minutes in the oil bath. Samples were transferred to fresh microcentrifuge tubes containing urea (f.c. 8 M urea upon dissolution).

Immunoprecipitation and limited proteolysis of Pus4-HA

For the LiP-MS experiment on isolated Pus4-HA, cell pellets from YDG1, YDG989, and YDG999 were lysed by cryogenic pulverization as described above (**Supplemental Table S4.3**). After clarification, lysates were normalized to 15 mg/mL total protein concentration in a total volume of 1 mL using measurements from a BCA assay. Clarified lysates were incubated with Pierce™ Anti-HA Magnetic Beads (1 mg beads, pre-equilibrated to native buffer, Thermo Fisher Scientific catalog# 88836) for 30 minutes with mixing at room temperature. The unbound fraction was then removed, and the beads were washed four times with 1 mL native buffer, and once with 1 mL Millipore water. Pus4-HA was eluted from the beads by adding 100 µL of a 2 mg/mL HA peptide (Thermo Fisher Scientific catalog# 26184) solution in native buffer and incubating for 10 min on a thermomixer set to 37°C and 700 rpm. After removing the elution to a separate microcentrifuge tube, the elution and collection was repeated once more with 150 µL HA peptide solution.

Preparation of samples for LC-MS

Samples in 8 M urea were reduced by addition of dithiothreitol (f.c. 10 mM, from freshly made stock) and incubating for 30 min at 37°C in a thermomixer set to 700 rpm. Samples were then alkylated by addition of iodoacetamide (f.c. 40 mM, from freshly made stock), incubated for 45 min at room temperature in the dark. A freshly prepared 100 mM ammonium bicarbonate solution was used to dilute sample urea concentration to 2 M, and samples were subjected to overnight digest with trypsin (New England Biolabs), added to a ratio of 1:50 trypsin:sample protein on a thermomixer set to 25°C and 700 rpm. Digests were terminated by addition of trifluoroacetic acid to a concentration of 1% (v:v). Samples were then desalted using Waters Sep-pak Vac Cartridges with 50 mg sorbent. Cartridges were placed in a vacuum manifold and conditioned with 2 x 1 mL of an 80% acetonitrile, 0.1% formic acid solution (Seppak elution buffer) and equilibrated with 4 x 1 mL of a 0.1% (v:v) aqueous formic acid solution (Seppak wash buffer). After loading of digested peptides, the sorbent was washed with 4 x 1 mL Seppak wash buffer, removed from the vacuum manifold, and eluted from the sorbent with 1 mL of Seppak elution buffer. Peptides were dried by evaporation in an Eppendorf SpeedVac concentrator and stored at -80°C until analysis by LC-MS.

LC/MS data acquisition for LiP-MS

Digested samples were resuspended in 0.1% aqueous formic acid solution, and peptide concentrations were normalized by absorbance at 280 nm (measured by nanodrop spectrophotometer) prior to LC-MS injection. Samples were chromatographically separated using a Vanquish HPLC system (Thermo Fisher) with mobile phases consisting of 0.1% formic acid in water (Buffer A) and 0.1% formic acid in acetonitrile (Buffer B). The effluent was

analyzed with an Orbitrap Ascend mass spectrometer (Thermo Fisher) in data-independent acquisition mode. MS1 spectra were collected with a scan range of 380-980 m/z, a resolution of 120,000, a maximum injection time of 251 ms, and an AGC target of 1E6. Data-independent MS2 spectra were acquired across the 380-980 m/z precursor scan range in isolation windows of 12 m/z units. Precursors were fragmented with an HCD collision energy of 30%. MS2 spectra were collected with a scan range of 150-2000 m/z, a resolution of 30,000, a maximum injection time of 59 ms, and an AGC target of 2.5E5.

Label Free Quantification and LiP-MS Data Analysis

For each of the three LiP-MS sub-experiments, two separate label-free quantifications (LFQ) were run: One including all trypsin-only samples, and one including all LiP samples. These were all run using the ‘DIA SpecLib Quant’ preset in Fragpipe. Briefly, a database search is done as described in the previous subsection to identify peptides from the LC-MS data. These are validated with Philosopher and used to construct a DIA-based empirical spectral library.

Precursor abundances are then quantified using the DIA-NN node (ver. 2.1.0) in Fragpipe^{186–189}, set to high-accuracy QuantUMS quantification strategy, and an FDR cutoff of 0.01.

Structural alteration of LiP-MS sites was assessed using FLiPPR¹⁹⁰. Precursor QuantUMS intensities were taken directly from the DIA-NN precursor matrix output for the LFQ corresponding to LiP samples. First, precursors are filtered based on missing intensity values: Precursors missing intensity values in a threshold number of samples (3 for N=4 mouse comparisons; 5 for N=10 human comparisons) are excluded from LiP analysis. If a precursor is missing intensity values in several samples below that threshold, those values are dropped, and summary statistics for said precursor are computed using only the remaining samples. If a

precursor has intensity values for all members of one group, and no members of the other, it is considered ‘all or nothing’, and intensity values are imputed gaussian-style with a mean of 104 and a standard deviation of 103. Fold-change and t-test p-values are computed for each precursor. If multiple charge states are detected for a given peptide, and the direction of fold-change is in agreement between all, they are combined into a single row whose fold-change is the median of the peptide’s detected precursors, and whose p-value is a combined p-value obtained by Fisher’s method. If the direction of fold-change between a peptide’s charge-states does not agree, the peptide is excluded from analysis. To account for the effect of a protein’s abundance on the measured abundance of its peptides, abundance values from the protein group matrix DIA-NN output from the LFQ corresponding to trypsin-only samples were used to compute fold-change and t-test p-values on the protein level. If a protein’s abundance had a fold-change of at least 2 and a p-value < 0.01 , its fold-change value is used to normalize the fold-change of all of its detected peptides for LiP-MS analysis. After filtering and normalization, peptide-level p-values are corrected by Benjamini-Hochberg (BH) method on a per-protein basis, to account for the fact that proteins with more detected peptides have more opportunities to be significant purely by chance. Peptides are considered structurally altered if their fold-change exceeds 2, and their adjusted p-value is < 0.05 . Proteins are considered structurally altered if they have at least 2 peptides that are structurally altered.

Preparation of WCL samples for TMT labeled mass spectrometry

From the -80°C freezer, strains were streaked to single colonies on YPD agar plates and grown for 2 days at 30°C . Five colonies of each strain were inoculated in 10 mL of YPD media and grown to saturation overnight. These cultures were then added to 140 mL of YPD media in

flasks and grown for 4-6 hours at 30°C at 225 rpm until OD₆₀₀ = 0.6-0.8. Cultures were spun down at 4200 rpm for 5 minutes to pellet cells, media was removed, and then pellets were flash frozen in liquid nitrogen, as described above, and stored at -80°C. Lysis via cryomill and protein estimation were performed as described above. Samples were adjusted to 1 µg/µl (200 µl total volume), flash frozen in liquid nitrogen, stored at -80°C or dry ice until TMT analysis at the IDeA National Resource for Quantitative Proteomics (Little Rock, AR).

Construction of mutants

All mutant strains were constructed using CRISPR transformations as previously described¹⁹¹. DNA oligos/gene blocks were purchased from Integrative DNA Technologies (IDT). Mutations were confirmed by Sanger sequencing of the entire coding region of the gene. Pus4 C-terminus mutants were constructed using the HA-tagged Pus4 strains (**Supplemental Table S4.3**).

Growth assay

From the -80°C freezer, strains were streaked to single colonies on YPD agar plates and grown for 2 days at 30°C. Single colonies were inoculated in wells of a 96-well plate containing 200 µl of CSM media (Sunrise catalog# 1001-100) in each well. Cells were grown for 2 days at 30°C. After remixing settled cells, 5 µl from each well was transferred (using multi-channel pipette) to a new 96-well plate containing 200 µl sterile nano pure water in each well, to dilute cells. Using the Singer Instruments ROTOR, the liquid dilution plate was pin-replicated into a 384-spot arrayed CSM agar media plate, or the same plate containing 10 mg/mL paromomycin sulfate (GoldBio catalog# P-700-100). This set up produced 4 technical replicates of each biological replicate. Colonies were grown for 72 hours at 30°C inside tupperware containers to retain

moisture. Colony size measurements were performed using a Singer Instruments PhenoBooth+ instrument. Plots and statistical analysis of raw data were generated using GraphPad Prism. The top and bottom rows of the 96-well plates were excluded in these experiments to minimize “edge effects” that propagate to the agar plates.

Whole genome sequencing (WGS)

Whole genome sequencing was performed by Preeti Bhattacharjee on one replicate each of three strain isolates of [*BIG*⁺]: YDG8 - [*BIG*⁺] MATa haploid, YDG109 - [*BIG*⁺] MATa haploid, YDG162 - [*BIG*⁺] MATa haploid (**Supplemental Table S4.3**). Non-prion strains with BY4741 and BY4742 background were sequenced as controls.

Sample preparation and gDNA extraction for WGS

A single colony of each strain was picked from a YPD plate and grown to saturation in 5 mL YPD culture overnight at 30°C on a rotator drum. Cells were harvested by centrifugation for 3 minutes at 4000 rpm and resuspended by pipetting in 200 µl TSENT buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). 0.3 g of acid-washed glass beads (Sigma-Aldrich, G8772), and 200 µl of 25:24:1 v/v Phenol:Chloroform:Isoamyl Alcohol (Sigma-Aldrich, 77617) was added. The tubes were vortexed for 2 minutes, then centrifuged for 5 minutes at 15,000 rpm. The upper aqueous layer was collected. This aqueous layer was ethanol precipitated - 500 µl of 100% room temperature ethanol (Sigma-Aldrich, E7023), and 10 µl 3M sodium acetate pH 5.2 (Millipore Sigma, 567422) were added, samples were vortexed and centrifuged for 5 minutes at 15,000 rpm. The supernatant was discarded, and 500 µl cold 70% ethanol was added, and the sample was centrifuged for 5 minutes at 15,000 rpm. The supernatant

was removed and the pellet was air-dried for 20 minutes. The DNA was resuspended in 50 µl nano pure water.

Library preparation for WGS

10 ng of DNA was transferred to a 384 well plate and dried in a speed-vac. Bead Linked Transposase (Illumina) was diluted 1:60 in a custom buffer (10 mM Tris-HCl, 5 mM MgCl₂, 8% PEG). 6 µl diluted beads were added to DNA and vortexed to resuspend DNA. Samples were incubated at 53°C for 30 minutes. Plates were spun down and placed on a magnet to gather beads. 5 µl of supernatant was removed and discarded. 1 µl of barcoded Illumina Nextera adapter primers (IDT), 5 µl 2X Equinox Master-mix (Watchmaker Genomics) and 3 µl Nuclease Free Water (NEB) added to each well. Samples were heated to 68°C for 3 minutes, then 98°C for 3 minutes. Samples were then amplified with 10 cycles of 98°C for 45 seconds, 62°C for 30 seconds, 68°C for 2 minutes; 68°C for 1 minutes; and held at 10°C. 20 µl Nuclease Free water was added to each well and mixed. 2 µl of each sample was pooled and cleaned twice with 0.8X volume Total Pure NGS beads (Omega Biosciences). A library run was finished on Illumina MiSeq Nano PE150. Read counts from MiSeq run were used to calculate a new pool with equal representation. The new pool was cleaned as above and run on the MiSeq to obtain a full set of reads.

WGS sequencing

Sequencing was performed on the Illumina MiSeq v2 with 250 bp paired-end reads, resulting in ~50X average read depth for each sample.

WGS Data Analysis

Alignment and indexing reads: Adaptors were removed and reads were trimmed for quality using Trimmomatic. Reads were aligned to the reference genome R64-5-1_20240529 using Bwa. We used samtools to make binary files for alignment viewing (.bam files), sorted them by coordinates, and indexed the files for visualization.

Variant calling: For variant calling, we used Freebayes

(<https://github.com/freebayes/freebayes.git>) to generate VCF files. Variants were annotated using snpEff (<https://pcingola.github.io>). We removed variants that were common across all strains sequenced, considering them ‘background’ mutations, since we were only interested in mutations that are unique to the putative prion strains. We filtered the VCF files as follows to identify strong unique variants: quality score ≥ 30 , read depth of variant (DP) ≥ 20 , alternate observations AO ≥ 10 . We manually inspected these variants and removed reads mapped to the telomeres and centromeres, TY elements, low complexity regions and long repeats since they are not reliably mapped by short read sequencing. We also disregarded loci annotated as dubious ORFs (in SGD) that are unlikely to encode functional protein.

Copy number variation (CNV) analysis: The cn.mops R package

(<https://git.bioconductor.org/packages/cn.mops>) was used for CNV analysis. The haplo.cn.mops() function was used, and the following parameters were used to optimize CNV calls for a haploid yeast genome with ~50X coverage: WL=500, priorImpact = 1, normType = "poisson", minWidth = 3, upperThreshold = 0.5, lowerThreshold = -0.5. Results were inspected manually by mapping the segments that showed change in copy number on IGV and identifying the gene or ORF. If the

segment overlapped completely with a gene, or the gene fell within the segment that has altered copy number, we considered that a copy number variant. If the segment covered less than half of a gene, we did not consider that a copy number variant.

Bridge to Chapter V:

In Chapter IV, I described our investigation of the putative yeast prion [*BIG*⁺]. Specifically, I investigated potential biochemical explanations for its activities or inheritance and attempted to identify other factors that could be involved in its functions, with the hopes of fully describing its molecular mechanism. Late into the project, we determined that a nonsense mutation of *YAK1* in our [*BIG*⁺] strains presented a major confounding variable for our analysis and interpretation of the phenotypes in these strains. In Chapter V, I summarize the largest takeaways from my graduate research and discuss the next steps for investigation.

CHAPTER V

CONCLUDING REMARKS

This dissertation explored several proposed functions for the yeast pseudouridine synthase, Pus4. In Chapter II, I summarized my work investigating how Pus4 binding and catalysis affected tRNA structural conformations. In Chapter III, I described the field's current biochemical understanding of prions, setting the stage for Chapter IV, in which I investigated whether an aggregate supported Pus4's ability to form the prion [*BIG*⁺], by searching for an altered biophysical state and identifying other protein factors and genes involved in [*BIG*⁺]. Overall, my work has better defined the activities of Pus4 and expanded the field's understanding of an unusual prion example.

Prior to the initiation of this study, the *E. coli* homolog TruB had a documented function as a tRNA chaperone, which researchers proposed might be a common function for many tRNA-modifying enzymes. This evidence motivated our investigation to get a better understanding of yeast Pus4's direct effects on tRNA structure. This question was challenging to pursue, since the modification deposited by Pus4 affects tRNA structure, and disentangling the effects from Pus4 catalysis and binding required careful experiment design. Additionally, bulk measurements are limited in their ability to detect changes in dwell time, dynamics, and lowly abundant yet relevant conformational states. Our collaboration with Dr. Julia Widom, whose lab primarily works in single-molecule techniques such as smFRET, provided the key expertise to investigate the specific effects of Pus4 on tRNA conformational states. This method, combined with biolayer interferometry, better clarified how binding and catalysis of this tRNA-modifying enzyme produces three distinct states for tRNAs before and after engagement with Pus4. We found that Pus4-binding induced more conformational states and facilitated initially unmodified

tRNA to more gradually approach the ensemble of structures that were rapidly attained by pre-modified tRNA. Overall, this study marks the first approach to combine optical binding assays and single molecule work to evaluate enzyme- and modification-dependent tRNA conformational dynamics.

The immediate steps following this study are to perform similar analysis using a catalytically impaired mutant of Pus4, such as recombinantly purified Pus4 R286K, or other mutants that might separate the binding, catalysis, and putative chaperone activities of the enzyme. We tested the tRNA dynamics of two different yeast tRNAs, and while the overall distributions and hierarchical pathways had features in common, they were not identical, suggesting that different tRNAs could explore different conformational dynamics. The analysis of other yeast tRNAs could help delineate which features are fundamental or unique to specific tRNA molecules that have unique combinations of RNA modifications that influence their structural dynamics. When Pus4 protein was added, additional mid-FRET transitional states were induced, allowing an unmodified and pre-modified substrate to converge on the same set of final states. A mutant incapable of catalysis would allow us to observe another layer of dynamics and add clarity to a model of interaction with this pseudouridine synthase. We could also repeat these experiments with the acceptor and donor fluorophores located on the D- and T-loops to assess local structural fluctuations at a site critical to the L-shape of tRNAs. This would also allow us to better infer the structural states that result from smFRET data. Our inability to “see” the structural conformations of these tRNAs is a current limitation of this dataset.

Some improvements in method development would greatly improve the information gained from these experiments. For example, it would be invaluable to be able to measure modification levels on the actual tRNAs used in smFRET. Developing this approach would pair

well with a dataset generated from a catalytic mutant of Pus4. Additionally, while we have ample evidence to support that Pus4 binding is occurring during smFRET, it would be useful to understand which smFRET states are when protein is bound or not. One approach is to covalently attach a third fluorophore to purified protein, allowing you to detect protein binding. This experimental design may supersede the BLI approach we took here, since the measurements would be at the single-molecule level rather than in bulk, and more comparable to the current set of tRNA smFRET experiments. It would take, however, significant reagent development to execute, in particular, where and how to apply the label on the exogenous Pus4 protein. Finally, Ψ_{55} is part of a modification circuit in the T-loop. Including other modifications of the T-loop in the experimental design would resolve their role in tRNA structural rearrangements, both with and without their depositing RNA-modifying enzymes. This work cannot be undertaken until preservation of m^1A_{58} can be achieved, since it spontaneously converts to m^6A during sample preparation of tRNAs. There remain several research questions that are motivating to pursue, especially after methodologies have developed further.

Pus4 was also proposed to be the protein basis of the yeast prion state [*BIG*⁺]. The initial efforts of the project sought to find the biophysical state of the prion, in the hopes that it would help explain the dramatic growth phenotypes that conferred competitive growth advantages to these cells. I performed biochemical analysis using several sedimentation techniques and a limited-proteolysis mass spectrometry (LiP-MS) approach and found no evidence of an altered structural state of Pus4. Additionally, we identified a causative nonsense mutation in the glucose-sensing protein kinase Yak1 that was clearly the source of [*BIG*⁺]'s paromomycin sensitivity. Its role in other [*BIG*⁺] phenotypes remains unclear. Collectively, my work has challenged the prion definition of [*BIG*⁺], but more work is needed before conclusive statements can be made.

Currently in the field of prions, the evidence for non-amyloid-forming prions is rather limited. A prion named $[GAR^+]$ is a complex of two proteins, Pma1 and Std1, resulting in a resistance to glucose-associated repression of alternative carbon sources. Std1 overexpression induces $[GAR^+]$, while Pma1 is required for propagation. The requirement of two protein factors, with separate contributions to a prion state is highly unusual. A non-amyloid explanation is possible since there is no detectable SDS-resistant species, and the state is not dependent on the amyloid remodeling chaperone Hsp104. Another example is $[SMAUG^+]$, which is driven by the RNA-binding protein Vts1, whose activity is enhanced in its prion state in contrast with classical prion examples. It was proposed that this prion propagates via condensates rather than an amyloid structure. This study was primarily performed in purified protein, however, and thus further experiments are needed to determine the structure of that prion state in the cell. Both studies suggested a non-amyloid state is likely responsible for the inheritance of their phenotypes, the strongest evidence being the exclusion of a detectable amyloid aggregate. In that sense, $[BIG^+]$ also meets this definition, since it has no detectable prion aggregate. While no evidence was found for an altered physical state of Pus4 in the $[BIG^+]$ state, it's "non-amyloid state" may persist in combination with another protein or RNA factor (like $[GAR^+]$), or in a difficult to detect state (like $[SMAUG^+]$), whether transient or lowly abundant. How $[BIG^+]$ fits into the category of non-amyloid-forming prions remains an important avenue of investigation.

Although much of my thesis work did not generate support for $[BIG^+]$'s prion definition, data from the previous 2021 study remains difficult to reconcile. Inheritance experiments showing non-mendelian inheritance through meiosis and mitosis, cytoductions, and chaperone curing all point to $[BIG^+]$ being a prion, one that is at least in part, Pus4-dependent. An alternative explanation is difficult to generate. Additionally, a description of *yak1* Δ strains in the

literature does not entirely agree with [*BIG*⁺] phenotypes, although there are some similarities. Future work will need to repeat some of these inheritance experiments with the *YAK1* Q578* mutation reverted to wild-type to clarify the role that this mutation may have on inheritance of this epigenetic state.

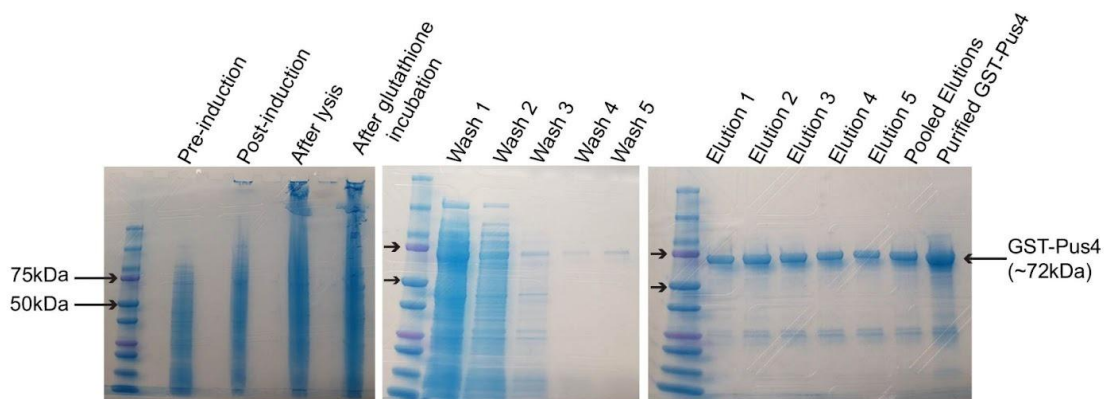
An unintended finding of this work was that the C-terminus of Pus4 may have a role in translation, potentially via catalysis. We recapitulated a result that *pus4Δ* has a growth impairment in the presence of the translation inhibitor paromomycin, suggesting that Pus4 has a connection to translation. A reduction of the negatively charged residues of the C-terminus of Pus4, in the context of [*BIG*⁺] and the *YAK1* Q578* mutation, resulted in a rescue of this phenotype. This new finding suggests that the C-terminus of Pus4 modulates this unknown role in translation. Whether this is through catalysis of Pus4's substrates, such as tRNAs or mRNAs, or via an unknown interaction remains an interesting future direction of this research. An approach such as a ribosome profiling may reveal which aspect of translation is affected by either *PUS4* or *YAK1* mutations/deletions, and potentially [*BIG*⁺].

Thus, my thesis work on the yeast pseudouridine synthase Pus4 has not only improved our understanding of its protein functions but advanced new methods to investigate them. My work has laid the groundwork for future investigations into this highly conserved enzyme, and other enzymes like it.

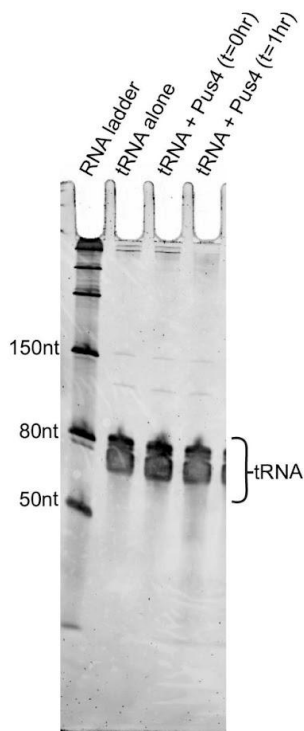
APPENDICES

SUPPLEMENT FOR CHAPTER II:

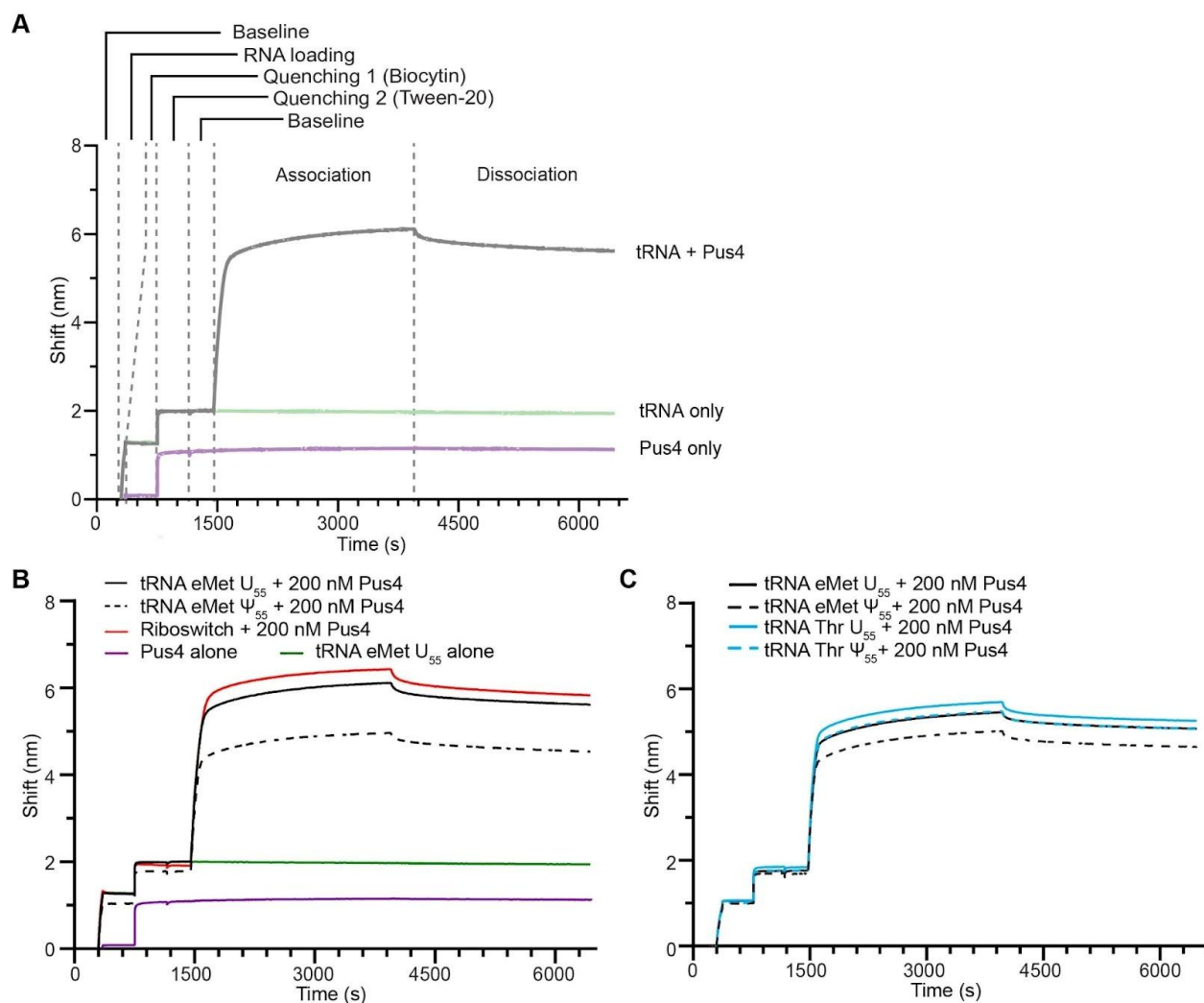
A



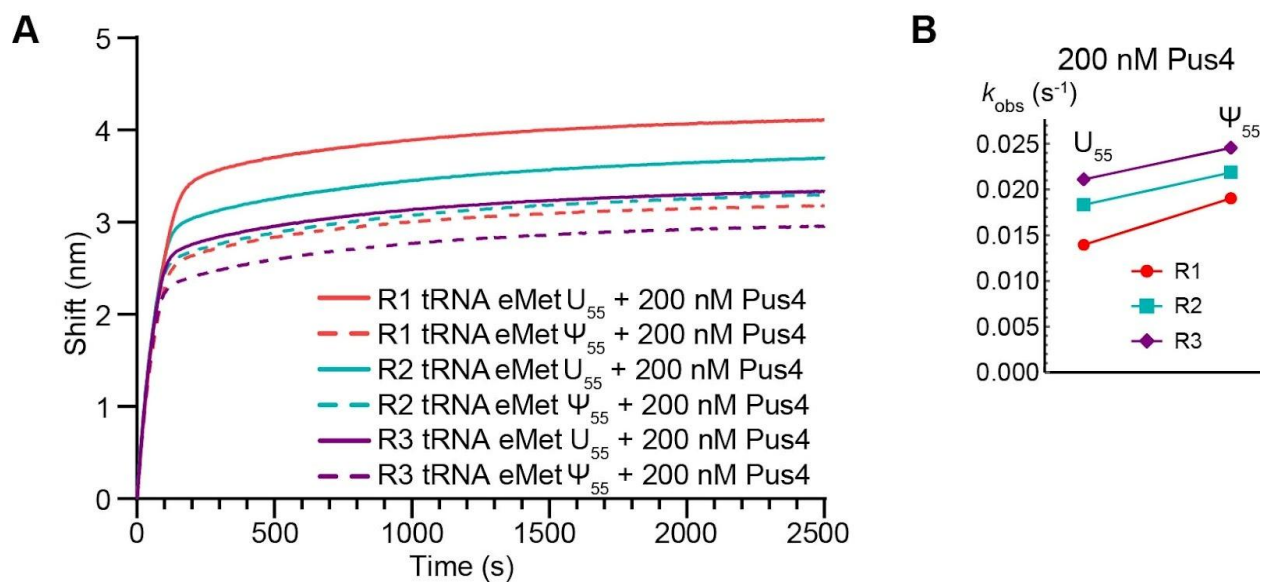
B



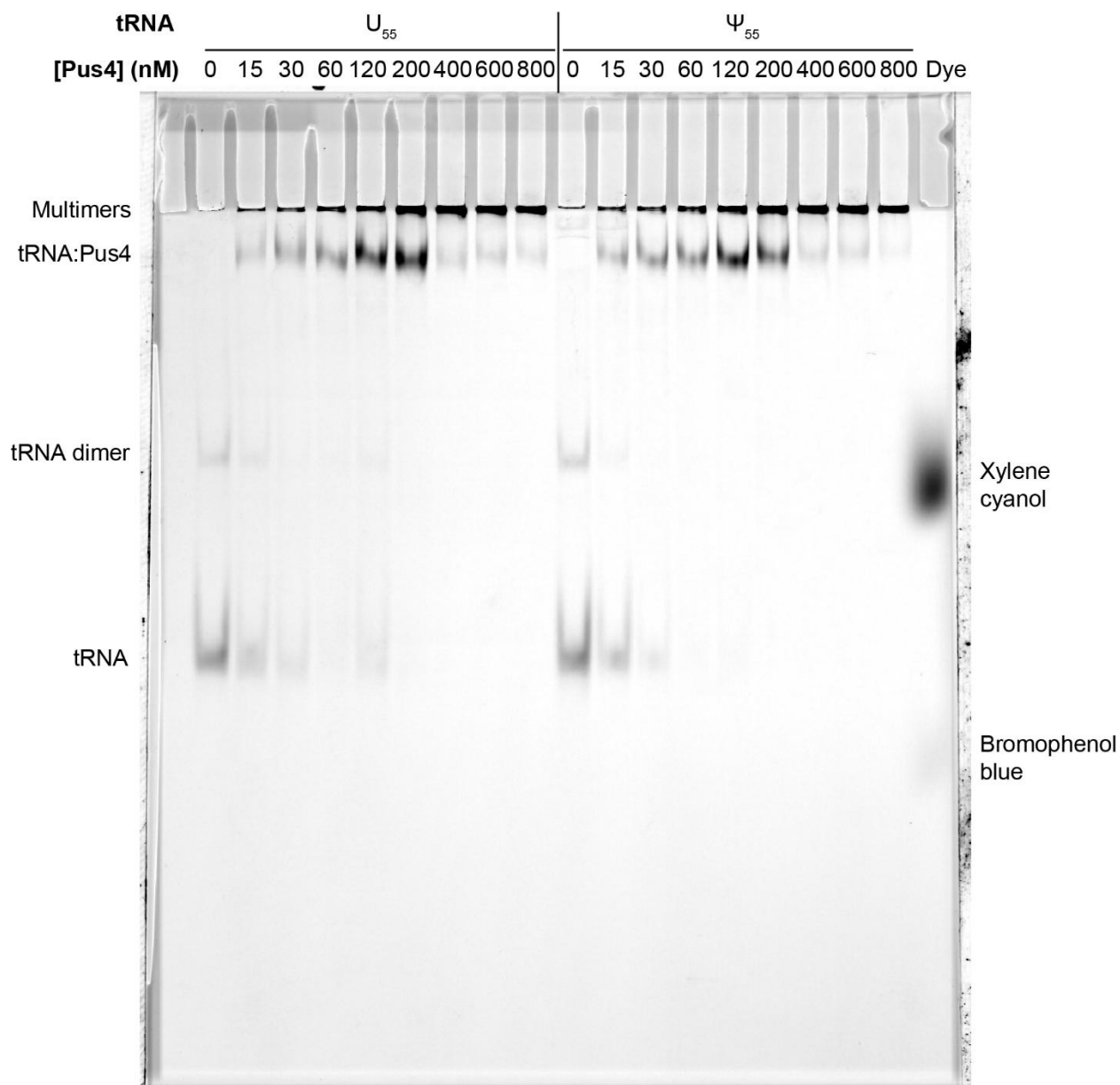
SUPPLEMENTAL FIGURE S2.1. Protein purification. (A) SDS-PAGE gels stained with GelCode Blue showing GST-Pus4 (72 kDa) present throughout purification. Upper and lower arrows indicate ladder standards of 75 and 50 kDa, respectively. (B) Urea PAGE gel showing the total yeast tRNA that remains without any appreciable degradation after incubation with purified Pus4 for 1 hour at room temperature.



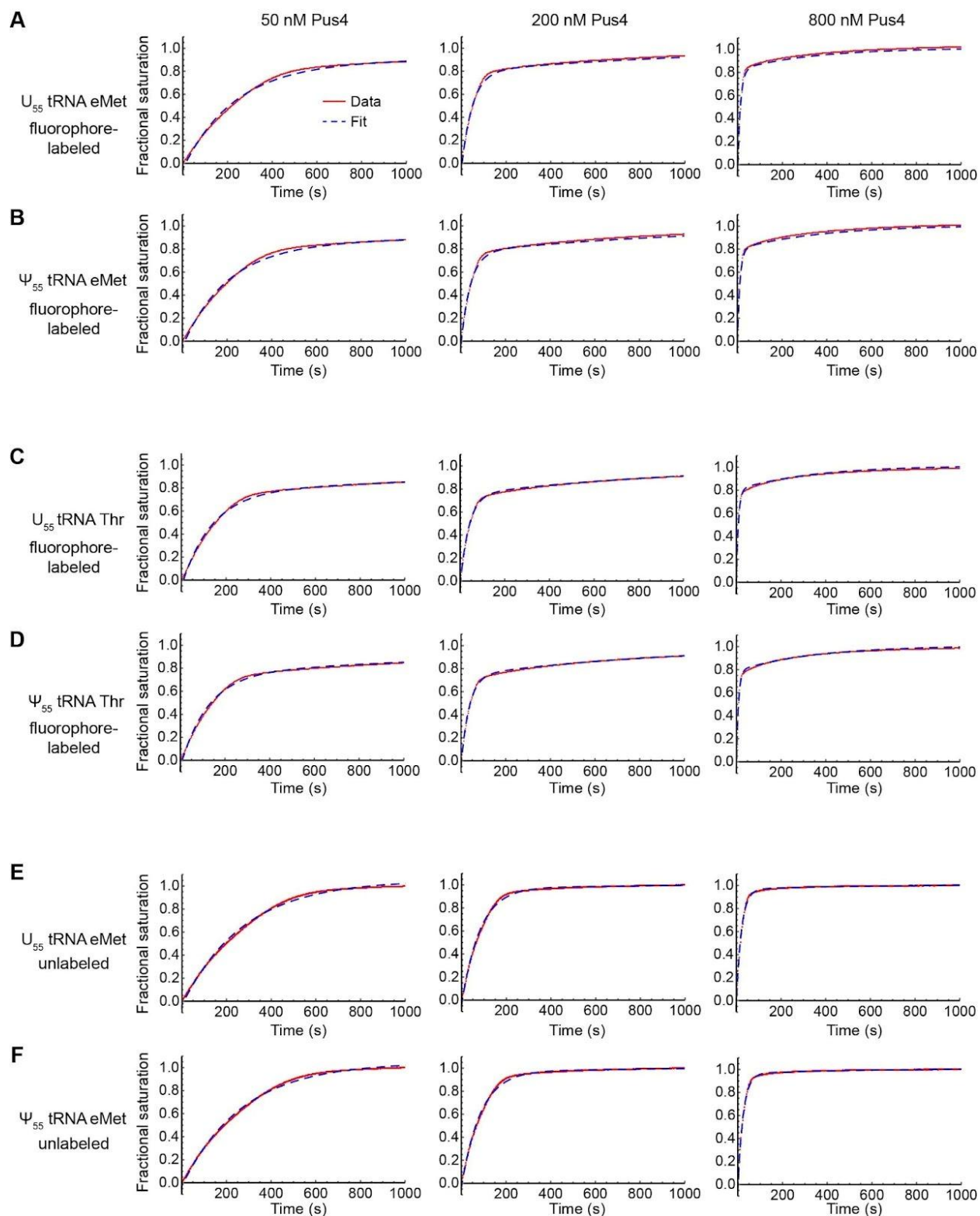
SUPPLEMENTAL FIGURE S2.2. Complete BLI traces of data from **Figure 2.2**. (A) Full BLI traces with each experimental step overlayed and labeled. Step (length of time): Baseline (300 s), RNA loading (~60 s), Quenching 1 (400 s), Quenching 2 (400 s), Baseline (300 s), Association (2500 s), Dissociation (2500 s). (B) Full BLI traces of 200 nM Pus4 showing association with U₅₅ (black solid) and Ψ₅₅ (black dashed) tRNA eMet, and NAD⁺ riboswitch (red). The traces from the reference probes of Pus4 alone (purple) and U₅₅ tRNA eMet alone (green) are also shown. (C) Same as B but with U₅₅ tRNA eMet (black solid), Ψ₅₅ tRNA eMet (black dashed), U₅₅ tRNA Thr (blue solid), and Ψ₅₅ tRNA Thr (blue dashed).



SUPPLEMENTAL FIGURE S2.3. BLI replicate data and resulting rates of fits to association phases of tRNA + Pus4 BLI curves. Each color represents a different BLI run. (A) Association phase of 200 nM Pus4 showing association with U_{55} (solid) and Ψ_{55} (dashed) tRNA eMet across three replicates (R1, R2, and R3). (B) k_{obs} determined from a bi-exponential fit to the entire association phases of three runs (R1, R2 and R3) of tRNA eMet + 200 nM Pus4 on different days. While the rates themselves are variable between runs, Ψ_{55} tRNA consistently exhibits a faster k_{obs} than U_{55} tRNA.

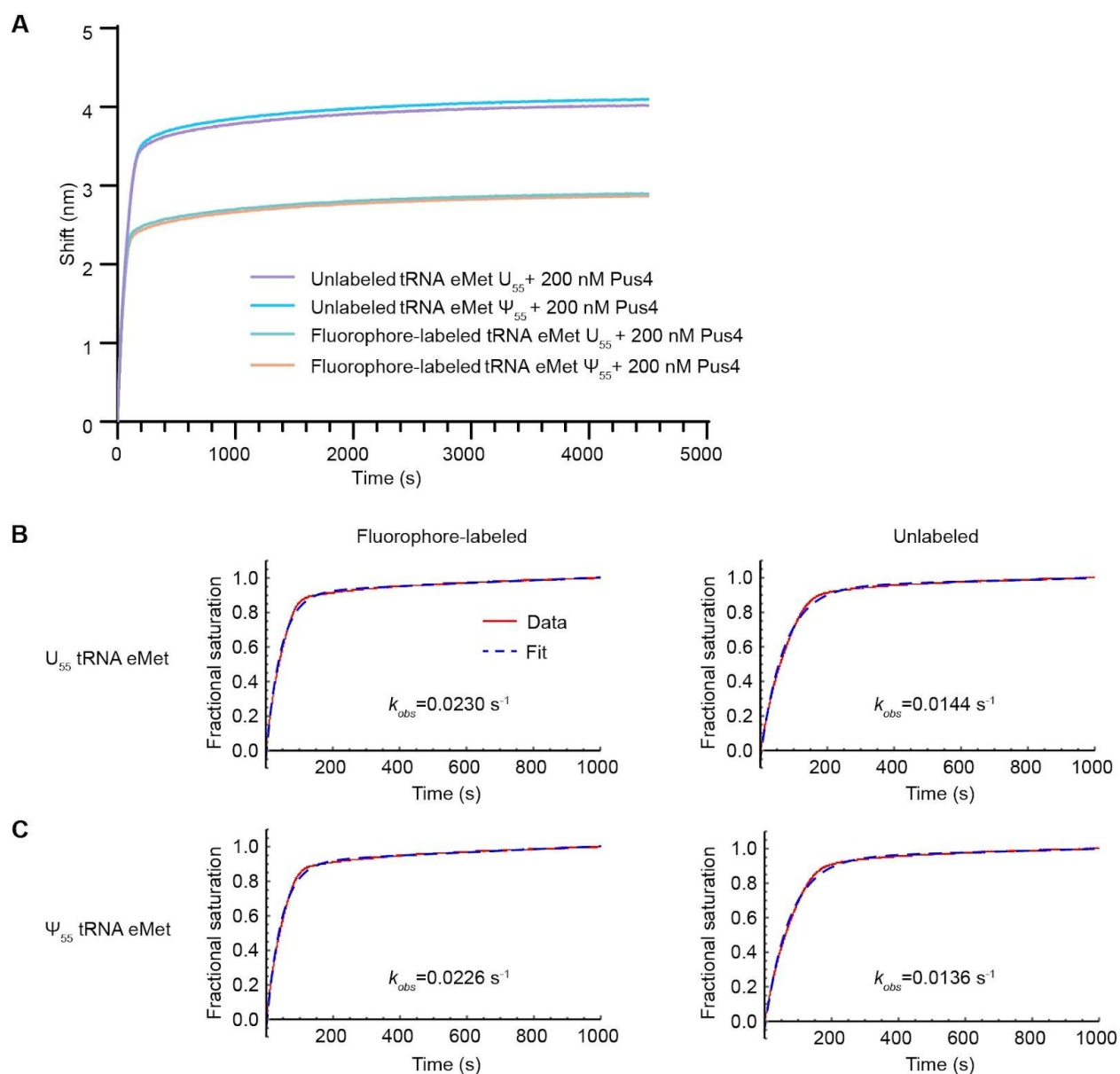


SUPPLEMENTAL FIGURE S2.4. Electrophoretic Mobility Shift Assay. U₅₅ or Ψ₅₅ tRNA eMet was incubated for 30 minutes in the presence of 0 to 800 nM Pus4 WT and the resulting protein-RNA assemblies were resolved on an 8% non-denaturing polyacrylamide gel. tRNA was visualized by detecting Cy5 fluorescence. The image was subjected to a 500-pixel rolling ball background subtraction. The slower-migrating band in the no-protein lanes may represent tRNA dimers. The far-right lane contains xylene cyanol and bromophenol blue as a reference.

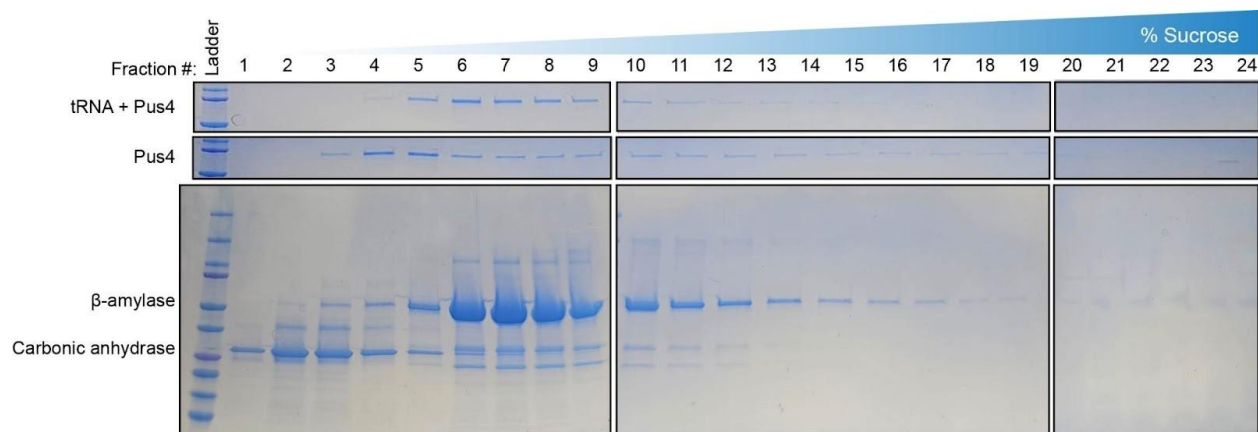


SUPPLEMENTAL FIGURE S2.5. Bi-exponential fits (blue dashed) to the association phases of BLI curves (red) at varying concentrations of Pus4. Left column: 50 nM; Middle: 200 nM;

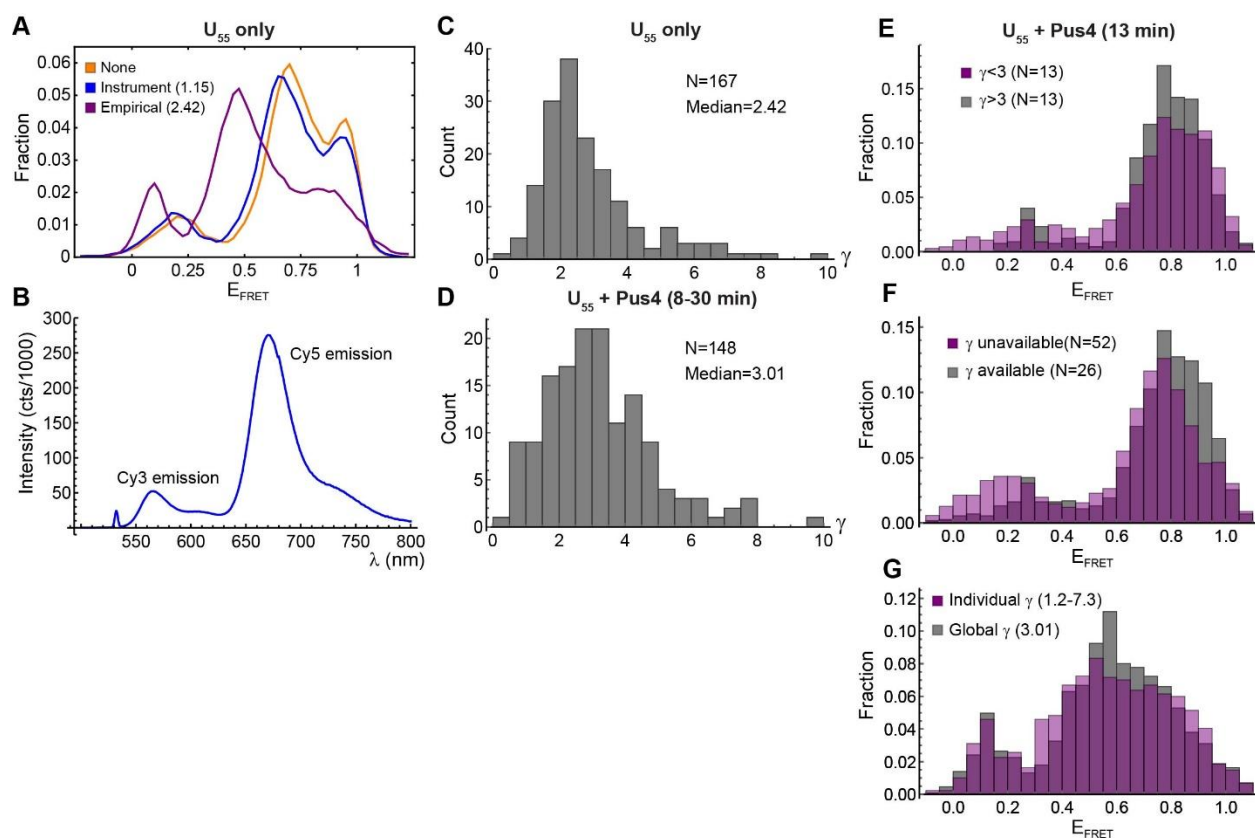
Right: 800 nM. (A) Fluorophore-labeled U₅₅ tRNA eMet. (B) Fluorophore-labeled Ψ_{55} tRNA eMet. (C) Fluorophore-labeled U₅₅ tRNA Thr. (D) Fluorophore-labeled Ψ_{55} tRNA Thr. (E) Unlabeled U₅₅ tRNA eMet. (F) Unlabeled Ψ_{55} tRNA eMet. The entire association phase was fitted for panels A-D, but the first 1000 s are displayed for ease of comparison.



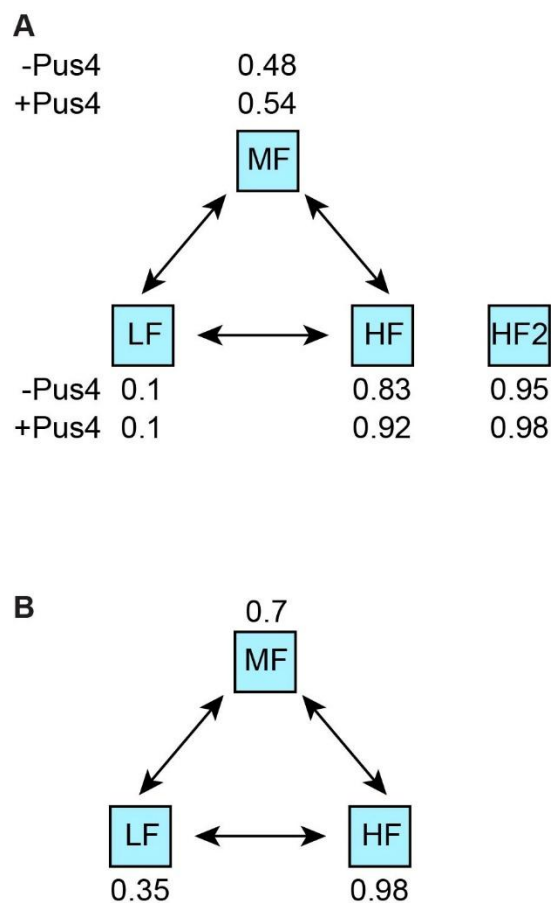
SUPPLEMENTAL FIGURE S2.6: Association phase of BLI curves and resulting fits of association to unlabeled and fluorophore-labeled tRNA eMet from within the same BLI run. (A) Association phase of 200 nM to unlabeled and fluorophore-labeled tRNA eMet. Traces are aligned to start at a shift of 0 nm. (B) Fits of the association phase of the BLI curve with U_{55} tRNA eMet. (C) Fits of the association phase of the BLI curve with Ψ_{55} tRNA eMet. The entire association phase was fitted, but the first 1000 s are displayed for ease of comparison. Pus4 shows a faster association rate to fluorophore-labeled tRNA eMet than unlabeled tRNA eMet, consistent with the comparison across BLI runs in **Figure 2.4**.



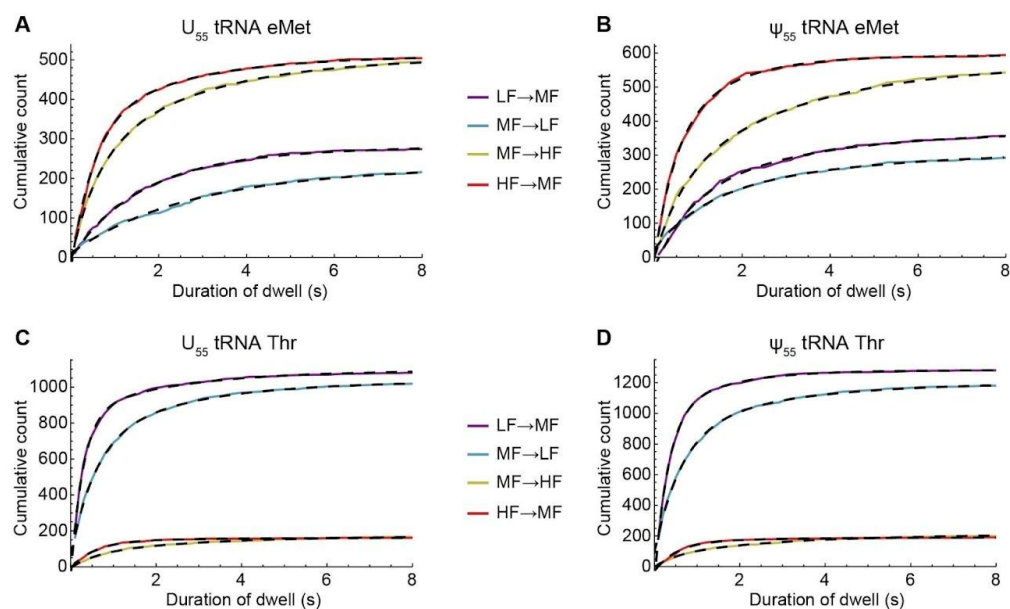
SUPPLEMENTAL FIGURE S2.7. Sucrose gradient ultracentrifugation. SDS-PAGE gels stained with GelCode Blue showing GST-Pus4 (72 kDa) with and without incubation with yeast tRNA as separated by a sucrose gradient (10–30% sucrose). Fraction 1 (low % sucrose) contains smaller complexes or monomers, while fraction 24 (high % sucrose) contains larger complexes. A control gradient was run in parallel containing purified β -amylase (tetramer - 200 kDa, monomer - 50 kDa) and carbonic anhydrase (26 kDa).



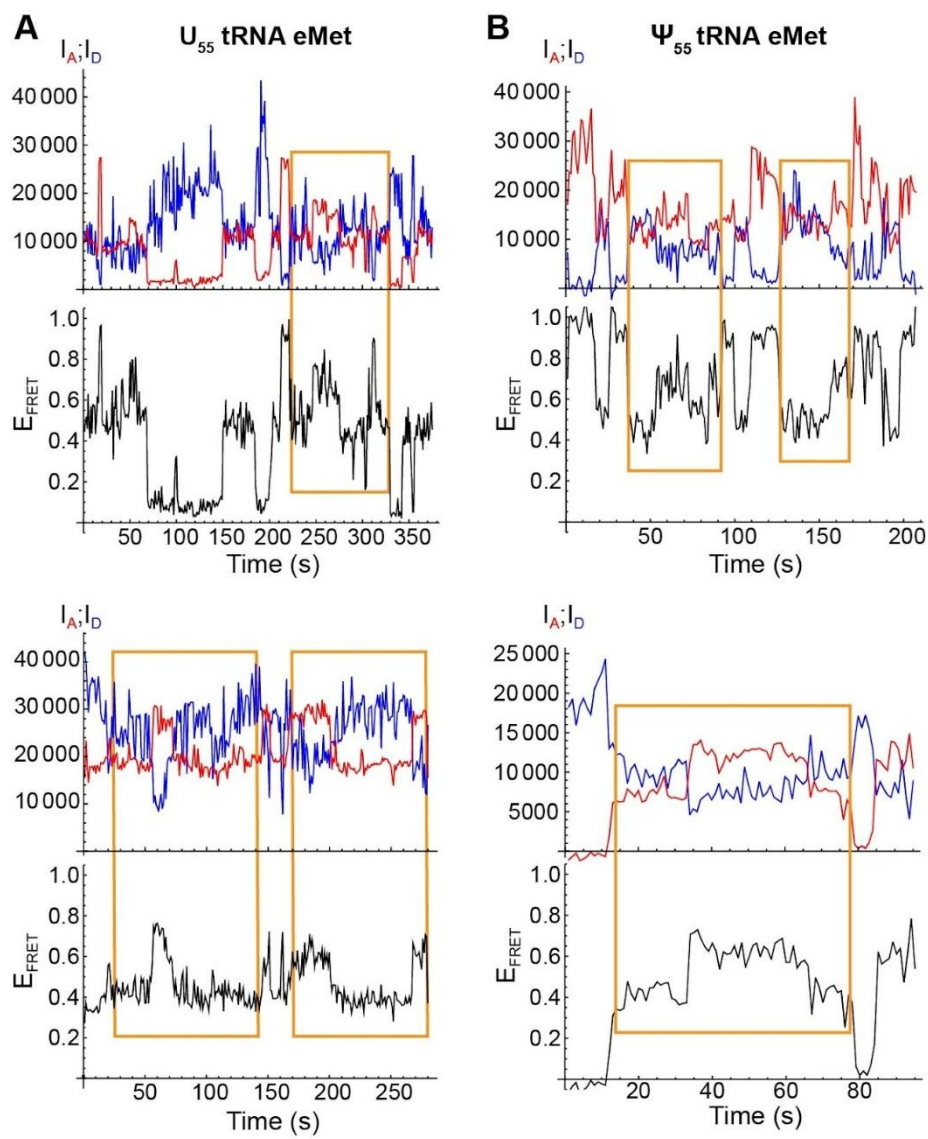
SUPPLEMENTAL FIGURE S2.8. Gamma factor correction. (A) Comparison of U_{55} tRNA eMet E_{FRET} histograms compiled from traces without γ factor correction (orange), traces corrected with a value of $\gamma=1.15$ calculated from the properties of the TIRF microscopy instrument's emission path optics and camera (blue) or traces corrected with empirically determined median value of $\gamma=2.42$ (purple, see panel C). (B) Bulk emission spectrum of U_{55} tRNA under excitation at 532 nm. Much brighter Cy5 than Cy3 emission indicates that the skew toward high apparent E_{FRET} is not instrument-specific. (C,D) Histograms of γ factor values obtained from U_{55} tRNA eMet traces recorded in the absence (C) and presence (D) of Pus4 (details in **Methods**). "N" indicates the number of traces included in each histogram. (E-G) E_{FRET} histograms justifying the use of a global γ factor correction (data on U_{55} tRNA eMet+Pus4). (E) Uncorrected traces with γ above the median (gray) have narrower features but a similar overall shape to traces with γ below the median. (F) Uncorrected traces for which γ could be calculated (gray) skew toward higher E_{FRET} than traces for which it could not be calculated, suggesting that limiting the analysis to molecules for which an individual γ can be calculated (those in which the acceptor fluorophore photobleaches before the donor) would distort the results. (G) Traces (N=26) corrected with individual values of γ yield a broader but otherwise similar histogram to traces corrected with the global value of $\gamma=3.01$.



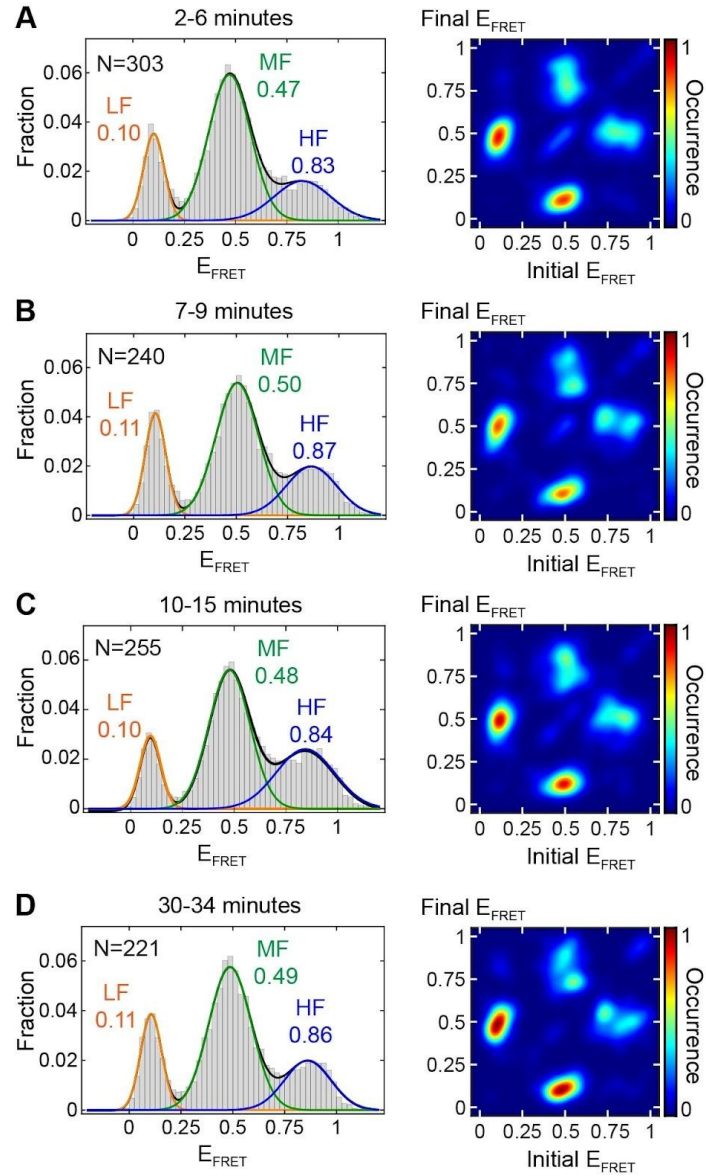
SUPPLEMENTAL FIGURE S2.9. Graphical depictions of the models used for hidden Markov modeling of (A) tRNA eMet and (B) tRNA Thr. The starting guess for the FRET efficiency of each state is indicated. HMM of tRNA eMet in the presence of Pus4 utilized the same model with different starting guesses (second line of numbers in panel A).



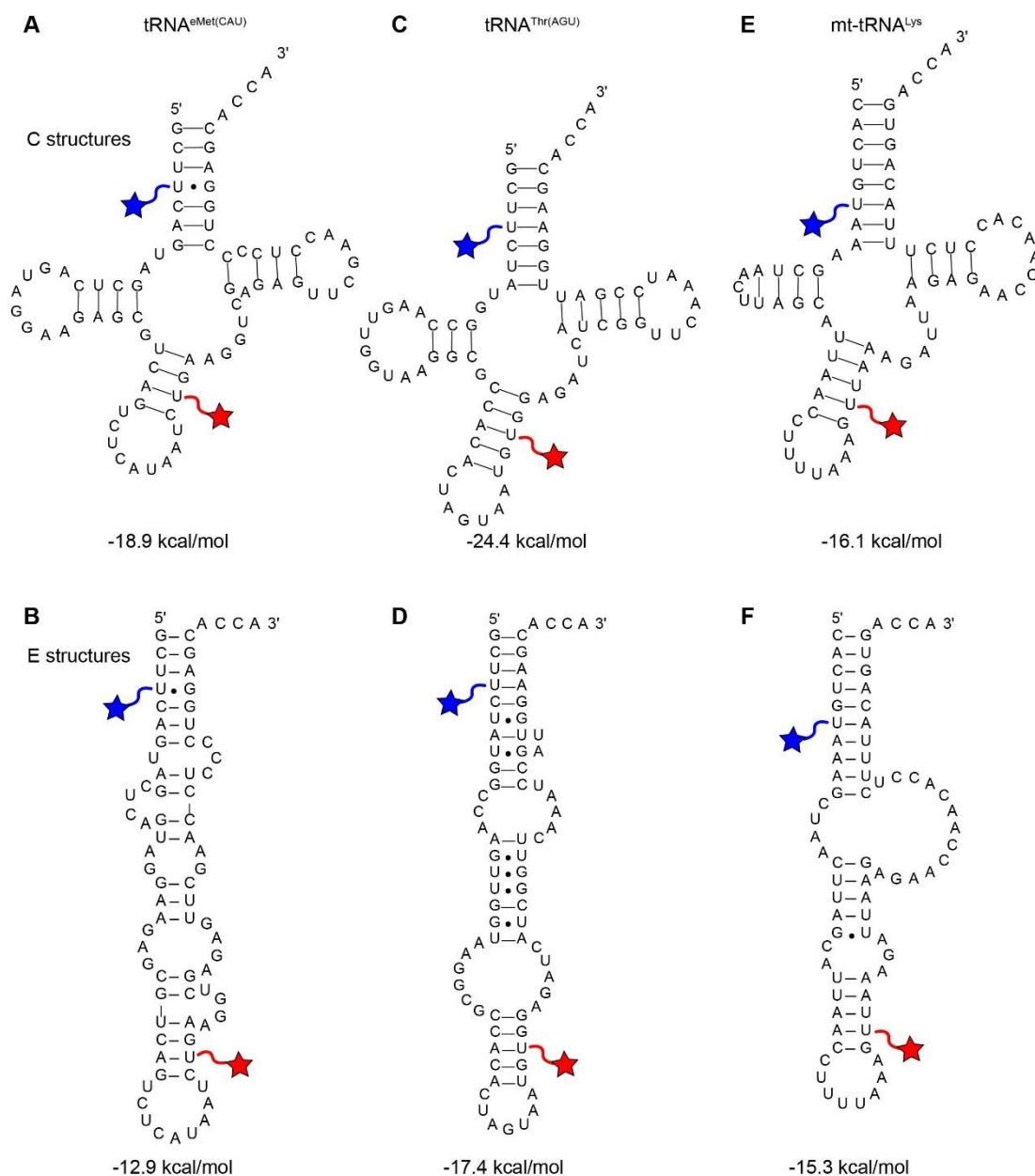
SUPPLEMENTAL FIGURE S2.10. Dwell times of tRNAs in each conformational state in the absence of Pus4. (A) Number of times U₅₅ tRNA eMet dwelled in a state for a duration less than or equal to the time indicated on the x-axis. Purple: dwell times in the LF state prior to transitioning to the MF state. Blue: dwell times in MF prior to transitioning to LF. Yellow: dwell times in MF prior to transitioning to HF. Red: Dwell times in HF prior to transitioning to MF. Bi-exponential fits are overlaid in dashed black lines. (B) Same as A but for Ψ₅₅ tRNA eMet. (C) Same as A but for U₅₅ tRNA Thr. (D) Same as A but for Ψ₅₅ tRNA Thr.



SUPPLEMENTAL FIGURE S2.11. Example traces showing interconversion between MF microstates (highlighted by orange boxes) in the presence of Pus4 for (A) U₅₅ tRNA eMet or (B) Ψ₅₅ tRNA eMet.



SUPPLEMENTAL FIGURE S2.12. U₅₅ tRNA eMet smFRET timecourse without the addition of Pus4. Histograms (left column) and TODPs (right column) recorded (A) 2–6 minutes after introduction of imaging buffer; (B) 7–9 minutes; (C) 10–15 minutes; (D) 30–34 minutes.



SUPPLEMENTAL FIGURE S2.13. Secondary structures and associated free energies at 25°C and 0.1 M salt predicted using the RNAfold web server (see **Methods** for details). (A) Predicted C structure of tRNA eMet. (B) Plausible E-like structure of tRNA eMet. (C) Predicted C structure of tRNA Thr. (D) Plausible E-like structure of tRNA Thr. (E) C structure of human mitochondrial tRNA^{Lys} from Voigts-Hoffmann *et al.* 2007. (F) E structure of human mitochondrial tRNA^{Lys} from Voigts-Hoffmann *et al.* 2007.

SUPPLEMENTAL TABLE S2.1. Yeast strains, plasmids and plasmid insert sequences.

Yeast strains

Strain	Description	From
YDG1	wild-type, BY4741	Horizon/Dharmacon Reagents
YDG127	<i>pus4</i> Δ::KanMX	Yeast Deletion Collection (Giaever <i>et al.</i> 2002)

Plasmids

Construct	Description	From
PDG322	To express WT Pus4 in <i>E. coli</i> ; pGEX-5X-3-ScPus4	This study, modified from plasmid gifted by Dr. Ute Kothe

Insert sequences

Sequence of expressed wild-type Pus4 protein (including GST tag, Factor Xa cleavage site, and Pus4 protein sequence):

ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTT
CTTTTGAATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGT
GATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTAT
TATATTGATGGTGATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCT
GACAAGCACACATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCT
TGAAGGAGCGGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGA
CTTTGAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTT
CGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCCATCCTGA
CTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGAT
GCGTTCCCAAATTAGTTTGTTTAAAAAACGTATTGAAGCTATCCCACAAATTGAT
AAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCAC
GTTTGGTGGTGGCGACCATCCTCCAAAATCGGATCTGATCGAAGGTCGTGGGATCCC
CAGGAATTCGGGGAACATGAATGGAATATTTGCTATTGAGAAGCCTAGCGGTATTA
CATCCAATCAGTTTATGCTAAAGTTGCAACATGCTTTAACTAAAAGTCAAGTATTCT
CGAAGGAAATTCAAAGGGCAACAGCGGAAAGAAAGCAGCAATATGAAAAACAGAC
AGGTAAGAAGGCCAGCAAGAGGAACTTCGTAAAGTCTCAAAGGTAAAGATGGGA
CACGGAGGCACTTTGGATCCGTTGGCCTCTGGTGTCTGGTAATTGGGATTGGAGCG
GGCACCAAAAACTTGCAAATTATCTTTCAGGCACCGTTAAAGTCTATGAAAGTGAA
GCCCTGTTTGGTGTTCCTACTTTCAGGAGATGTAGAAGGTGAGATTTTGTCCCAG
AACTCCGTTAAACATTTAAATTTTCGATGATTTAAAGACTGTGGAGGAAAAATTTGTT
GGACAATTAAAGCAGACGCCGCCAATATACGCAGCTTTGAAGATGGATGGCAAACC
CCTGCATGAATATGCTCGAGAAGGAAAGCCTTTACCTAGAGCCATTGAGCCCAGAC

AAGTTACTATTTATGACTTGAAGGTGTTTTTCAGACTCTCTTAAACGTGATCATGACTA
TCCCTTATTGAGGCCCACTACAGAAGAAGCTGTTGATACCGTGAAAAATTTGAATGC
AAACATGCTAAACGATGTTTTATACTTTTCAAAAGAATATACAGAGAAGCACGGCCT
TGATTCTGAGGTGGCAAAGGTAGAAGAACCATTTCCGCTGAGTGAACAAGAAGAGC
AAGAAATCCAAAAAGAAGGAGATTCTTATAGGGCTCCAAAATTGCACTTCAAAGCT
AATGTGTCTTCTGGAACATACATAAGGTCCTTAGTGAGCGACATCGGTAAATCTATG
AGGAGCTCATGTTATATGGTGAAATTGATACGTTTGCAACAGCAGGACTGGTCTCTT
GAAAAAAATAATGTGTTCCAATTAACGGATTTTACTGAAAGAGACGAGAAGGTGTG
GAGTAAGGTACTGGAGAAGGTTCTAGACGAGGGAGCAACTGTTGATGTTATAGAAG
AGCTAAAGAAAGCAGAAAAGGAAATACCAGCGGACGTGAAGGAATGTATAGTTTC
GAGTGATCAACCTGGTGATGAGGCTACGGCTGAAACAATTGAAACTGCTAACGCCG
AAGAACATTCTAATACACTAAAAAGAAAAATCGAACAGGTGTAA

SUPPLEMENTAL TABLE S2.2. Fluorophore intensities.

The mean intensity across all frames of all donor or acceptor traces within each analyzed movie was determined. The table reports the mean and standard deviation of this value across the indicated number of movies. Time ranges indicate the time window following injection of imaging buffer containing OSS either with or without Pus4 during which all movies in a condition were recorded. Measurements are on tRNA eMet unless otherwise noted.

Condition	Donor intensity	Acceptor intensity	Movies
U ₅₅ only	2500±150	3300±340	3
U ₅₅ + Pus4 2–6 min.	9400±1670	9200±970	5
U ₅₅ + Pus4 7–9 min.	10000±2760	10000±1290	4
U ₅₅ + Pus4 10–15 min.	12700±950	11000±1260	4
U ₅₅ + Pus4 30–34 min.	11000±1080	11000±1070	3
Ψ ₅₅ only	2500±480	2800±400	3
Ψ ₅₅ + Pus4 2–6 min.	10800±500	10000±1720	3
Ψ ₅₅ + Pus4 7–9 min.	10700±800	12100±620	2
Ψ ₅₅ + Pus4 10–15 min.	11300±930	11700±110	3
Ψ ₅₅ + Pus4 30–34 min.	12000±1620	11400±850	3
U ₅₅ alone 2–6 min.*	4600±280	4000±620	2
U ₅₅ alone 7–9 min.*	4270	4430	1
U ₅₅ alone 10–15 min.*	4130±280	4320±270	2
U ₅₅ alone 30–34 min.*	4500±520	3930±30	2
Ψ ₅₅ alone 10–15 min.*	4320±50	4100±80	2

U ₅₅ tRNA ^{Thr} 10–15 min.*	3200±100	5100±160	2
Ψ ₅₅ tRNA ^{Thr} 10–15 min.*	3400±340	5500±230	3

*Recorded with an electron multiplication (EM) gain setting of 450. All others recorded with a setting of 300.

SUPPLEMENTAL TABLE S2.3. Oligonucleotides

Description	Sequence (5' to 3')	Supplier and Purification	Nucleic Acid
eMet CAU 5' oligo	biotin- GCUU[Cy3]CAGUAGCUCAGUAGGAAGAGCGU CAGUCUCAUAAU	Dharmacon, HPLC	RNA
eMet CAU 3' oligo	phosphate- CU[Cy5]GAAGGUCGAGAGUUCGAACCUC CCC UGGAGCACCA	Dharmacon, HPLC	RNA
eMet CAU 3' with Ψ	phosphate- CU[Cy5]GAAGGUCGAGAGUΨCGAACCUC CCC UGGAGCACCA	Dharmacon, HPLC	RNA
Thr AGU 5' oligo	biotin- GCUU[Cy3]CUAUGGCCAAGUUGGUAAGGCGC CACACUAGUAAU	Dharmacon, HPLC	RNA
Thr AGU 3' oligo	phosphate- GU[Cy5]GGAGAUCAUCGGUUC AAAUCCGAUU GGAAGCACCA	Dharmacon, HPLC	RNA
Thr AGU 3' with Ψ	phosphate- GU[Cy5]GGAGAUCAUCGGUΨCAA AUCCGAUU GGAAGCACCA	Dharmacon, HPLC	RNA
Splint for 5' and 3' portions of tRNA eMet	GGTGGGTCTGCTTTGGTGCTCCAGGGGAGGT TCGAACTCTCGACCTTCAGATTATGAGACTG ACGCTCTTCCTACTGAGCTACTGAAGCATTTT GGGTAC	IDT, PAGE	DNA
Splint for 5' and 3' portions of tRNA Thr	GGTGGGTCTGCTTTGGTGCTTCCAATCGGATT TGAACCGATGATCTCCACATTACTAGTGTGG CGCCTTACCAACTTGGCCATAGAAGCATTTTG GGTAC	IDT, PAGE	DNA
NAD ⁺ riboswitch	biotin- AUCUAUAGAGCGUU(Cy5)GCGUCCGAAAGUC UAAACAGACACGGCUCUUUAAAAACAAAAG GAGA(Cy3)	Dharmacon, HPLC	RNA

eMet CAU, no fluorophores	biotin- GCUUCAGUAGCUCAGUAGGAAGAGCGUCAG UCUCAUAAUCUGAAGGUCGAGAGUUCGAAC CUCCCCUGGAGCACCA	Dharmacon, HPLC	RNA
eMet CAU with Ψ, no fluorophores	biotin- GCUUCAGUAGCUCAGUAGGAAGAGCGUCAG UCUCAUAAUCUGAAGGUCGAGAGUΨCGAAC CUCCCCUGGAGCACCA	Dharmacon, HPLC	RNA

SUPPLEMENTAL TABLE S2.4. Direct RNA Sequencing Data Files

Sample is the name of the sample as referred to in Dennis and Conoan Nieves *et al.* 2025.

Fast5 and FastQ file are the names of the files containing the basecalled sequencing data for the specified sample. Files from this study are available at the European Nucleotide Archive (<https://www.ebi.ac.uk/ena>) with study accession number PRJEB85651. Files from Shaw and Thomas *et al.* 2024 reanalyzed in this study are available with study accession number PRJEB68201.

Accession	Sample	Fast5 file	FastQ file
PRJEB856 51	WT Pus4 <i>in vitro</i>	20240718_RNA002_UO _sCer_tRNA_pus4deletio n_in_vitro_WT_pus4.tar. gz	20240718_RNA002_UO_sCer_tRNA_p us4deletion_in_vitro_WT_pus4_noU.fas tq.gz
PRJEB856 51	WT Pus4 no MgCl ₂ <i>in vitro</i>	20240813_RNA002_UO _sCer_tRNA_pus4deletio n_in_vitro_WT_pus4_no MgCl ₂ .tar.gz	20240813_RNA002_UO_sCer_tRNA_p us4deletion_in_vitro_WT_pus4_noMgC l_noU.fastq.gz
PRJEB682 01	<i>in vivo</i> WT replicate 1	WT_tRNAs_rep1_7_22_ 21_fast5s.tar.gz	WT_tRNAs_rep1_7_22_noU.fastq.gz
PRJEB682 01	<i>in vivo</i> WT replicate 2	WT_tRNAs_rep2_8_6_2 1_fast5s.tar.gz	WT_tRNAs_rep2_8_6_21 _noU.fastq.gz
PRJEB682 01	<i>in vivo</i> WT replicate 3	WT_tRNAs_rep3_9_17_ 21_fast5s.tar.gz	WT_tRNAs_rep3_9_17_21_noU.fastq.g z
PRJEB682 01	<i>in vivo</i> <i>pus4</i> Δ replicate 1	pus4_tRNAs_rep1_06_30 _21_fast5s.tar.gz	pus4_tRNAs_rep1_06_30_21_noU.fastq .gz
PRJEB682 01	<i>in vivo</i> <i>pus4</i> Δ replicate 2	pus4_tRNAs_rep2_07_19 _21_fast5s.tar.gz	pus4_tRNAs_rep2_07_19_21_noU.fastq .gz

PRJEB682	<i>in vivo</i>	pus4_tRNAs_rep3_10_26	pus4_tRNAs_rep3_10_26_21_noU.fastq
01	<i>pus4</i> Δ	_21_fast5s.tar.gz	.gz
	replicate 3		

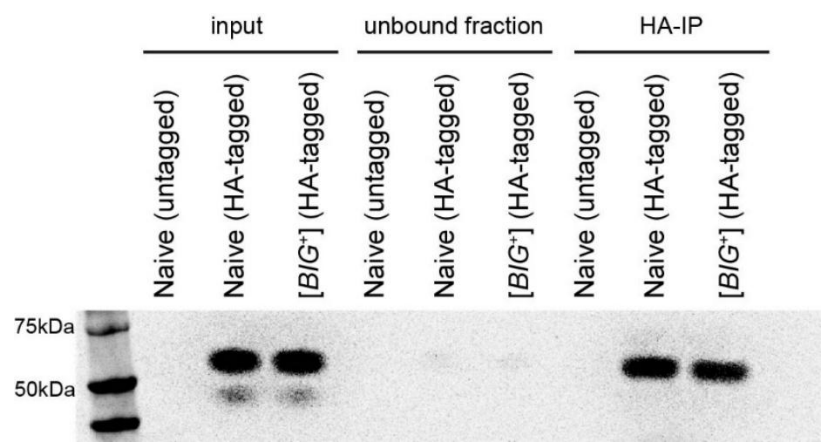
SUPPLEMENT REFERENCES:

Giaever G, Chu AM, Ni L, Connelly C, Riles L, Véronneau S, Dow S, Lucau-Danila A, Anderson K, André B, et al. 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**: 387–391..

Shaw EA, Thomas NK, Jones JD, Abu-Shumays RL, Vaaler AL, Akeson M, Koutmou KS, Jain M, Garcia DM. 2024. Combining Nanopore direct RNA sequencing with genetics and mass spectrometry for analysis of T-loop base modifications across 42 yeast tRNA isoacceptors. *Nucleic Acids Res* **52**: 12074–12092.

Voigts-Hoffmann F, Hengesbach M, Kobitski AY, van Aerschot A, Herdewijn P, Nienhaus GU, Helm M. 2007. A Methyl Group Controls Conformational Equilibrium in Human Mitochondrial tRNA^{Lys}. *J Am Chem Soc* **129**: 13382–13383.

SUPPLEMENT FOR CHAPTER IV:



SUPPLEMENTAL FIGURE 4.1: Anti-HA western blot of HA-IP of naïve and [BIG⁺] lysates. 5 mg of total protein loaded into the input lanes.

Supplemental Table S4.1: Summary of differentially expressed genes in the LiP-MS HA-IP experiment (adj. p-value < 0.01). Primarily ribosomal proteins were pulled out in both samples, but other proteins included IMD4, HSP150, YCR087C-A, TY1B-H, and CMD1. There were no significantly differentially expressed genes when comparing naïve to $[BIG^+]$. Albumin was a significant contaminant, likely due to the bead pulldown.

Protein	Naïve (YDG1 vs YDG989) Adjusted P-value	$[BIG^+]$ (YDG1 vs YDG999) Adjusted P-value	Naïve vs $[BIG^+]$ (YDG989 vs YDG999) Adjusted P-value
ALB	8.85E-07	5.87E-06	0.362
PUS4	8.85E-07	7.60E-07	0.987
RPL33A	1.85E-06	7.60E-07	0.987
RPL1A	1.90E-06	7.60E-07	0.987
RPL34B	2.88E-06	3.89E-06	0.987
RPL21A	4.64E-06	3.96E-06	0.987
RPL15A	5.19E-06	9.13E-06	0.987
IMD4	1.37E-05	2.75E-05	0.987
RPL4A	5.41E-05	2.36E-05	0.987
RPS5	5.41E-05	3.20E-05	0.987
RPL37A	8.02E-05	3.36E-05	0.987
RPL32	0.000326	0.000147	0.987
RPL6B	0.00068	0.000439	0.987
RPS25B	0.00073	0.00122	0.987
RPL24A	0.000995	0.000301	0.987
RPL3	0.000995	0.000439	0.987
HSP150	0.000995	0.00032	0.987
YCR087C-A	0.00155	0.00237	0.987
RPL5	0.00188	0.00111	0.987
RPL23A	0.00267	0.00276	0.987
TY1B-H	0.00311	0.00572	0.987
RPL13B	0.0037	0.00356	0.987
RPL6A	0.0037	0.000939	0.987
RPN13	0.00384	0.00182	0.987
CMD1	0.00408	0.00839	0.987
RPL10	0.00639	0.00279	0.987
RPP2B	0.00708	0.00664	0.987
RPL16B	0.00708	0.00468	0.987
RPL11A	0.00794	0.00279	0.987
RPL8B	0.00848	0.00772	0.987
RPL17A	0.0087	0.00302	0.987
RPL19A	0.0087	0.00402	0.987
RPL16A	0.0087	0.00468	0.987

SUPPLEMENTAL TABLE S4.2: Proteins and RNAs with increased or decreased levels in a *YAK1* Q578* strain compared to a WT strain (Brion *et al.* 2020). The overlapped hits with the downregulated proteins from the WCL-MS IDeA dataset are denoted with asterisks (five proteins in total).

RNA and Protein Lower	RNA and Protein Higher	Protein only lower	Protein only higher
HSP12	PET10	HBT1*	PAB1
CWP1	SBP1	HSP26*	PAL1
RTN2*	YER079W	YPR127W	PRO3
UGA2	ADH5	SEC26	SHM1
MSC1*	HSP78	RTN1	PBP1
GTT1	ILV2	GCN1	PWP1
CTT1*	ASN1	URA2	YRA1
KAP123	YDR391C	DBP6	SSC1
GPP2	YGL117W	ALY2	YLR287C
VMA13	YGL039W	SAC1	IDH1
YGP1	AIM17	RPB2	PIN4
RPL43A	YNL134C	FAS1	MOT2
FAA1	ZTA1	RPN2	FRS2
AAH1	GCY1	ILS1	RNR2
CRH1	YHB1	CLU1	CDC11
YPT7		RPO21	DPH5
SCW10		NDE1	REH1
DOT6		UGA1	BDF1
RPA135		SEC21	ARA1
YFR006W		CDC60	AIM9
URA1		COP1	TAL1
ACO1		SEC7	SSE1
GRE2		CHC1	BEM1
YCP4		NEW1	LYS4
FAP7		XRN1	NUP57
PIL1		ADE6	CCT2
PSA1		VAS1	URE2
		ACC1	SRP54
		RAT1	SHM2
		FAS2	SSQ1
		PFK1	TAF8
		URB1	GOR1
		MDN1	ZEO1
		TCB3	MRD1
		RRP5	SSZ1
		RPA190	SET5

		RPG1	NNR2
		RPN1	CIT1
		ARO1	PTC5
		FUN12	PRX1
		SCS2	PGI1
		UBA1	RET2
		PFK2	ENP2
		PRB1	CCT8
		EIS1	ZDS1
		MAK16	CDC48
		ADE1	RPN9
		RHO3	VMA5
		AIM45	DUS3
			TOM70
			MVD1
			TYS1
			THR4
			UTP7
			HXK1
			MRP51
			RSM24
			HEL2
			ILV1
			ZPS1

SUPPLEMENTAL TABLE S4.3: Yeast strains used in this study.

Strain	Description	Source
YDG1	Wild-type, BY4741	Horizon/Dharmacon Reagents
YDG109	[<i>BIG</i> ⁺], Strain A (Figure 4.5)	Garcia & Campbell <i>et al.</i> , 2021
YDG127	<i>pus4</i> Δ, replaced with KanMX	Yeast Deletion Collection (Giaver, G. <i>et al.</i> , 2002)
YDG162	[<i>BIG</i> ⁺], Strain B (Figure 4.5)	Garcia & Campbell <i>et al.</i> , 2021
YDG219	<i>pus4</i> Δ, Pus4 replaced with Ura3	Garcia & Campbell <i>et al.</i> , 2021
YDG989	C-terminus 3xHA-tagged Pus4, Naive	CRISPR on YDG1
YDG999	C-terminus 3xHA-tagged Pus4, [<i>BIG</i> ⁺]	CRISPR on YDG162
YDG1146	Naïve KRK Deletion of Pus4, C-terminus 3xHA-tagged	CRISPR on YDG989
YDG1147	[<i>BIG</i> ⁺] KRK Deletion of Pus4, C-terminus 3xHA-tagged	CRISPR on YDG999
YDG1148	Naïve No Charge mutant, DERKH residues mutated to A of C-terminus of Pus4, C-terminus 3xHA-tagged	CRISPR on YDG989
YDG1149	[<i>BIG</i> ⁺] No Charge mutant, DERKH residues mutated to A of C-terminus of Pus4, C-terminus 3xHA-tagged	CRISPR on YDG999
YDG1150	Naïve Full C-terminus Deletion of Pus4, C-terminus 3xHA-tagged	CRISPR on YDG989
YDG1151	[<i>BIG</i> ⁺] Full C-terminus Deletion of Pus4, C-terminus 3xHA-tagged	CRISPR on YDG999

YDG1152	Naïve C-terminus Helix 2 Deletion of Pus4, C-terminus 3xHA-tagged	CRISPR on YDG989
YDG1153	[<i>BIG</i> ⁺] C-terminus Helix 2 Deletion of Pus4, C-terminus 3xHA-tagged	CRISPR on YDG999
YDG1178	rhy1248 prion strain containing [<i>RNQ</i> ⁺] [<i>PSI</i> ⁺] [<i>mot3</i> ⁻]	Halfmann Lab (Stowers Institute)
YDG1270	<i>pus4</i> Δ, Pus4 replaced with His3	CRISPR on YDG127
YDG1289	Nonsense mutant of Pus4	CRISPR on YDG1
YDG1338	Naïve All Negative C-terminus mutant, 3xHA-tagged on C-terminus, all positively charged residues (DE) mutated to Lysine (K)	CRISPR on YDG1
YDG1339	[<i>BIG</i> ⁺] All Negative C-terminus mutant, 3xHA-tagged on C-terminus, all positively charged residues (DE) mutated to Lysine (K)	CRISPR on YDG162
YDG1340	Naïve All Positive C-terminus mutant, 3xHA-tagged on C-terminus, all negatively charged residues (KRH) mutated to Aspartic Acid (D)	CRISPR on YDG1
YDG1341	[<i>BIG</i> ⁺] All Positive C-terminus mutant, 3xHA-tagged on C-terminus, all negatively charged residues (KRH) mutated to Aspartic Acid (D)	CRISPR on YDG162
YDG1364	[<i>BIG</i> ⁺] with 3xHA tag added on C-terminus of Yak1	CRISPR on YDG109
YDG1365	Naïve with 3xHA tag added on C-terminus of Yak1	CRISPR on YDG1
YDG1398	YDG109 with repair to eliminate nonsense mutation (Q578*) in <i>YAK1</i>	CRISPR on YDG109
YDG1399	YDG162 with repair to eliminate nonsense mutation (Q578*) <i>YAK1</i>	CRISPR on YDG162
YDG1400	YDG1 with nonsense mutation (Q578*) added in <i>YAK1</i>	CRISPR on YDG1

SUPPLEMENT REFERENCES:

Brion, C., Lutz, S. M. & Albert, F. W. Simultaneous quantification of mRNA and protein in single cells reveals post-transcriptional effects of genetic variation. *eLife* **9**, e60645 (2020).

Garcia, D. M. *et al.* A prion accelerates proliferation at the expense of lifespan. *eLife* **10**, (2021).

Giaever, G. *et al.* Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**, 387–391 (2002).

REFERENCES

1. Liu, W.-W. *et al.* RNA modifications in cellular metabolism: implications for metabolism-targeted therapy and immunotherapy. *Sig Transduct Target Ther* **9**, 70 (2024).
2. Cohn, W. E. & Volkin, E. Nucleoside-5'-Phosphates from Ribonucleic Acid. *Nature* **167**, 483–484 (1951).
3. Pan, T. Modifications and functional genomics of human transfer RNA. *Cell Res* **28**, 395–404 (2018).
4. Lucas, M. C. *et al.* Quantitative analysis of tRNA abundance and modifications by nanopore RNA sequencing. *Nat Biotechnol* **42**, 72–86 (2024).
5. Shaw, E. A. *et al.* Combining Nanopore direct RNA sequencing with genetics and mass spectrometry for analysis of T-loop base modifications across 42 yeast tRNA isoacceptors. *Nucleic Acids Research* **52**, 12074–12092 (2024).
6. Sahin, U. *et al.* BNT162b2 vaccine induces neutralizing antibodies and poly-specific T cells in humans. *Nature* **595**, 572–577 (2021).
7. Karikó, K. *et al.* Incorporation of Pseudouridine Into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability. *Molecular Therapy* **16**, 1833–1840 (2008).
8. Tomikawa, C. Pseudouridine Modifications in Transfer RNA and tRNA Pseudouridine Synthases. *Journal of Molecular Biology* **437**, 169183 (2025).
9. Biela, A. D. *et al.* Determining the effects of pseudouridine incorporation on human tRNAs. *The EMBO Journal* **44**, 3553–3585 (2025).

10. Becker, H. F., Motorin, Y., Planta, R. J. & Grosjean, H. The yeast gene YNL292w encodes a pseudouridine synthase (Pus4) catalyzing the formation of Ψ 55 in both mitochondrial and cytoplasmic tRNAs. *Nucleic Acids Res* **25**, 4493–4499 (1997).
11. Reinsch, J. L. & Garcia, D. M. Concurrent detection of chemically modified bases in yeast mitochondrial tRNAs by Nanopore direct RNA sequencing. 2025.05.09.653160 Preprint at <https://doi.org/10.1101/2025.05.09.653160> (2025).
12. Schwartz, S. *et al.* Transcriptome-wide Mapping Reveals Widespread Dynamic-Regulated Pseudouridylation of ncRNA and mRNA. *Cell* **159**, 148–162 (2014).
13. Carlile, T. M. *et al.* Pseudouridine profiling reveals regulated mRNA pseudouridylation in yeast and human cells. *Nature* **515**, 143–146 (2014).
14. Begik, O. *et al.* Quantitative profiling of pseudouridylation dynamics in native RNAs with nanopore sequencing. *Nat Biotechnol* **39**, 1278–1291 (2021).
15. Keffer-Wilkes, L. C., Veerareddygar, G. R. & Kothe, U. RNA modification enzyme TruB is a tRNA chaperone. *Proceedings of the National Academy of Sciences* **113**, 14306–14311 (2016).
16. Hoang, C., Hamilton, C. S., G.Mueller, E. & Ferré-D’Amaré, A. R. Precursor complex structure of pseudouridine synthase TruB suggests coupling of active site perturbations to an RNA-sequestering peripheral protein domain. *Protein Science* **14**, 2201–2206 (2005).
17. Pan, H., Agarwalla, S., Moustakas, D. T., Finer-Moore, J. & Stroud, R. M. Structure of tRNA pseudouridine synthase TruB and its RNA complex: RNA recognition through a combination of rigid docking and induced fit. *Proceedings of the National Academy of Sciences* **100**, 12648–12653 (2003).

18. Hoang, C. & Ferré-D'Amaré, A. R. Cocystal Structure of a tRNA Ψ 55 Pseudouridine Synthase: Nucleotide Flipping by an RNA-Modifying Enzyme. *Cell* **107**, 929–939 (2001).
19. Abramson, J. *et al.* Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**, 493–500 (2024).
20. Wright, J. R., Keffer-Wilkes, L. C., Dobing, S. R. & Kothe, U. Pre-steady-state kinetic analysis of the three *Escherichia coli* pseudouridine synthases TruB, TruA, and RluA reveals uniformly slow catalysis. *RNA* **17**, 2074–2084 (2011).
21. Chou, K. Y. *et al.* Histone modification in *Saccharomyces cerevisiae*: A review of the current status. *Computational and Structural Biotechnology Journal* **21**, 1843–1850 (2023).
22. Fuchs, S. M. & Quasem, I. Budding yeast as a model to study epigenetics. *Drug Discovery Today: Disease Models* **12**, 1–6 (2014).
23. Lasserre, J.-P. *et al.* Yeast as a system for modeling mitochondrial disease mechanisms and discovering therapies. *Dis Model Mech* **8**, 509–526 (2015).
24. Malina, C., Larsson, C. & Nielsen, J. Yeast mitochondria: an overview of mitochondrial biology and the potential of mitochondrial systems biology. *FEMS Yeast Res* **18**, foy040 (2018).
25. Foury, F., Roganti, T., Lecrenier, N. & Purnelle, B. The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Letters* **440**, 325–331 (1998).
26. Westermann, B. Mitochondrial inheritance in yeast. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1837**, 1039–1046 (2014).
27. Reichert, P. & Caudron, F. Mnemons and the memorization of past signaling events. *Current Opinion in Cell Biology* **69**, 127–135 (2021).

28. Caudron, F. & Barral, Y. A Super-Assembly of Whi3 Encodes Memory of Deceptive Encounters by Single Cells during Yeast Courtship. *Cell* **155**, 1244–1257 (2013).
29. Cox, B. S. Ψ , A cytoplasmic suppressor of super-suppressor in yeast. *Heredity* **20**, 505–521 (1965).
30. Nakayashiki, T., Kurtzman, C. P., Edskes, H. K. & Wickner, R. B. Yeast prions [URE3] and [PSI⁺] are diseases. *PNAS* **102**, 10575–10580 (2005).
31. True, H. L. & Lindquist, S. L. A yeast prion provides a mechanism for genetic variation and phenotypic diversity. *Nature* **407**, 477–483 (2000).
32. Suzuki, G., Shimazu, N. & Tanaka, M. A Yeast Prion, Mod5, Promotes Acquired Drug Resistance and Cell Survival Under Environmental Stress. *Science* **336**, 355–359 (2012).
33. Brown, J. C. S. & Lindquist, S. A heritable switch in carbon source utilization driven by an unusual yeast prion. *Genes & Development* **23**, 2320–2332 (2009).
34. Halfmann, R. *et al.* Prions are a common mechanism for phenotypic inheritance in wild yeasts. *Nature* **482**, 363–368 (2012).
35. Yuan, A. H. & Hochschild, A. A bacterial global regulator forms a prion. *Science* **355**, 198–201 (2017).
36. Chakrabortee, S. *et al.* Luminidependens (LD) is an Arabidopsis protein with prion behavior. *PNAS* **113**, 6065–6070 (2016).
37. Nan, H., Chen, H., Tuite, M. F. & Xu, X. A viral expression factor behaves as a prion. *Nature Communications* **10**, 359 (2019).
38. Prusiner, S. B. Novel proteinaceous infectious particles cause scrapie. *Science* **216**, 136–144 (1982).

39. Chakrabortee, S. *et al.* Intrinsically Disordered Proteins Drive Emergence and Inheritance of Biological Traits. *Cell* **167**, 369-381.e12 (2016).
40. Alberti, S., Halfmann, R., King, O., Kapila, A. & Lindquist, S. A Systematic Survey Identifies Prions and Illuminates Sequence Features of Prionogenic Proteins. *Cell* **137**, 146–158 (2009).
41. Derkatch, I. L., Bradley, M. E., Hong, J. Y. & Liebman, S. W. Prions Affect the Appearance of Other Prions: The Story of [PIN⁺]. *Cell* **106**, 171–182 (2001).
42. Garcia, D. M. *et al.* A prion accelerates proliferation at the expense of lifespan. *eLife* **10**, (2021).
43. Rintala-Dempsey, A. C. & Kothe, U. Eukaryotic stand-alone pseudouridine synthases – RNA modifying enzymes and emerging regulators of gene expression? *RNA Biology* **14**, 1185–1196 (2017).
44. Mukhopadhyay, S., Deogharia, M. & Gupta, R. Mammalian nuclear TRUB1, mitochondrial TRUB2, and cytoplasmic PUS10 produce conserved pseudouridine 55 in different sets of tRNA. *RNA* **27**, 66–79 (2021).
45. Song, J. *et al.* Differential roles of human PUS10 in miRNA processing and tRNA pseudouridylation. *Nature Chemical Biology* **16**, 160–169 (2020).
46. Lorenz, C., Lünse, C. E. & Mörl, M. tRNA Modifications: Impact on Structure and Thermal Adaptation. *Biomolecules* **7**, 35 (2017).
47. Zhang, J. & Ferré-D’Amaré, A. R. The tRNA Elbow in Structure, Recognition and Evolution. *Life* **6**, 3 (2016).
48. Biela, A. *et al.* The diverse structural modes of tRNA binding and recognition. *Journal of Biological Chemistry* **299**, (2023).

49. Robertus, J. D. *et al.* Structure of yeast phenylalanine tRNA at 3 Å resolution. *Nature* **250**, 546–551 (1974).
50. Shi, H. & Moore, P. B. The crystal structure of yeast phenylalanine tRNA at 1.93 Å resolution: a classic structure revisited. *RNA* **6**, 1091–1105 (2000).
51. Cappannini, A. *et al.* MODOMICS: a database of RNA modifications and related information. 2023 update. *Nucleic Acids Research* **52**, D239–D244 (2024).
52. Carlile, T. M. *et al.* mRNA structure determines modification by pseudouridine synthase 1. *Nat Chem Biol* **15**, 966–974 (2019).
53. Lin, T.-Y. *et al.* The molecular basis of tRNA selectivity by human pseudouridine synthase 3. *Molecular Cell* **84**, 2472-2489.e8 (2024).
54. Purchal, M. K. *et al.* Pseudouridine synthase 7 is an opportunistic enzyme that binds and modifies substrates with diverse sequences and structures. *Proceedings of the National Academy of Sciences* **119**, e2109708119 (2022).
55. Behm-Ansmant, I. *et al.* The *Saccharomyces cerevisiae* U2 snRNA:pseudouridine-synthase Pus7p is a novel multisite–multisubstrate RNA:Ψ-synthase also acting on tRNAs. *RNA* **9**, 1371–1382 (2003).
56. Grünberg, S. *et al.* The structural basis of mRNA recognition and binding by yeast pseudouridine synthase PUS1. *PLOS ONE* **18**, e0291267 (2023).
57. Roovers, M. *et al.* Formation of the conserved pseudouridine at position 55 in archaeal tRNA. *Nucleic Acids Research* **34**, 4293–4301 (2006).
58. Gurha, P., Joardar, A., Chaurasia, P. & Gupta, R. Differential Roles of Archaeal Box H/ACA Proteins in Guide RNA-Dependent and Independent Pseudouridine Formation. *RNA Biology* **4**, 101–109 (2007).

59. Ansmant, I., Massenet, S., Grosjean, H., Motorin, Y. & Branlant, C. Identification of the *Saccharomyces cerevisiae* RNA:pseudouridine synthase responsible for formation of Ψ 2819 in 21S mitochondrial ribosomal RNA. *Nucleic Acids Research* **28**, 1941–1946 (2000).
60. Ansmant, I., Motorin, Y., Massenet, S., Grosjean, H. & Branlant, C. Identification and Characterization of the tRNA: Ψ 31-Synthase (Pus6p) of *Saccharomyces cerevisiae* *. *Journal of Biological Chemistry* **276**, 34934–34940 (2001).
61. Zhou, J., Liang, B. & Li, H. Structural and functional evidence of high specificity of Cbf5 for ACA trinucleotide. *RNA* **17**, 244–250 (2011).
62. Tillault, A.-S., Schultz, S. K., Wieden, H.-J. & Kothe, U. Molecular Determinants for 23S rRNA Recognition and Modification by the *E. coli* Pseudouridine Synthase RluE. *Journal of Molecular Biology* **430**, 1284–1294 (2018).
63. Schaening-Burgos, C., LeBlanc, H., Fagre, C., Li, G.-W. & Gilbert, W. V. RluA is the major mRNA pseudouridine synthase in *Escherichia coli*. *PLOS Genetics* **20**, e1011100 (2024).
64. Urban, A., Behm-Ansmant, I., Branlant, C. & Motorin, Y. RNA Sequence and Two-dimensional Structure Features Required for Efficient Substrate Modification by the *Saccharomyces cerevisiae* RNA: Ψ -Synthase Pus7p*. *Journal of Biological Chemistry* **284**, 5845–5858 (2009).
65. Lecointe, F. *et al.* Characterization of Yeast Protein Deg1 as Pseudouridine Synthase (Pus3) Catalyzing the Formation of Ψ 38 and Ψ 39 in tRNA Anticodon Loop*. *Journal of Biological Chemistry* **273**, 1316–1323 (1998).
66. Motorin, Y. *et al.* The yeast tRNA:pseudouridine synthase Pus1p displays a multisite substrate specificity. *RNA* **4**, 856–869 (1998).

67. Gurha, P. & Gupta, R. Archaeal Pus10 proteins can produce both pseudouridine 54 and 55 in tRNA. *RNA* **14**, 2521–2527 (2008).
68. Phannachet, K. & Huang, R. H. Conformational change of pseudouridine 55 synthase upon its association with RNA substrate. *Nucleic Acids Research* **32**, 1422–1429 (2004).
69. Gu, X., Yu, M., Ivanetich, K. M. & Santi, D. V. Molecular Recognition of tRNA by tRNA Pseudouridine 55 Synthase. *Biochemistry* **37**, 339–343 (1998).
70. Gutsell, N. *et al.* Deletion of the Escherichia coli pseudouridine synthase gene truB blocks formation of pseudouridine 55 in tRNA in vivo, does not affect exponential growth, but confers a strong selective disadvantage in competition with wild-type cells. *RNA* **6**, 1870–1881 (2000).
71. Gc, K., Gyawali, P., Balci, H. & Abeysirigunawardena, S. Ribosomal RNA Methyltransferase RsmC Moonlights as an RNA Chaperone. *ChemBioChem* **21**, 1885–1892 (2020).
72. Johansson, M. J. O. & Byström, A. S. Dual function of the tRNA(m(5)U54)methyltransferase in tRNA maturation. *RNA* **8**, 324–335 (2002).
73. Copela, L. A., Chakshusmathi, G., Sherrer, R. L. & Wolin, S. L. The La protein functions redundantly with tRNA modification enzymes to ensure tRNA structural stability. *RNA* **12**, 644–654 (2006).
74. Keffer-Wilkes, L. C., Soon, E. F. & Kothe, U. The methyltransferase TrmA facilitates tRNA folding through interaction with its RNA-binding domain. *Nucleic Acids Research* **48**, 7981–7990 (2020).
75. Voigts-Hoffmann, F. *et al.* A Methyl Group Controls Conformational Equilibrium in Human Mitochondrial tRNA^{Lys}. *J. Am. Chem. Soc.* **129**, 13382–13383 (2007).

76. Dammertz, K., Hengesbach, M., Helm, M., Nienhaus, G. U. & Kobitski, A. Yu. Single-Molecule FRET Studies of Counterion Effects on the Free Energy Landscape of Human Mitochondrial Lysine tRNA. *Biochemistry* **50**, 3107–3115 (2011).
77. Kobitski, A. Yu., Hengesbach, M., Helm, M. & Nienhaus, G. U. Sculpting an RNA Conformational Energy Landscape by a Methyl Group Modification—A Single-Molecule FRET Study. *Angewandte Chemie International Edition* **47**, 4326–4330 (2008).
78. Kobitski, A. Y. *et al.* Single-Molecule FRET Reveals a Cooperative Effect of Two Methyl Group Modifications in the Folding of Human Mitochondrial tRNA^{Lys}. *Chemistry & Biology* **18**, 928–936 (2011).
79. Chan, P. P. & Lowe, T. M. GtRNAdb: a database of transfer RNA genes detected in genomic sequence. *Nucleic Acids Research* **37**, D93–D97 (2009).
80. Chan, P. P. & Lowe, T. M. GtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes. *Nucleic Acids Research* **44**, D184–D189 (2016).
81. Barraud, P. *et al.* Time-resolved NMR monitoring of tRNA maturation. *Nat Commun* **10**, 3373 (2019).
82. Macon, J. B. & Wolfenden, Richard. 1-Methyladenosine. Dimroth rearrangement and reversible reduction. *Biochemistry* **7**, 3453–3458 (1968).
83. Arluison, V., Buckle, M. & Grosjean, H. Pseudouridine synthetase *pus1* of *Saccharomyces cerevisiae*: kinetic characterisation, tRNA structural requirement and real-time analysis of its complex with tRNA. *Journal of Molecular Biology* **289**, 491–502 (1999).
84. Fabrini, R. *et al.* Monomer-dimer equilibrium in glutathione transferases: a critical re-examination. *Biochemistry* **48**, 10473–10482 (2009).

85. Conoan Nieves, N. E. & Widom, J. R. Ligand binding characteristics of an NAD⁺ riboswitch revealed by FRET and biolayer interferometry. *Chem. Commun.*
<https://doi.org/10.1039/D4CC04261A> (2024) doi:10.1039/D4CC04261A.
86. Panchapakesan, S. S. S., Corey, L., Malkowski, S. N., Higgs, G. & Breaker, R. R. A second riboswitch class for the enzyme cofactor NAD⁺. *RNA* **27**, 99–105 (2021).
87. Lovejoy, A. F., Riordan, D. P. & Brown, P. O. Transcriptome-Wide Mapping of Pseudouridines: Pseudouridine Synthases Modify Specific mRNAs in *S. cerevisiae*. *PLOS ONE* **9**, e110799 (2014).
88. McCann, J. J., Choi, U. B., Zheng, L., Weninger, K. & Bowen, M. E. Optimizing Methods to Recover Absolute FRET Efficiency from Immobilized Single Molecules. *Biophysical Journal* **99**, 961–970 (2010).
89. Blanco, M. & Walter, N. G. Chapter 9 - Analysis of Complex Single-Molecule FRET Time Trajectories. in *Methods in Enzymology* (ed. Walter, N. G.) vol. 472 153–178 (Academic Press, 2010).
90. Cole, P. E., Yang, S. K. & Crothers, D. M. Conformational changes of transfer ribonucleic acid. Equilibrium phase diagrams. *Biochemistry* **11**, 4358–4368 (1972).
91. Bhaskaran, H., Taniguchi, T., Suzuki, T., Suzuki, T. & Perona, J. J. Structural Dynamics of a Mitochondrial tRNA Possessing Weak Thermodynamic Stability. *Biochemistry* **53**, 1456–1465 (2014).
92. Hwang, H., Kim, H. & Myong, S. Protein induced fluorescence enhancement as a single molecule assay with short distance sensitivity. *Proceedings of the National Academy of Sciences* **108**, 7414–7418 (2011).

93. Kim, S. H. *et al.* The General Structure of Transfer RNA Molecules. *Proceedings of the National Academy of Sciences* **71**, 4970–4974 (1974).
94. Helm, M. *et al.* The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial tRNA. *Nucleic Acids Res* **26**, 1636–1643 (1998).
95. Yamagami, R., Sieg, J. P., Assmann, S. M. & Bevilacqua, P. C. Genome-wide analysis of the in vivo tRNA structurome reveals RNA structural and modification dynamics under heat stress. *Proceedings of the National Academy of Sciences* **119**, e2201237119 (2022).
96. Cole, P. E. & Crothers, D. M. Conformational changes of transfer ribonucleic acid. Relaxation kinetics of the early melting transition of methionine transfer ribonucleic acid (*Escherichia coli*). *Biochemistry* **11**, 4368–4374 (1972).
97. Biedenbänder, T. *et al.* RNA modifications stabilize the tertiary structure of tRNA^{fMet} by locally increasing conformational dynamics. *Nucleic Acids Research* **50**, 2334–2349 (2022).
98. Hua, B. *et al.* An improved surface passivation method for single-molecule studies. *Nat Methods* **11**, 1233–1236 (2014).
99. Nicolai, C. & Sachs, F. SOLVING ION CHANNEL KINETICS WITH THE QuB SOFTWARE. *Biophys. Rev. Lett.* **08**, 191–211 (2013).
100. Milescu, L. S., Nicolai, C. & Bannen, J. QuB Software. (2000).
101. Qin, F. Restoration of Single-Channel Currents Using the Segmental k-Means Method Based on Hidden Markov Modeling. *Biophysical Journal* **86**, 1488–1501 (2004).
102. Kuzmenkina, E. V., Heyes, C. D. & Nienhaus, G. U. Single-molecule Förster resonance energy transfer study of protein dynamics under denaturing conditions. *Proceedings of the National Academy of Sciences* **102**, 15471–15476 (2005).

103. Gruber, A. R., Lorenz, R., Bernhart, S. H., Neuböck, R. & Hofacker, I. L. The Vienna RNA Websuite. *Nucleic Acids Research* **36**, W70–W74 (2008).
104. McCann, H. *et al.* R2DT: a comprehensive platform for visualizing RNA secondary structure. *Nucleic Acids Research* **53**, gkaf032 (2025).
105. Balbirnie, M., Grothe, R. & Eisenberg, D. S. An amyloid-forming peptide from the yeast prion Sup35 reveals a dehydrated β -sheet structure for amyloid. *PNAS* **98**, 2375–2380 (2001).
106. Fitzpatrick, A. W. P. *et al.* Atomic structure and hierarchical assembly of a cross- β amyloid fibril. *Proc Natl Acad Sci U S A* **110**, 5468–5473 (2013).
107. Glover, J. R. *et al.* Self-seeded fibers formed by Sup35, the protein determinant of [PSI⁺], a heritable prion-like factor of *S. cerevisiae*. *Cell* **89**, 811–819 (1997).
108. King, C.-Y. *et al.* Prion-inducing domain 2-114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments. *Proceedings of the National Academy of Sciences* **94**, 6618–6622 (1997).
109. Barnhart, M. M. & Chapman, M. R. Curli Biogenesis and Function. *Annual Review of Microbiology* **60**, 131–147 (2006).
110. Eisenberg, D. & Jucker, M. The Amyloid State of Proteins in Human Diseases. *Cell* **148**, 1188–1203 (2012).
111. Kaye, R. *et al.* Common Structure of Soluble Amyloid Oligomers Implies Common Mechanism of Pathogenesis. *Science* **300**, 486–489 (2003).
112. Steiner, J. A., Quansah, E. & Brundin, P. The concept of alpha-synuclein as a prion-like protein: ten years after. *Cell and Tissue Research* **373**, 161–173 (2018).

113. Jucker, M. & Walker, L. C. Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. *Nature* **501**, 45–51 (2013).
114. Kitamoto, T. *et al.* Amyloid plaques in Creutzfeldt-Jakob disease stain with prion protein antibodies. *Ann Neurol* **20**, 204–208 (1986).
115. Garcia, D. M. & Jarosz, D. F. Rebels with a cause: molecular features and physiological consequences of yeast prions. *FEMS Yeast Research* **14**, 136–147 (2014).
116. Holmes, D. L., Lancaster, A. K., Lindquist, S. & Halfmann, R. Heritable remodeling of yeast multicellularity by an environmentally responsive prion. *Cell* **153**, 153–165 (2013).
117. Chakravarty, A. K., Smejkal, T., Itakura, A. K., Garcia, D. M. & Jarosz, D. F. A Non-amyloid Prion Particle that Activates a Heritable Gene Expression Program. *Mol Cell* **77**, 251-265.e9 (2020).
118. Harvey, Z. H., Chakravarty, A. K., Futia, R. A. & Jarosz, D. F. A Prion Epigenetic Switch Establishes an Active Chromatin State. *Cell* **180**, 928-940.e14 (2020).
119. Lacroute, F. Non-Mendelian Mutation Allowing Ureidosuccinic Acid Uptake in Yeast. *J Bacteriol* **106**, 519–522 (1971).
120. Wickner, R. B. [URE3] as an altered URE2 protein: evidence for a prion analog in *Saccharomyces cerevisiae*. *Science* **264**, 566–569 (1994).
121. Derkatch, I. L., Bradley, M. E., Zhou, P., Chernoff, Y. O. & Liebman, S. W. Genetic and Environmental Factors Affecting the De Novo Appearance of the [Psi(+)] Prion in *Saccharomyces Cerevisiae*. *Genetics* **147**, 507–519 (1997).
122. Coustou, V., Deleu, C., Saupe, S. & Begueret, J. The protein product of the het-s heterokaryon incompatibility gene of the fungus *Podospora anserina* behaves as a prion analog. *Proc Natl Acad Sci U S A* **94**, 9773–9778 (1997).

123. Du, Z., Park, K.-W., Yu, H., Fan, Q. & Li, L. Newly identified prion linked to the chromatin-remodeling factor Swi1 in *Saccharomyces cerevisiae*. *Nat Genet* **40**, 460–465 (2008).
124. Patel, B. K., Gavin-Smyth, J. & Liebman, S. W. The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion. *Nat Cell Biol* **11**, 344–349 (2009).
125. Rogoza, T. *et al.* Non-Mendelian determinant [ISP+] in yeast is a nuclear-residing prion form of the global transcriptional regulator Sfp1. *Proc Natl Acad Sci U S A* **107**, 10573–10577 (2010).
126. Volkov, K. V. *et al.* Novel non-Mendelian determinant involved in the control of translation accuracy in *Saccharomyces cerevisiae*. *Genetics* **160**, 25–36 (2002).
127. Saifitdinova, A. F. *et al.* [NSI+]: a novel non-Mendelian nonsense suppressor determinant in *Saccharomyces cerevisiae*. *Curr Genet* **56**, 467–478 (2010).
128. Uversky, V. N., Gillespie, J. R. & Fink, A. L. Why are natively unfolded proteins unstructured under physiologic conditions? *Proteins: Structure, Function, and Genetics* **41**, 415–427 (2000).
129. Li, L. & Lindquist, S. Creating a protein-based element of inheritance. *Science* **287**, 661–664 (2000).
130. Stein, K. C. & True, H. L. Extensive diversity of prion strains is defined by differential chaperone interactions and distinct amyloidogenic regions. *PLoS Genet* **10**, e1004337 (2014).
131. Ba, A. N. N. *et al.* Proteome-Wide Discovery of Evolutionary Conserved Sequences in Disordered Regions. *Science Signaling* **5**, (2012).

132. Oldfield, C. J., Uversky, V. N., Dunker, A. K. & Kurgan, L. Chapter 1 - Introduction to intrinsically disordered proteins and regions. in *Intrinsically Disordered Proteins* (ed. Salvi, N.) 1–34 (Academic Press, 2019). doi:10.1016/B978-0-12-816348-1.00001-6.
133. Hervás, R. *et al.* Cryo-EM structure of a neuronal functional amyloid implicated in memory persistence in *Drosophila*. *Science* **367**, 1230–1234 (2020).
134. Van Melckebeke, H. *et al.* Atomic-resolution three-dimensional structure of HET-s(218–289) amyloid fibrils by solid-state NMR spectroscopy. *J Am Chem Soc* **132**, 13765–13775 (2010).
135. Wasmer, C. *et al.* Amyloid Fibrils of the HET-s(218–289) Prion Form a β Solenoid with a Triangular Hydrophobic Core. *Science* **319**, 1523–1526 (2008).
136. Kraus, A. *et al.* High-resolution structure and strain comparison of infectious mammalian prions. *Molecular Cell* **81**, 4540–4551.e6 (2021).
137. Amenitsch, H., Benetti, F., Ramos, A., Legname, G. & Requena, J. R. SAXS structural study of PrP(Sc) reveals ~11 nm diameter of basic double intertwined fibers. *Prion* **7**, 496–500 (2013).
138. Sim, V. L. & Caughey, B. Ultrastructures and strain comparison of under-glycosylated scrapie prion fibrils. *Neurobiology of Aging* **30**, 2031–2042 (2009).
139. Spagnolli, G. *et al.* Full atomistic model of prion structure and conversion. *PLoS Pathog* **15**, e1007864 (2019).
140. Terry, C. *et al.* Structural features distinguishing infectious ex vivo mammalian prions from non-infectious fibrillar assemblies generated in vitro. *Sci Rep* **9**, 376 (2019).
141. Vázquez-Fernández, E. *et al.* The Structural Architecture of an Infectious Mammalian Prion Using Electron Cryomicroscopy. *PLoS Pathog* **12**, e1005835 (2016).

142. Si, K. & Kandel, E. R. The Role of Functional Prion-Like Proteins in the Persistence of Memory. *Cold Spring Harb Perspect Biol* **8**, a021774 (2016).
143. Jackson, G. S. *et al.* Reversible Conversion of Monomeric Human Prion Protein Between Native and Fibrillogenic Conformations. *Science* **283**, 1935–1937 (1999).
144. Bradley, M. E. & Liebman, S. W. The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [PSI⁺] prions. *Molecular Microbiology* **51**, 1649–1659 (2004).
145. Collins, S. R., Douglass, A., Vale, R. D. & Weissman, J. S. Mechanism of Prion Propagation: Amyloid Growth Occurs by Monomer Addition. *PLoS Biology* **2**, e321 (2004).
146. Krishnan, R. & Lindquist, S. L. Structural insights into a yeast prion illuminate nucleation and strain diversity. *Nature* **435**, 765–772 (2005).
147. Tessier, P. M. & Lindquist, S. Prion recognition elements govern nucleation, strain specificity and species barriers. *Nature* **447**, 556–561 (2007).
148. Villali, J. *et al.* Nucleation seed size determines amyloid clearance and establishes a barrier to prion appearance in yeast. *Nature Structural & Molecular Biology* **27**, 540–549 (2020).
149. Keefer, K. M., Stein, K. C. & True, H. L. Heterologous prion-forming proteins interact to cross-seed aggregation in *Saccharomyces cerevisiae*. *Sci Rep* **7**, 5853 (2017).
150. Serio, T. R. [PIN⁺]ing down the mechanism of prion appearance. *FEMS Yeast Res* **18**, (2018).
151. Chernoff, Y. O., Lindquist, S. L., Ono, B., Inge-Vechtomov, S. G. & Liebman, S. W. Role of the Chaperone Protein Hsp104 in Propagation of the Yeast Prion-Like Factor [*psi*⁺]. *Science* **268**, 880–884 (1995).

152. Shorter, J. & Lindquist, S. Hsp104 Catalyzes Formation and Elimination of Self-Replicating Sup35 Prion Conformers. *Science* **304**, 1793–1797 (2004).
153. Shorter, J. & Lindquist, S. Destruction or Potentiation of Different Prions Catalyzed by Similar Hsp104 Remodeling Activities. *Molecular Cell* **23**, 425–438 (2006).
154. Shorter, J. & Lindquist, S. Hsp104, Hsp70 and Hsp40 interplay regulates formation, growth and elimination of Sup35 prions. *EMBO J* **27**, 2712–2724 (2008).
155. Bradley, M. E., Edskes, H. K., Hong, J. Y., Wickner, R. B. & Liebman, S. W. Interactions among prions and prion ‘strains’ in yeast. *Proc Natl Acad Sci U S A* **99 Suppl 4**, 16392–16399 (2002).
156. Derkatch, I. L., Chernoff, Y. O., Kushnirov, V. V., Inge-Vechtomov, S. G. & Liebman, S. W. Genesis and Variability of [*PSI*] Prion Factors in *Saccharomyces cerevisiae*. *Genetics* **144**, 1375–1386 (1996).
157. Tanaka, M., Chien, P., Naber, N., Cooke, R. & Weissman, J. S. Conformational variations in an infectious protein determine prion strain differences. *Nature* **428**, 323–328 (2004).
158. Schlumpberger, M., Prusiner, S. B. & Herskowitz, I. Induction of distinct [URE3] yeast prion strains. *Mol Cell Biol* **21**, 7035–7046 (2001).
159. Wickner, R. B., Son, M. & Edskes, H. K. Prion Variants of Yeast are Numerous, Mutable, and Segregate on Growth, Affecting Prion Pathogenesis, Transmission Barriers, and Sensitivity to Anti-Prion Systems. *Viruses* **11**, E238 (2019).
160. Derdowski, A., Sindi, S. S., Klaips, C. L., DiSalvo, S. & Serio, T. R. A Size Threshold Limits Prion Transmission and Establishes Phenotypic Diversity. *Science* **330**, 680–683 (2010).

161. Alberti, S. Phase separation in biology. *Current Biology* **27**, R1097–R1102 (2017).
162. Franzmann, T. M. *et al.* Phase separation of a yeast prion protein promotes cellular fitness. *Science* **359**, eaao5654 (2018).
163. Toyama, B. H., Kelly, M. J. S., Gross, J. D. & Weissman, J. S. The structural basis of yeast prion strain variants. *Nature* **449**, 233–237 (2007).
164. Kryndushkin, D. S., Alexandrov, I. M., Ter-Avanesyan, M. D. & Kushnirov, V. V. Yeast [PSI⁺] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. *J Biol Chem* **278**, 49636–49643 (2003).
165. Kushnirov, V. V., Dergalev, A. A. & Alexandrov, A. I. Proteinase K resistant cores of prions and amyloids. *Prion* **14**, 11–19 (2020).
166. Xue, C., Lin, T. Y., Chang, D. & Guo, Z. Thioflavin T as an amyloid dye: fibril quantification, optimal concentration and effect on aggregation. *R Soc Open Sci* **4**, 160696 (2017).
167. Yakupova, E. I., Bobyleva, L. G., Vikhlyantsev, I. M. & Bobylev, A. G. Congo Red and amyloids: history and relationship. *Biosci Rep* **39**, BSR20181415 (2019).
168. Halfmann, R. & Lindquist, S. Screening for Amyloid Aggregation by Semi-Denaturing Detergent-Agarose Gel Electrophoresis. *JoVE (Journal of Visualized Experiments)* e838 (2008) doi:10.3791/838.
169. Hanna-Addams, S. & Wang, Z. Use of Two Dimensional Semi-denaturing Detergent Agarose Gel Electrophoresis to Confirm Size Heterogeneity of Amyloid or Amyloid-like Fibers. *J Vis Exp* 57498 (2018) doi:10.3791/57498.
170. Greene, L., Park, Y.-N., Masison, D. & Eisenberg, E. Application of GFP-Labeling to Study Prions in Yeast. *Protein & Peptide Letters* **16**, 635–641 (2009).

171. Patino, M. M., Liu, J.-J., Glover, J. R. & Lindquist, S. Support for the Prion Hypothesis for Inheritance of a Phenotypic Trait in Yeast. *Science* **273**, 622–626 (1996).
172. Khan, T. *et al.* Quantifying Nucleation In Vivo Reveals the Physical Basis of Prion-like Phase Behavior. *Mol Cell* **71**, 155-168.e7 (2018).
173. Newby, G. A. *et al.* A Genetic Tool to Track Protein Aggregates and Control Prion Inheritance. *Cell* **171**, 966-979.e18 (2017).
174. Liebman, S. W., Bagriantsev, S. N. & Derkatch, I. L. Biochemical and genetic methods for characterization of [PIN+] prions in yeast. *Methods* **39**, 23–34 (2006).
175. Tanaka, M., Collins, S. R., Toyama, B. H. & Weissman, J. S. The physical basis of how prion conformations determine strain phenotypes. *Nature* **442**, 585–589 (2006).
176. Zhou, F., Zhang, H., Kulkarni, S. D., Lorsch, J. R. & Hinnebusch, A. G. eIF1 discriminates against suboptimal initiation sites to prevent excessive uORF translation genome-wide. *RNA* **26**, 419–438 (2020).
177. Garrett, S., Menold, M. M. & Broach, J. R. The *Saccharomyces cerevisiae* YAK1 Gene Encodes a Protein Kinase That Is Induced by Arrest Early in the Cell Cycle. *Molecular and Cellular Biology* **11**, 4045–4052 (1991).
178. Moriya, H. *et al.* Yak1p, a DYRK family kinase, translocates to the nucleus and phosphorylates yeast Pop2p in response to a glucose signal. *Genes Dev.* **15**, 1217–1228 (2001).
179. Lee, P., Cho, B.-R., Joo, H.-S. & Hahn, J.-S. Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. *Molecular Microbiology* **70**, 882–895 (2008).

180. Lee, P., Paik, S.-M., Shin, C.-S., Huh, W.-K. & Hahn, J.-S. Regulation of yeast Yak1 kinase by PKA and autophosphorylation-dependent 14-3-3 binding. *Molecular Microbiology* **79**, 633–646 (2011).
181. Brion, C., Lutz, S. M. & Albert, F. W. Simultaneous quantification of mRNA and protein in single cells reveals post-transcriptional effects of genetic variation. *eLife* **9**, e60645 (2020).
182. Alamgir, M., Eroukova, V., Jessulat, M., Xu, J. & Golshani, A. Chemical-genetic profile analysis in yeast suggests that a previously uncharacterized open reading frame, YBR261C, affects protein synthesis. *BMC Genomics* **9**, 583 (2008).
183. Cao, L. *et al.* Chronological Lifespan in Yeast Is Dependent on the Accumulation of Storage Carbohydrates Mediated by Yak1, Mck1 and Rim15 Kinases. *PLOS Genetics* **12**, e1006458 (2016).
184. Dennis, E. M. *et al.* Chronology of tRNA structural dynamics prior to and during interaction with a pseudouridine synthase. 2025.05.06.652307 Preprint at <https://doi.org/10.1101/2025.05.06.652307> (2025).
185. To, P., Whitehead, B., Tarbox, H. E. & Fried, S. D. Nonrefoldability is Pervasive Across the E. coli Proteome. *J. Am. Chem. Soc.* **143**, 11435–11448 (2021).
186. Kong, A. T., Leprevost, F. V., Avtonomov, D. M., Mellacheruvu, D. & Nesvizhskii, A. I. MSFragger: ultrafast and comprehensive peptide identification in mass spectrometry-based proteomics. *Nat Methods* **14**, 513–520 (2017).
187. Yu, F., Haynes, S. E. & Nesvizhskii, A. I. IonQuant Enables Accurate and Sensitive Label-Free Quantification With FDR-Controlled Match-Between-Runs. *Molecular & Cellular Proteomics* **20**, 100077 (2021).

188. Yu, F. *et al.* Fast Quantitative Analysis of timsTOF PASEF Data with MSFragger and IonQuant. *Molecular & Cellular Proteomics* **19**, 1575–1585 (2020).
189. Yu, F. *et al.* Analysis of DIA proteomics data using MSFragger-DIA and FragPipe computational platform. *Nat Commun* **14**, 4154 (2023).
190. Manriquez-Sandoval, E., Brewer, J., Lule, G., Lopez, S. & Fried, S. D. FLiPPR: A Processor for Limited Proteolysis (LiP) Mass Spectrometry Data Sets Built on FragPipe. *J. Proteome Res.* **23**, 2332–2342 (2024).
191. Anand, R., Memisoglu, G. & Haber, J. Cas9-mediated gene editing in *Saccharomyces cerevisiae*. <https://www.protocols.io/view/cas9-mediated-gene-editing-in-saccharomyces-cerevi-d29u8h6w> (2017).