MISFOLDED BUT NOT MALICIOUS: PRION PROTEINS IN BUDDING YEAST

by

MIKALA CAPAGE

A THESIS

Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

May 2022

An Abstract of the Thesis of

Mikala Capage for the degree of Bachelor of Arts (or Science)
in the Department of Biology to be taken June 2022

Title: Misfolded but not Malicious: Prion Proteins in Budding Yeast

Approved: _____*David Garcia, Assistant Professor*_____
Primary Thesis Advisor

Prion proteins, although frequently associated with neurodegenerative diseases, are not universally harmful to cells. Instead, prions may serve as a beneficial epigenetic mechanism, allowing cells to alter their phenotype to adapt to adverse environmental conditions. Prions form when a protein adopts alternate and stable folding conformation. The Garcia Lab aims to identify beneficial prions using the budding yeast, Saccharomyces cerevisiae. We are particularly interested in prion conformations of RNA modifying enzymes (RMEs), because these proteins can affect the expression of many genes simultaneously. After screening hundreds of yeast strains, the Garcia Lab identified six strains of yeast – associated with potentially alternate conformations of the RMEs Abd1, Cet1, Ppm2, Pus4, Pus6 and Trm5 – that exhibited resistance to harmful chemicals. Extensive tests are needed to confirm that their resistance to stress is caused by a prion-based conformation of the RNA modifying enzymes. Here, experiments describing the meiotic inheritance, protein dependance, and cytoplasmic inheritance of these resistance phenotypes are presented. The initial results are key to attributing the previously identified growth states to a prion conformation of each of the six RNA modifying enzymes. The Garcia lab will continue to investigate these putative prions in future experiments to determine the mechanism for resistance. This research represents an important contribution to our understanding of prions as a protein-based epigenetic mechanism and their effects on key cell processes.

Acknowledgements

I would like to thank Professor David Garcia, Professor George von Dassow, and Professor Michael Moffitt for serving on my thesis committee, representing both the Biology Department and the Clark Honors College. I would like to especially recognize and thank Dr. Garcia for his outstanding mentorship and support over the last three years in every step in the research process. I am incredibly lucky to have been a part of the Garcia Lab at this early stage in my academic career. Additionally, I would like to thank Dr. Melissa Graboyes for her guidance and for seeing a scholar in me before I saw it in myself. I would also like to thank Ms. Miriam Jordan for providing this excellent template and logistical support throughout the thesis process. Finally, I would like to thank my family for always standing behind me and supporting me every step of the way.

Table of Contents

List of Figures

List of Tables

Introduction

This thesis project aims to identify novel prion proteins in the proteome of the budding yeast, *Saccharomyces cerevisiae*. Prions are alternately folded versions of proteins that can spontaneously arise in cells. They are self-templating, meaning they cause the naïve (or standardly folded proteins) to adopt their altered conformation and they are heritable, passing down to future generations of cells.

Prion proteins represent a novel and exciting area of research that involves a wide range of concepts such as epigenetics, evolution and adaptation, and protein folding. In the Garcia Lab, a particular emphasis is placed on prion conformations of the enzymes that carry out modifications to RNA molecules (called RNA-modifying enzymes, or RMEs). The experiments described in this thesis are all performed with the model organism, *Saccharomyces cerevisiae*, a budding yeast. This single-celled, eukaryotic model organism is ideal for large scale genetic and epigenetic experiments because it is genetically tractable, shares many of genes with humans, and is easy to grow, store, and revive. High through-put assays, in which many experiments are carried out in parallel, are also easy to perform making this system ideal for the projects described here.

This project builds on the previous work of Dr. David Garcia, who performed an initial screen of RNA-modifying enzymes to determine which enzymes are capable of driving heritable, prion-like traits. To perform this screen, each RNA-modifying enzyme was transiently overexpressed in a different yeast strain and the strains of yeast were then grown in the presence of close to 60 chemical stressors. These two techniques – transient overexpression coupled with exposure to stress – have been shown to

increase the chances that the candidate protein switches into an altered prion-based conformation (Tyedmers et al., 2008; Wickner et al., 2006). This screen produced millions of data points which were analyzed further to identify candidate strains – yeast strains we believed to show resistance to the chemical stressors caused by a prion conformation of the particular enzyme. There are six candidate strains of interest, each associated with a putative prion conformation of a distinct RNA-modifying enzyme (see Table 1).

RNA-Modifying Enzyme	*Function*	*Human Ortholog*
Abd1	mRNA methyltransferase	Rnmt
Cet1	5’ mRNA triphosphatase	Rngtt
Ppm2	tRNA methyltransferase	Lcmt2
Pus4	tRNA pseudouridine synthase	TruB1/TruB2
Pus6	tRNA pseudouridine synthase	Rpusd2
Trm5	tRNA methyltransferase	Trmt5

Table 1. RNA-Modifying Enzymes

However, there are a number of other mechanisms that could be causing the resistance phenotypes observed in these six strains, such as genetic mutations or mutated mitochondria. Simply observing resistance phenotypes in strains of yeast that were exposed to conditions known to induce prions is not enough to conclude that a prion protein is present in the cells.

Thus, it is necessary to perform a variety of genetic experiments to confirm that the resistance phenotypes are caused by prion proteins, which are inherited via non-

DNA-based mechanisms. These experiments are designed to distinguish between traits caused by prion proteins and those caused by other mechanisms. Prions display distinct patterns of meiotic inheritance, dependence on the gene that encodes the protein believed to be in a prion conformation, and cytoplasmic inheritance. Testing if the candidate strains of yeast exhibit these traits is essential in determining if the observed resistance phenotypes are caused by a prion protein. To answer these questions, I collaborated closely with Jacob Evarts (CHC, ’21), a former undergraduate researcher in the Garcia Lab, who carried out the initial computational analysis as well as many of the growth assays presented here.

The original design of the screen performed by Dr. Garcia and one other student created strains that had experienced transient overexpression of 72 RNA-modifying enzymes while undergoing exposure to close to 60 chemical stressors. A matched set of control strains of yeast were stressed under the same conditions but did not experience any transient overexpression These strains are used in this project to control for the effects the chemical stress alone may have had on the cells. These control strains are referred to as “Plate A” strains, while the experimental strains are “Plate B” strains. Additional controls include naïve strain of yeast with no prior overexpression or exposure to stress.

While prions have been traditionally thought of as agents of deadly diseases, research has shown that some prions may be beneficial in adapting a cell to changes in its environment. However, many open questions remain regarding the role of beneficial protein-based epigenetic mechanisms. This project adds to our understanding of prions by identifying and confirming novel yeast prions, which opens up numerous avenues

for future research to understand how prions behave, inherit, and alter phenotypes in an epigenetic manner.

Research Questions

1. Are the resistance phenotypes dominant in a diploid background?
2. What are the meiotic inheritance patterns of the resistance phenotypes?
3. Are the resistance phenotypes dependent on the gene that encodes the protein in question?
4. What are the cytoplasmic inheritance patterns for the resistance phenotypes?
5. Challenges to studying essential genes – how do we modify methods to test the prion-like nature of essential proteins?

Literature Review

The current field of prion protein research got its start from the discovery of related neurodegenerative diseases in sheep, cows, and humans that evaded medical understanding and the discovery of traits in budding yeast cells that couldn't be attributed to any known mechanism. While seemingly unconnected, these two distinct areas of research were drawn together through the discovery of a phenomenon called a prion protein – an alternately folded version of a protein that behaves differently than the typically folded protein does (Prusiner, 1982; Shorter & Lindquist, 2005).

These specific instances of neurodegeneration observed in animals and humans were eventually explained by the prion conformation of the protein PrP (first identified, and thus named, for its prion state). In its naïve conformation (denoted PrP^{C}), the protein is a plasma membrane protein but when it adopts an alternate conformation (denoted PrP^{SC}), the prion aggregates in cells of the central nervous system (Shorter & Lindquist, 2005). It is this prion form that causes severe disease (Shorter & Lindquist, 2005).

As Prusiner details in his 1982 paper "Novel Proteinaceous Infectious Particles Cause Scrapie", many other theories for the cause of the neurodegenerative symptoms were posited, including parasites, viruses, and aggregating polysaccharides, among other suggestions (Prusiner, 1982). Similarly, yeast researchers had observed traits in some yeast strains that did not behave as if they were caused by known mechanisms in yeast. Attributing the traits in yeast to alternately folded conformations of known proteins reconciled "a panoply of otherwise unreconcilable data" (Shorter & Lindquist, 2005).

Although prion research in humans has exclusively stayed focused on PrP and its disease-causing potential, the field of basic research into prion proteins using yeast as a model organism has advanced beyond investigating prions as solely disease-causing agents. Some research now supports the idea that prions can serve as a beneficial epigenetic mechanism that allow cells to rapidly respond to changes in their environment.

This line of thinking regarding prions has been shaped by the discovery and characterization of several yeast prions, including [*PSI*$^{+}$], [*GAR*$^{+}$], and [*BIG*$^{+}$]. These prions cause vastly different traits in yeast cells. [*PSI*$^{+}$] is the alternate form of a protein called Sup35, a yeast translation terminator (Halfmann et al., 2012). In the altered conformation however, [*PSI*$^{+}$] congregates into amyloid fibers and doesn't terminate translation (Byers & Jarosz, 2014). As a result, translational readthrough is increased and in a single step, a vast amount of genetic variation that lies downstream of stop codons is potentially revealed in the protein C-terminus. The genetic variation unlocked by the [*PSI*$^{+}$] may help yeast cells adapt to rapid and adverse changes in environmental conditions. This theory was supported by the finding that induction of the [*PSI*$^{+}$] prion increases upon exposure to environmental stressors (Tyedmers et al., 2008).

While [*PSI*$^{+}$] has been shown to be induced by environmental stressors, [*GAR*$^{+}$] is a prion that is induced by chemical signals secreted by bacteria growing and living near yeast cells in the wild (Jarosz, Brown, et al., 2014). These chemical signals change yeast from being metabolic specialists, feeding only on glucose and producing ethanol as a byproduct, to metabolic generalists, metabolizing any carbon source that is available (Jarosz, Lancaster, et al., 2014). The trait associated with [*GAR*$^{+}$] benefits the

yeast cells, allowing them to get more energy from available carbon sources and benefits the bacteria living with the yeast, which cannot survive in environments with higher ethanol levels produced when yeast cells metabolize glucose exclusively (Jarosz, Lancaster, et al., 2014).

The prion [*BIG*$^{+}$] is an alternate conformation of the yeast protein Pus4, a pseudouridine synthase that changes the canonical base uridine in position 55 on tRNAs into a pseudouridine. The name of the prion stands for Better In Growth, and the prion is named as such because the cells containing this prion are bigger in size and grow faster than their naïve counterparts. This increased size comes at a cost however, as [*BIG*$^{+}$] cells have shorter lifespans than naïve cells. This trade-off between size and lifespan may be beneficial in some environments and this prion is being further investigated in the Garcia Lab (Garcia et al., 2021).

These examples show that prions can cause an array of diverse traits in yeast cells. In addition to identifying traits that prions can cause, prion discovery has established properties that distinguish them from nucleic acid mutations, mitochondrial mutations, or other extrachromosomal traits. In "How to find a prion: [URE3], [PSI^{+}], and [beta]" Wickner et al outlined a select few properties shared by yeast prion proteins, including dependence on the gene that encodes the underlying protein and increased occurrence through overexpression (Wickner et al., 2006). These two properties of prions highlight the connections between a prion protein and the naïve conformation of the underlying protein. Additionally, Wickner et al describes cytoplasmic transfer as the method for passing on all non-chromosomal genetic elements, meaning that the ability to pass from cell to cell through mixing cytoplasm is a feature of prion proteins, but also

a feature of plasmids, viruses, or mitochondrial defects (Wickner et al., 2006). Thus, while all prions should be heritable through cytoplasmic mixing, this doesn't guarantee that a prion protein is causing the observed trait.

Methods

Extensive research into prion proteins has identified key properties shared by prions, some of which were discussed in the literature review. In this project, yeast strains that exhibit resistance to chemical stressors have been identified as likely containing a prion protein. However, more evidence is needed to fully support this claim. By comparing the properties of these putative strains to established prions, we can begin to attribute the resistance to an alternate conformation of an RME. Below, each property of prions and an associated method to test this property are explained.

Growth Assays

The six candidate strains in this experiment display resistance to the effects of growth-inhibiting chemical stressors (namely, cycloheximide). This resistance phenotype is assessed in growth assays, which measure the growth of various strains of yeast in liquid cultures over time. The original strains of yeast were assessed for their ability to grow better than a control strain in the presence of chemical stressors. Thus, the continued presence or disappearance of the resistance phenotype is determined for the various progenitor strains created in each step of the experiment, with different hypothesis about what will happen to the resistance phenotype if the trait is prion-based or not.

The basic protocol for a growth assay involves inoculating hundreds of samples of liquid media with either experimental or control strains of yeast. Some of the liquid samples of media also contain varying concentrations of the chemical stressor, cycloheximide, and are called a “stress condition”, while other samples do not contain the chemical and are called the “unstressed condition”. These samples of inoculated

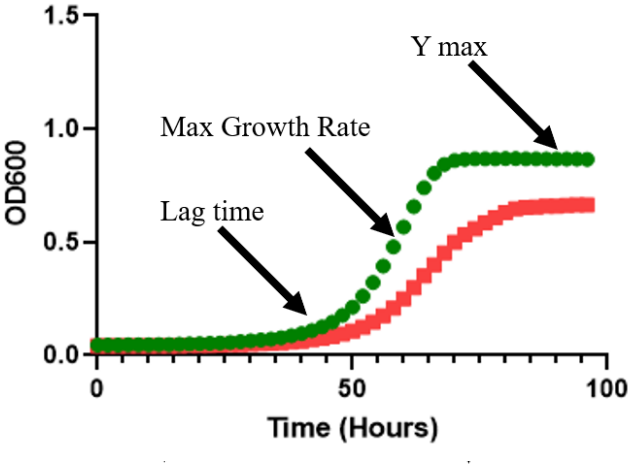


Figure 1. Sample growth curves. The green line would be considered the resistant strain while the red line would be produced by a sensitive or control strain

media are allowed to grow at 30C for four days, with the optical density of the sample measured every two hours. The optical density is a proxy for cell growth, with samples with a higher optical density corresponding to strains that exhibited better growth.

After four days of continual measurements, growth curves are constructed for each strain. Figure 1 exemplifies a typical graph that results from these assays, with growth curves for two strains of yeast on the same graph. Three regions of interest are noted on the figure: the lag time, the maximum growth rate, and the Y max. These components of the curve correspond to phases of culture growth and are points of comparison between an experimental and a control strain to determine if the experimental strain exhibits the "better growth" associated with the resistance phenotype.

To visualize and analyze the growth assay data, this project relies on two pieces of software: Wellcompare, a Python program written by Jacob Evarts for analyzing the meiotic inheritance data, and PRISM for the other experiments, including the protein dependency and the cytoplasmic inheritance experiments.

Diploid Dominance and Meiotic Inheritance

To determine the meiotic inheritance pattern for the resistance phenotypes identified in the screen, diploid strains were created by crossing the haploid strain from the screen with a naïve haploid strain of the opposite mating type (Figure 2). Diploid strains contain two copies of each gene in the organism's genome and are the only cells that can undergo meiosis – a process that forms four genetically unique haploid spores – by first duplicating its genome and then dividing two successive times.

Creating diploid strains from the experimental haploid strains was thus an essential step in determining the meiotic inheritance pattern of the resistance phenotypes, but it also provided data of its own. Traits caused by prion proteins are dominant in a diploid background, so testing these strains to see if they retained their characteristic resistance is one early piece of data that indicated if the resistance phenotypes were prion-like in nature.

Figure 2. Patterns of meiotic inheritance for genetic mutations and prion-based traits. Adapted from Charkrabortee et al 2016

After creating the diploid strains, the process of sporulating and dissecting could begin. Diploid yeast cells can undergo meiosis but are only likely to do so in nutrient-poor conditions in which the cells are not likely to survive. Growing the diploid cells in nutrient rich media, then quickly transferring them to a nutrient poor media increases the frequency at which the cells undergo meiosis and form a tetrad – four genetically unique spores encased in an ascus wall that can hibernate for months with very few nutrients. These tetrads package

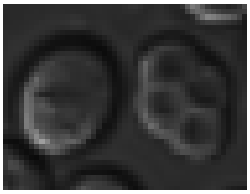
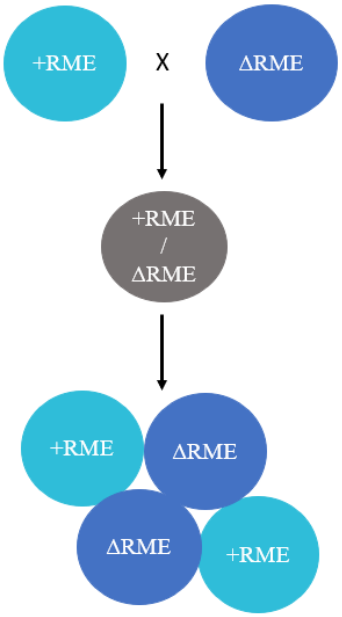


Figure 3. DIC Image of Tetrad (right) and Diploid Cells (left)

the meiotic offspring together and thus can be used to determine how the resistance phenotypes in this experiment inherit through meiosis.



2:2 Pattern

After sporulating the diploid strains to create tetrads, the culture is mixed with chemicals to partially digest the ascus coating surrounding the tetrad. Next, using a dissection microscope outfitted with thin glass needle, each spore from the tetrad is separated from its neighbors and grown into a colony on a solid agar plate. These colonies are preserved, maintaining the sets of haploid cells that originated from a single diploid cell at the beginning of the experiment. Comparing the growth of the experimental meiotic offspring against the growth of control strains can determine if the experimental strains inherited the resistance phenotype in patterns consistent with prion proteins (Figure 2).

Protein Dependency

To determine if the resistance phenotype is dependent on the continued presence of the protein, diploid strains of yeast that are heterozygous for the gene that encodes the RME in question are sporulated and dissected, and the haploid offspring are tested in growth assays to determine which strains retain the resistance phenotype.

To create the diploid strains, the original haploid strain of yeast was crossed with a haploid strain that had the gene encoding the RME deleted and replaced with a gene that makes the strains resistant to the antibiotic kanamycin. These heterozygotic diploids were sporulated and dissected as described above. Mendelian genetics governs the inheritance of the gene encoding the protein, so half of the resulting meiotic offspring have the gene for the RME and half of the strains do not (Figure 4). Analysis of the tetrad spores were split, with the +RME strains compared against the ΔRME strains. If the resistance phenotype is dependent on the underlying protein, as is expected if it is caused by a prion conformation of that enzyme, it will not be present in the ΔRME spores.

Figure 4. Schematic showing the inheritance pattern for prion-like traits in a protein dependency experiment

Cytoplasmic Inheritance

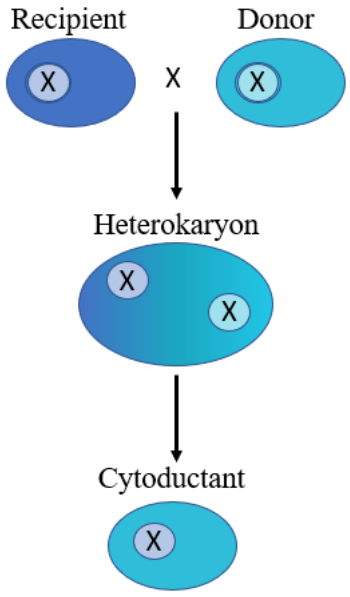


Figure 5. Inheritance scheme for cytoductions

A cytoduction is a technique in yeast genetics that essentially separates the fusing of nuclei from the mixing of cytoplasm during cell mating. Cytoductions can thus be used to determine if the resistance phenotypes can be inherited through the cytoplasm alone and without the nucleus. This protocol takes advantage of a mutant strain of yeast missing karyogamy genes, which allow two haploid nuclei to fuse into one diploid nucleus. By crossing the haploid strains of yeast with Δ*kar* mutant strain, the cytoplasm can be mixed without the nuclei fusing (Figure 5). If the resistance phenotypes are caused

by an agent in the cytoplasm, the resistance phenotype will show up in the next generation of cells. If the resistance phenotype disappears, it means the trait was caused by mutation in the nucleic acid genome and thus not by a prion protein.

This experiment can also be used to determine the protein dependency of the resistance phenotypes by cytoducing into a strain of yeast missing the RME gene. In this case, the resistance phenotype should disappear as the strain would lack the continued expression of the RME. By cytoducing into recipient strains with and without the RME gene, this experiment can answer questions about the cytoplasmic inheritance of the resistance phenotypes and provides another way to assess the protein dependency of these strains.

Constraints in testing essential enzymes

This project investigates putative prions of six RNA-modifying enzymes. Half of these enzymes (Pus4, Pus6, and Ppm2) are not essential for the growth and survival of the yeast cells. Experiments that involve deleting copies of the gene that encode these three enzymes, including the experiments to determine protein dependency and cytoplasmic inheritance, are feasible because the enzymes are not essential. Importantly, these experiments cannot be performed the same way for the three putative prion strains that involve essential enzymes, as deleting the gene renders the strains inviable. These enzymes are Abd1, Cet1, and Trm5.

This limitation is not unique to this project and was addressed by David Breslow and others in the creation of a library of yeast strains with hypomorphic alleles for most of the essential genes in the yeast genome (Breslow et al., 2008). Hypomorphic alleles have reduced gene function compared to the wildtype allele. In the case of this library,

the hypomorphic alleles are termed “DAmP alleles”, standing for Decreased Abundance through mRNA Perturbation.

The DAmP library contains strains of yeast that have a gene that confers resistance to the antibiotic kanamycin inserted into the 3’ untranslated region of the given protein coding gene (Figure 6). The presence of this cassette both reduces the expression of the protein gene and serves as a marker for the reduced expression. By limiting the function of the gene, this library can approximate the effect of deleting a gene, while leaving enough function to keep the strains viable (Breslow et al., 2008).

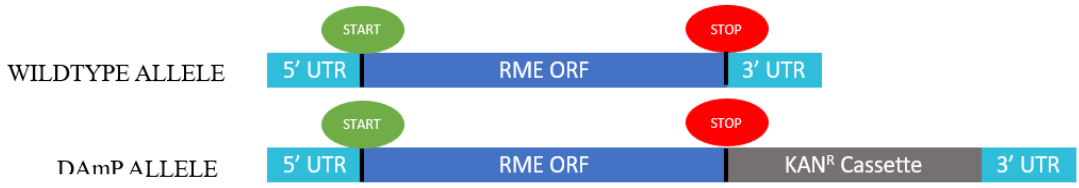


Figure 6. Schematic comparing the constructs for wildtype and DAmP alleles. Adapted from Breslow et al.

For this thesis, DAmP strains were purchased for the three essential enzymes. The purchased strains did not have the specific combination of auxotrophic markers and mating types needed for experiments in this thesis (see Table 2). To construct the yeast strains that could be used for the projects described here, a variety of genetic alterations were made to the DAmP strains, which are detailed below.

	Cytoductions	*Δ RME dissections*
Strain characteristics	Mat a, lysine+, methionine-	Mat α, lysine-, methionine+

Table 2. Strain characteristics for DAmP strains

Spore Enrichment

The spore enrichment protocol is used to create DAmP haploid spores of specific mating types and with specific auxotrophic markers by sporulating the DAmP diploid strains (as described earlier), selectively killing any remaining diploid cells with a potent substance called diethyl ether, then dissolving the ascus wall that binds the haploid spores together. The remaining solution contains haploid spores which are plated on YPD plates to grow into colonies. Finally, liquid cultures are inoculated with one colony per culture and frozen at -80C. The protocol used here is modeled off two protocols in "Ether-zymolyase ascospore isolation procedure: an efficient protocol for ascospores isolation in Saccharomyces cerevisiae yeast" (Bahulul et al) and "Mapping causal variants with single nucleotide resolution reveals biochemical drivers of phenotypic change" (She and Jarosz).

A spore enrichment protocol is similar to a tetrad sporulation and dissection protocol as both protocols can be used to create meiotic offspring from diploid yeast cells, but the spore enrichment protocol does so in a high through-put manner, allowing the isolation of hundreds of meiotic spores at once. However, the protocol isn't designed to keep sets of four meiotic spores together – all spores are released from the tetrads and mixed in the solution before being plated and grown into colonies. Both methods to isolate meiotic progeny have their advantages and are employed in this project for different aims.

Mating Type Assay

The spore enrichment protocol creates a variety of haploid spores of different mating types with different sets of auxotrophic markers. These spores then need to be tested to determine which spores fit the criteria laid out in Table 1 and thus can be used for future experiments. First, the mating type of each of the spores was determined by testing the ability of experimental spores to successfully mate to spores of a known mating type. The mating type of the experimental spores can be determined by working backwards from evidence of successful matings, which is assessed by the ability of mated spores to grow on agar plates containing no supplemental amino acids. This assay was used to quickly determine the mating type for ~75 strains of yeast collected from the spore enrichment protocol.

Technical language

Growth Assays

Frozen stocks were used to inoculate liquid YPD media and the strains were growth at 30C for three days. Cultures were then pin-replicated to 96-well plates containing CSM media and the chemical stressors at set concentrations (0.8ug/mL for haploid cells and roughly 1.6ug/mL for diploid cells) or just CSM media as a control. Cultures were allowed to grow for four days at 30C, with the optical density of the sample measured every two hours. Data points were compiled, analyzed, and visualized using one of two software packages: Wellcompare, a Python program written by Jacob Evarts, or PRISM.

Creation of Diploid Strains

Strains with previous overexpression of an RNA-modifying enzyme were crossed with a strain of the opposition mating type on YPD agar overnight. Cells were streaked to two rounds of dual selection plates to select for diploid cells. Single colonies were selected and stored in 25% glycerol at -80 °C for future experiments.

Tetrad Sporulation and Dissections

Diploid strains were streaked to YPD plates from glycerol stocks and grown at 30 °C for two days before single colonies were transferred to pre-sporulation media to grow for two additional days at 25 °C. Cells were then pelleted, washed two times with sporulation media, and resuspended in sporulation media. Strains were left to sporulate for six days at 25 °C, at which point an adequate number of tetrads had formed. Next, 500uL of each sporulated culture was then pelleted and resuspended in solution of 0.1M KPO4/1.2M sorbitol, 100T Zymolyase, and 1M ß-mercaptoethanol, which is designed to digest the ascus wall surrounding the haploid spores. Digestion was stopped after 8 minutes by placing the sample on ice and diluting it with a 0.1M KPO4/1.2M sorbitol solution. A small amount of the digested solution was then streaked to a YPD agar plate for dissections.

Dissections were performed with a Singer MSM200 dissection microscope. Dissected tetrads were grown for at least three days at 30 °C. For tetrads with 100% colony grown (all four spores from a single tetrad produced viable colonies), individual liquid YPD cultures were inoculated with the colonies and grown at 30 °C overnight. Tetrads were confirmed by testing the segregation of auxotrophic markers and frozen

into 25% glycerol stocks at -80 °C. Each tetrad growth was then measured under stress and compared against tetrads from control strains.

Cytoductions

Forward cytoductions

Strains with previous overexpression of an RNA-modifying enzyme were crossed with a petite *kar1-15* deletion strain on YPD overnight, then streaked to a Glycerol -met plate to select for heterokaryons and diploids. Colonies that grew on these plates were then screened on two successive rounds of dual selection plates to screen out diploids. Colonies determined to be haploids were grown overnight in liquid YPD and frozen in 25% glycerol at -80C.

deltaRME petite strains

Strains that are missing the RME gene were grown in YPD + ethidium bromide for 2 days, pelleted, washed with YPD, and resuspended in YPD + ethidium bromide to be grown for 2 more days. The culture was pelleted and resuspended in YPD, then plated to YPD agar plates to grow into colonies. Two colonies per strain were screened on solid agar plates with glycerol, and confirmed petite strains were collected, grown overnight in liquid YPD and frozen in 25% glycerol at -80C.

Reverse cytoductions

Forward cytoductants were crossed with the appropriate deltaRME petite strain and grown overnight on a YPD agar plate. Heterokaryons and diploid cells were selected for using a Glycerol -lys plate. Colonies that grew on these plates were either then screened on two successive rounds of dual selection plates to screen out diploids, or one round of a lead (Pb^{2+}) + YPD plate to distinguish diploids from haploid strains.

Colonies determined to be haploids were grown overnight in liquid YPD and frozen in 25% glycerol at -80C.

Spore Enrichment

Diploid DAmP yeast strains were sporulated following the same protocol described above. Instead of being dissected, 500mL of the sporulated cultures were pelleted and resuspended in a 1:1 diethyl ether:water solution and placed on an vigorous agitator for 1 hour to kill remaining diploid cells. The culture was then pelleted and washed three times with water. Cells were resuspended in a solution of 1M sorbitol with 1mg/mL of zymolyase for 1 hour at room temperature with gentle agitation. Next, the cells were pelleted, washed with water, serially diluted, and plated on YPD plates. Colonies were grown at 30C for 2 days. 24 colonies per strain, for a total of 72 colonies were used to inoculate liquid cultures. Liquid cultures were grown overnight, then frozen in 25% glycerol at -80C.

Mating Type Assay

Haploid DAmP spores were revived in liquid YPD media for three days at 30C, diluted 10-fold, then mixed in equal proportions with a similarly diluted liquid culture of one of two mating type testing strains. Cells were allowed to mate in liquid culture overnight at 30C, before being diluted again and pin-replicated to SD-minimum plates (agar with no supplemental amino acids) and YPD plates. Colonies were grown for 2-3 days at 30C and assessed for growth as evidence as a successful mating.

Results

Diploid Inheritance Patterns

The first experiments in this project set out to determine if the resistance phenotypes were dominant in a diploid background. I created the experimental and control diploids yeast strains, and the assays to analyze the growth of the strains were performed by Jacob Evarts.

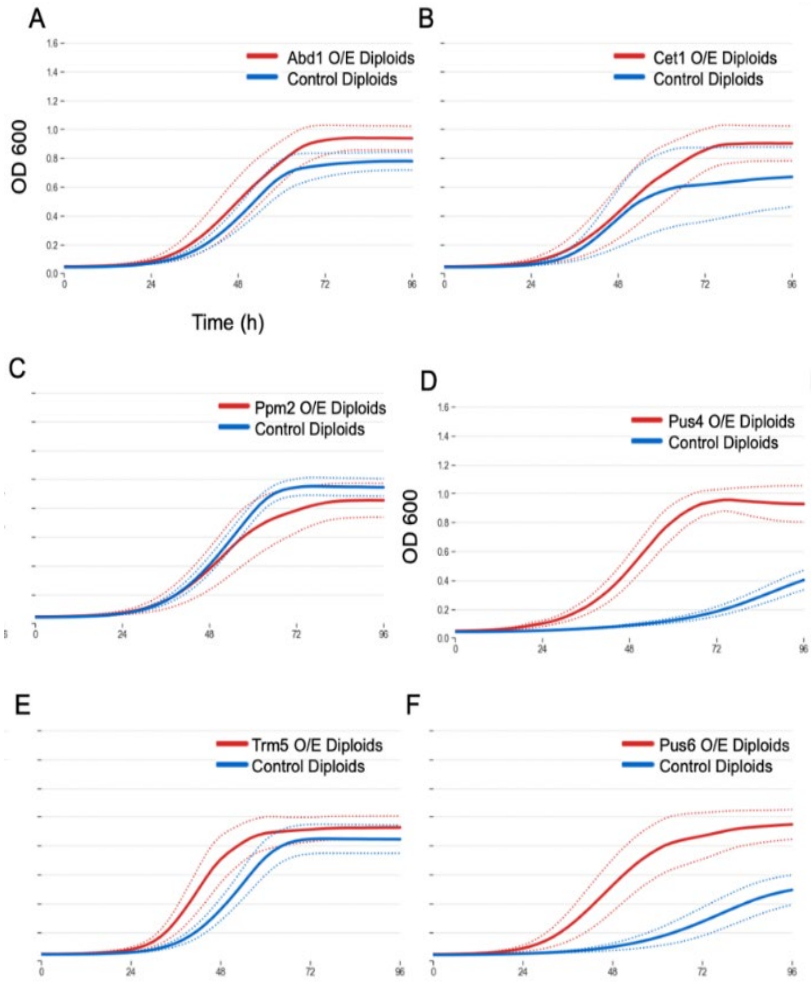


Figure 7. Graphs from growth assays to determine the inheritance pattern of the diploid strains

These assays showed that the resistance phenotypes were dominant in five of the six strains in this experiment (Figure 7). The resistance phenotype is apparent in that the strains associated with a putative prion grew better than the naïve control strains, exhibiting higher Y-max values and shorter lag times (although these metrics have not been quantified). However, the

diploid strain associated with Ppm2 failed to show the resistance phenotype associated with the original haploid strain, as seen in the graph in Figure 7C.

Meiotic Inheritance Patterns

Next, the sets of meiotic progenies were tested in growth assays to determine how the resistance phenotype inherits through meiosis. I performed the sporulations and dissections needed to create the experimental and control sets of meiotic progenies, and the assays to analyze the growth of the strains were performed by Jacob Evarts. Numerous growth assays were performed to determine the meiotic inheritance of the strains and Table 3 displays the inheritance patterns for each strain.

	Number Abd1 Tetrads	*Number Cet1 Tetrads*	*Number Ppm2 Tetrads*	*Number Pus6 Tetrads*	*Number Trm5 Tetrads*
4:0	4	1	0	0	0
3:1	0	1	0	2	1
2:2	0	0	3	0	1
1:3	0	0	1	2	0
0:4	0	0	2	0	0
Total Number of Tetrads showing Prion-like Inheritance	4	2	0	2	1

Table 3. Meiotic inheritance patterns for putative prion strains

The tetrads associated with Pus4 were not run in the same growth assay as the other tetrads, due to contamination. However, the tetrads were separately tested as part of the initial growth assays for the protein dependency experiment. These growth assays indicated a 4:0 inheritance pattern for the Pus4 tetrads, and this result is included in the summary table in the discussion.

The strains associated with Abd1, Cet1, Pus4, Pus6, and Trm5 showed evidence of non-Mendelian inheritance, with at least one and sometimes multiple tetrads showing non-Mendelian inheritance patterns. However, the strain associated with Ppm2 did not show any evidence of an inheritance pattern consistent with prion proteins, and instead had multiple tetrads that exhibited the 2:2 inheritance pattern consistent with a genetic mutation.

Current Research

At this point in the project, the group of six candidate strains were split into two groups: those with putative prion conformations of essential enzymes (Abd1, Cet1, Trm5) and non-essential enzymes (Ppm2, Pus4, Pus6). The experiments to determine protein dependency and cytoducability first continued with the non-essential enzymes, while the DAmP strains associated with the essential enzymes were created for use in these experiments (see Methods).

Protein Dependency

The tetrad sporulations and dissections necessary to determine the protein dependency of the resistance phenotypes for the Ppm2, Pus4, and Pus6 are complete. Currently, growth assays are being performed on the sets of meiotic progenies from the candidate strains Pus4 and Ppm2. If the resistance phenotypes exhibit patterns of inheritance consistent with a prion protein, it is expected that the resistance phenotype can only be propagated into the half of the meiotic progeny that retain the protein-coding gene of interest. Thus, the tetrads from the heterozygous diploids are expected to exhibit a 2:2 inheritance pattern.

Cytoductions

The cytoductions and following growth assays are currently in progress and will likely be completed in the coming months. This experiment required creating over 250 strains of yeast that would serve as experimental strains and various control strains (with many replicates of each one). Only 42 strains remain to be made. After strains are constructed, they will be tested in growth assays like the other assays performed here.

The goal will be to compare the growth dynamics for control strains and experimental strains to determine if the experimental strains retain and lose the resistance phenotype when expected.

A prion-like strain is expected to retain the associated phenotype even if the cytoplasm of the parent strain is the only component of the cell that gets passed on. Additionally, the phenotype is expected to be lost if the gene that encodes the protein is not expressed in the final strain. This is because prions are protein dependent and are present in the cytoplasm of the cell. In contrast, a non-prion-like strain may be caused by a mutation in the cell's nucleic acid genome and would thus lose the trait if only the cytoplasm is inherited into the next generation (see Figure 5).

Discussion

The experiments described here aimed to determine if the resistance phenotypes observed in six strains of yeast can be attributed to a prion conformation of a previously overexpressed enzyme. Prions display inheritance patterns that distinguish them from non-prion traits. These differences were exploited in experiments that test various inheritance patterns to compare them against established properties of prions. Here, the six candidate strains are broken into three groups to discuss the results, summarized in Table 4.

Pus4

The resistance phenotype in the strain associated with the enzyme Pus4 showed prion-like dominance in a diploid background and non-Mendelian inheritance through meiosis. These two results are consistent with the hypothesis that Pus4 has switched into a prion conformation in the experimental strains. These results are also consistent with previous findings from the Garcia Lab that show Pus4 can maintain a prion conformation that results in a resistance phenotype (Garcia et al., 2021). For this project, Pus4 served as a positive control, and the experiments performed here replicated prior findings regarding this enzyme.

Pus6

The resistance phenotype in the strain associated with the enzyme Pus6 showed the same prion-like dominance in a diploid background and non-Mendelian inheritance through meiosis as the Pus4 strain. These results are also consistent with hypothesis that Pus6 is in a prion conformation. However, the protein dependency of the resistance

phenotype for the Pus6 strain could not be completed through the tetrad sporulation and dissection method. Although Pus6 is not essential for cell viability, heterozygous Pus6 diploids rarely sporulated to produce viable, four-spore tetrads.

Determining if the resistance phenotype is dependent on continual Pus6 expression will instead be assessed through cytoductions. If the resistance phenotype does not propagate through the cytoplasm into a strain that is lacking the Pus6 gene (the ΔPus6 strains), then it can be concluded that the resistance phenotype is dependent on Pus6 expression. This experiment will be key in supporting the hypothesis that a prion conformation of Pus6 gives rise to the observed resistance phenotypes, although the dominance and non-Mendelian inheritance patterns strongly support this hypothesis as well.

Ppm2

The resistance phenotype associated with Ppm2 failed to display prion-like patterns of inheritance in the experiments conducted here. The trait was not dominant in a diploid background, as would be expected of a prion protein. Additionally, the meiotic progeny for Ppm2 tetrads showed a 2:2 inheritance pattern, with only half of the spores inheriting the resistance phenotype. This pattern of inheritance is consistent with traits caused by genetic mutations, not an extranuclear prion protein. These results strongly indicate that the resistance phenotypes observed in Ppm2 strains is not prion-based and instead likely caused by a genetic mutation.

Abd1, Cet1, Trm5

Like Pus4 and Pus6, the resistance phenotypes associated with the enzymes Abd1, Cet1, and Trm5 showed dominance in a diploid background and non-Mendelian patterns of meiotic inheritance. Both results are strong indications that the proteins in these strains are likely in a prion conformation, and that this altered conformation is giving rise to the observed resistance phenotypes. However, because these enzymes are essential, the experiments that require reduced expression of these enzymes (such as the protein dependency experiments and the cytoductions) could not be completed without first taking the additional step of preparing the DAmP strains that are needed to mimic the protein deletion. After these strains are completed, the ΔRME dissections and cytoductions will proceed with these three strains. These two experiments will provide additional insight into the nature of the resistance phenotypes observed in these strains.

RME	*Dominant in Diploid*	*Non-Mendelian Meiotic Inheritance*	*Prion Like*
Abd1	✓	✓	✓
Cet1	✓	✓	✓
Ppm2	✗	✗	✗
Pus4	✓	✓	✓
Pus6	✓	✓	✓
Trm5	✓	✓	✓

Table 4. Summary of prion-like traits in the six putative prion strains

From the six initial strains that displayed resistance to the effects of harmful chemical stressors, five of those strains exhibit inheritance patterns that are consistent with prion proteins (Table 4). In contrast, the Ppm2 strain displayed inheritance patterns that are more consistent with a genetic mutation and not a prion protein. Future research will continue to investigate the five putative prions identified and confirmed by the experiments described here, while the strain associated with Ppm2 will not be pursued further.

This project started by screening all 72 RNA modifying enzymes in the yeast proteome to determine their ability to drive heritable, prion-like phenotypes in response to stress. The experiments presented here, both completed and ongoing, suggest that five of the original 72 enzymes can maintain an alternate, prion conformation. Adding five prions to the list of established yeast prions represents a significant expansion in our understanding of prion biology in yeast. Additionally, as this screen yielded a roughly 7% prion protein frequency within one class of enzymes, it stands to reason that prions may be equally present in the yeast proteome as a whole. Screens of other classes of enzymes modeled after the screen performed for this project may identify a number of additional prions and continue to inform our understanding of the role of prion proteins in biological processes.

Future Directions

Future research on this project will continue in two directions. First, additional experiments may be necessary to confirm the presence of a prion protein in the five strains identified in this thesis. For example, mutated mitochondria may be the root cause of the observed resistance phenotypes and not a prion protein. Mitochondria exist in the cytoplasm of cells and are inherited into future generations through random distribution in dividing cells, much like prion proteins. Distinguishing between a mitochondrial trait and a prion protein can be achieved by selectively and extensively mutating the mitochondria in the original haploid strains from the screen so they cannot function (creating petite strains). Mitochondria from a naïve strain are then reintroduced through cell mating, and the resulting strains are tested to determine if the resistance phenotype is preserved (Chakrabortee et al., 2016). This experiment would be completed using many of the same techniques described here, including the creation of petite strains, cell mating, and tetrad sporulations and dissections.

In addition to further experiments to confirm the prion-based nature of the resistance phenotypes, the more exciting avenues for future research will begin to investigate the biochemical nature of the prion proteins and elucidate the mechanism by which the altered protein conformation confers resistance to the cells. Importantly, prions are altered version of existing proteins, and in the case of many established prions the associated phenotypes are connected to the original function of the protein. In the case of the enzymes in this project, the chemical modifications catalyzed by these enzymes are known, so one place to begin future research will be to investigate if the modification activity changes when the protein is in its alternate conformation. This

could include changes to the target, the extent of the modification, or to the conditions in which the modification takes place. Additionally, it is possible that these enzymes have additional functions beyond their RNA modification activity that are not yet characterized. Research in this area might involve determining additional functions of the enzymes, then investigating how these novel functions differ in strains containing a prion conformation of the enzyme.

References

Breslow, D. K., Cameron, D. M., Collins, S. R., Schuldiner, M., Stewart-Ornstein, J., Newman, H. W., Braun, S., Madhani, H. D., Krogan, N. J., & Weissman, J. S. (2008). A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. *Nature Methods*, *5*(8), 711–718. https://doi.org/10.1038/nmeth.1234

Byers, J. S., & Jarosz, D. F. (2014). Pernicious Pathogens or Expedient Elements of Inheritance: The Significance of Yeast Prions. *PLOS Pathogens*, *10*(4), e1003992. https://doi.org/10.1371/journal.ppat.1003992

Chakrabortee, S., Byers, J. S., Jones, S., Garcia, D. M., Bhullar, B., Chang, A., She, R., Lee, L., Fremin, B., Lindquist, S., & Jarosz, D. F. (2016). Intrinsically Disordered Proteins Drive Emergence and Inheritance of Biological Traits. *Cell*, *167*(2), 369-381.e12. https://doi.org/10.1016/j.cell.2016.09.017

Garcia, D. M., Campbell, E. A., Jakobson, C. M., Tsuchiya, M., Shaw, E. A., DiNardo, A. L., Kaeberlein, M., & Jarosz, D. F. (2021). A prion accelerates proliferation at the expense of lifespan. *ELife*, *10*, e60917. https://doi.org/10.7554/eLife.60917

Halfmann, R., Jarosz, D. F., Jones, S. K., Chang, A., Lancaster, A. K., & Lindquist, S. (2012). Prions are a common mechanism for phenotypic inheritance in wild yeasts. *Nature*, *482*(7385), 363–368. https://doi.org/10.1038/nature10875

Jarosz, D. F., Brown, J. C. S., Walker, G. A., Datta, M. S., Ung, W. L., Lancaster, A. K., Rotem, A., Chang, A., Newby, G. A., Weitz, D. A., Bisson, L. F., & Lindquist, S. (2014). Cross-Kingdom Chemical Communication Drives a Heritable, Mutually Beneficial Prion-Based Transformation of Metabolism. *Cell*, *158*(5), 1083–1093. https://doi.org/10.1016/j.cell.2014.07.025

Jarosz, D. F., Lancaster, A. K., Brown, J. C. S., & Lindquist, S. (2014). An Evolutionarily Conserved Prion-like Element Converts Wild Fungi from Metabolic Specialists to Generalists. *Cell*, *158*(5), 1072–1082. https://doi.org/10.1016/j.cell.2014.07.024

Prusiner, S. B. (1982). Novel Proteinaceous Infectious Particles Cause Scrapie. *Science*, *216*(4542), 136–144. https://doi.org/10.1126/science.6801762

Shorter, J., & Lindquist, S. (2005). Prions as adaptive conduits of memory and inheritance. *Nature Reviews Genetics*, *6*(6), 435–450. https://doi.org/10.1038/nrg1616

Tyedmers, J., Madariaga, M., & Lindquist, S. (2008). Prion Switching in Response to Environmental Stress. *PloS Biology*, *6*(11), e294. https://doi.org/10.1371/journal.pbio.0060294

Wickner, R. B., Edskes, H. K., & Shewmaker, F. (2006). How to find a prion: [URE3], [PSI+] and [β]. *Methods*, *39*(1), 3–8. https://doi.org/10.1016/j.ymeth.2006.04.009