PUTATIVE PRION STATE DYNAMICS IN RNA-MODIFYING ENZYMES

by

JACOB EVARTS

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Approved: _____*David Garcia, Ph.D.*_____
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Prions are primarily associated with fatal neurodegenerative diseases, such as Mad Cow disease and Creutzfeldt-Jakob disease. Recent evidence, however, suggests that prions also comprise an additional class of epigenetic mechanism that is biologically beneficial. From an evolutionary standpoint, the ability to change phenotypes without requiring changes to the genome, as prions do, would be hugely beneficial in fluctuating environments. Through overexpressing proteins and introducing environmental stressors, two techniques known to increase *de novo* prion formation, we performed a large-scale screen of many RNA-modifying enzymes in budding yeast to test if they harbor beneficial prionogenic behavior. From this screen, we have identified five induced prion-like states. We show that many of these putative prions have characteristics consistent with prion-based epigenetics. Prion-based inheritance expands on the central dogma of biology, supporting the idea that prions are an epigenetic mechanism for passing on heritable traits.

Acknowledgements

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This thesis is dedicated to the memory of Cecilia.

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Introduction

Mad cow disease, sheep scrapie, and Creutzfeldt-Jakob in humans are all mammalian neurodegenerative diseases that appear to be caused by infectious proteins called prions (Derkatch, 1996). These mammalian diseases were the original area of research into prions, and still shape the way prions are viewed today. Prions are alternative forms of cellular proteins that can induce normal, or naïve, proteins to adopt a new, altered structural state. In this sense, prions are a self-templating form of epigenetic regulation, or the regulation of phenotypes without changes to the genome (Wickner, 1994). Once a sufficient number of proteins are in a prion state, they have a tendency to aggregate, falling into two categories: amyloids and non-amyloid forming prions. Thus, prion-based diseases occur when a prion protein is introduced and induces conformational changes in a host's naïve proteins, which in turn agglomerate and lead to neurodegeneration.

Not all prions are agents of disease and degeneration, however. Some yeast prions have been shown to bring about beneficial phenotypes, such as resistance to environmental stress (Suzuki et al., 2012). Prion heritability allows species variation at the level of protein conformations, which is significant because it was previously believed that species

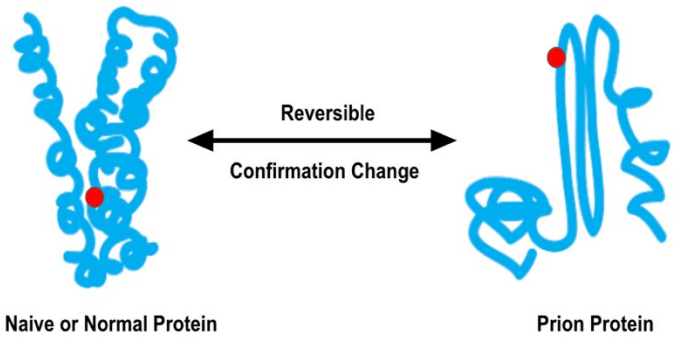

Fig 1. Protein conformational change between its naïve and prion states. These shape changes can expose previously hidden active sites or binding sites on the protein, potentially changing its function.

variation was only heritable through DNA. (Wickner, 1994). Researchers have shown that two yeast prions in particular, [URE3] and [*PSI*$^+$], are important mechanisms of inheritance (Derkatch, 1996; Wickner, 1994).

The Garcia Lab has been investigating novel ways to identify new yeast prions and understand the mechanisms of expression and inheritance behind them. The yeast *Saccharomyces cerevisiae* is an optimal model organism because genetic modifications are easily introduced to the cells, they share thousands of genes with humans, and experiments can be parallelized in large scale. High-throughput growth assays allow for proteome-wide screens to be analyzed, reducing the time necessary to uncover unique phenotypes.

Background

Yeast Prion Discovery

Yeast prion research started with the identification of the [*PSI*$^+$] (originally known as the factor ψ^+), referred to as a non-Mendelian factor by Liebman *et al.* (Liebman *et al.,* 1975), in some strains of *Saccharomyces cerevisiae.* It was initially found that the [*PSI*$^+$] factor increased the effectiveness of specific translational suppressors (Cox and Young, 1971), and it was later confirmed that suppression of certain stop codon nonsense mutations increased in ψ^+ strains (Liebman and Sherman, 1979). Lindquist and colleagues went on to confirm that the [*PSI*$^+$] factor was a prion-like aggregate of Sup35, a cellular protein that functions as a translation release factor in its naive state. Strains that contained [*PSI*$^+$] were phenotypically similar to strains in which the Sup35 had been mutated (Patino *et al.*, 1996), suggesting that the prion version of Sup35 was loss-of-function even with no change to its gene sequence.

Furthermore, Cox and colleagues demonstrated that [*PSI*$^{+}$]/ψ^{+} was not related to any extrachromosomal DNA or RNA (Cox *et al.*, 1988).

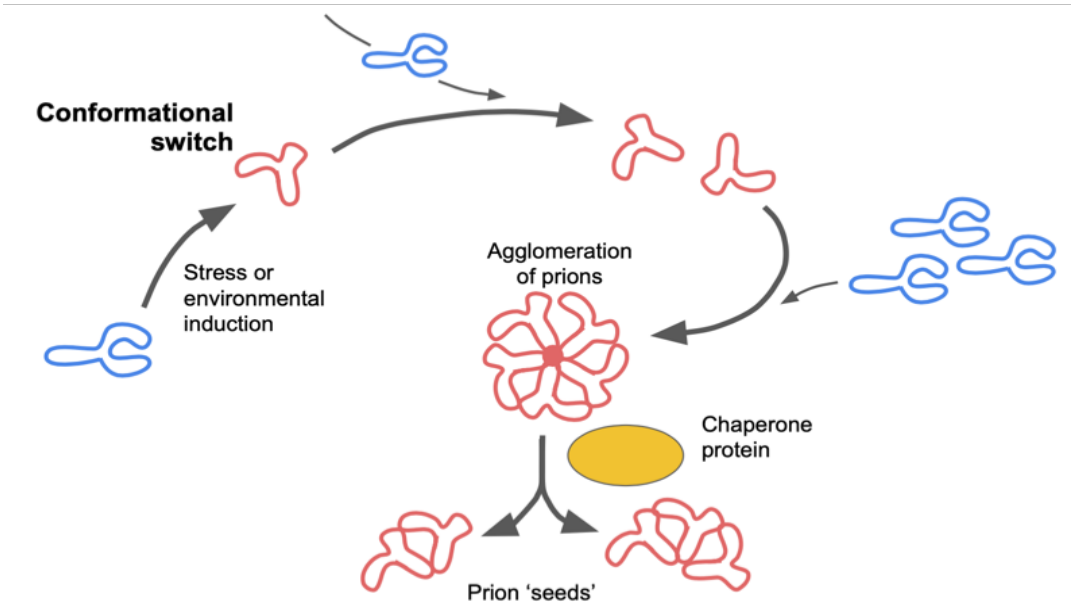

Fig 2. Model for prion formation and agglomerations from a stock of naïve proteins. Dispersal of the prion agglomeration to progeny or other individuals, called seeding, is facilitated by chaperone proteins.

Similarly, Wickner found that [URE3] was a prionic form of the protein Ure2p, responsible for allowing cells to metabolically shift to using ureidosuccinate in the presence of ammonium ions, despite ammonium usually repressing the uptake of ureidosuccinate in favor of more readily available nitrogen sources. Wickner also described how overexpression of the naïve Ure2p protein increased the frequency with which a yeast strain became [URE3] by up to 100-fold (Wickner, 1994).

Chaperone Dependency

In 1995, it was discovered that the chaperone heat-shock protein 104 (HSP104) plays a significant role in the propagation of the [*PSI*$^{+}$] factor in yeast (Chernoff *et al.,*

1995). Heat-shock proteins are well-conserved chaperone proteins that aid in folding new proteins or refolding proteins that have lost their typical conformation (Taipale et al., 2010, Garcia & Jarosz, 2014). Lindquist and colleagues propsed a hypothesis that some chaperone proteins put proteins into a sort of unstable transition state, from which they could either convert back to naïve proteins or convert to prion form with some small spontaneous probability or aided by existing prions. Additionally, chaperones are also responsible for breaking aggregates of prions into smaller seeds, allowing for propagation to other organisms or into progeny (Patino *et al.*, 1996). For example, these prion seeds are passed to the daughter cells during yeast cell division and act as templates for future prion transformations (Chakrabortee *et al.*, 2016). Although Hsp104 must be present in the cell for the propagation of [*PSI*$^{+}$], overexpression of Hsp104 leads to the elimination of the [*PSI*$^{+}$] factor from the strain (Chernoff *et al.,*1995). Loss-of-function mutations to the Hsp104 gene or inhibition of the corresponding protein has thus been found to be an effective "cure" of some yeast prions (Wickner, 1994; Lindquist *et al.,* 1996). Interestingly, the chaperone proteins only need to be transiently disrupted to eliminate the prion (Chakrabortee *et al.*, 2016).

Non-Mendelian Inheritance

In addition to being chaperone-dependent, a hallmark of previously identified prion-states is their non-Mendelian inheritance (Shorter and Lindquist, 2005). Since prion inheritance in yeast is not tied to chromosomal segregation, it is common to see prions passed to most or all meiotic progeny (4:0 or 3:1 in meiotic progeny). This pattern is due to prions being proteins, which localize in the cytoplasm of the cell. When a cell divides, the resulting daughter cells receive cytoplasm from the original

parent cell. Prions, being cytoplasmic elements, are distributed to all daughter cells and used as templates for further prion production. Traits that arise from mutations in the genome, in contrast, are generally passed on to half of the progeny (2:2 inheritance pattern).

Because of their unique inheritance mechanisms, prions also tend to be dominant in diploid cells created through mating of strains with and without the prion (Chakrabortee *et al.*, 2016). Thus, a key aspect of these prion phenotypes is that they do not depend on genotype, making them epigenetic elements.

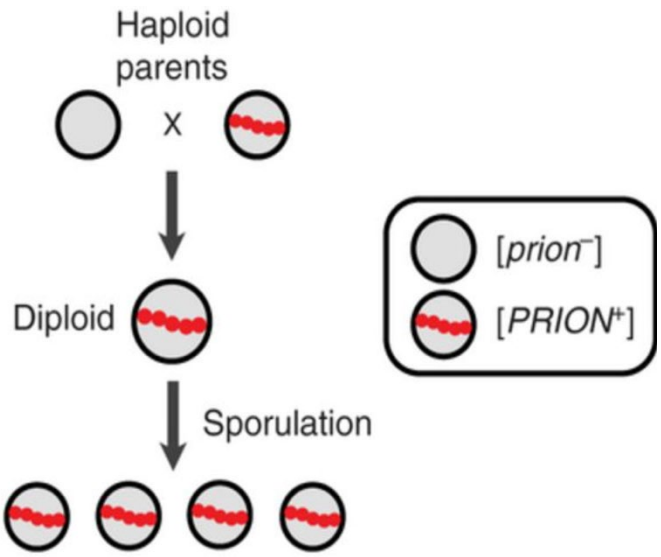

Fig 3. Prion inheritance in yeast diploids and spores. Prion phenotypes are dominant when crossed with a naïve strain. Adapted from Garcia & Jarosz, 2014

Prion-based information transfer has recently been found to play a much more significant role in trait heritability than previously thought and provides many beneficial phenotypic changes, such as resistance to acute environmental stress (Chakrabortee *et al.*, 2016; Halfmann *et al.,* 2012; Yuan and Hochschild, 2017). Here, we propose that prions appear to be a relatively common form of inheritance in RNA-modifying enzymes in budding yeast.

Spontaneous Prion Formation

While considering how to increase the rate of *de novo* prion formation, or the formation of the original prion protein that can then template further copies, it is important to consider why prions might exist as an adaptive mechanism. When a cell is under acute stress, whether exposure to a chemical or extreme temperature, it needs a fast phenotypic change to adapt. Genetic adaptation through mutations is a relatively slow process and has a high probability of decreasing the fitness of a cell instead of increasing it (Garcia and Jarosz, 2014). Prions allow for much faster phenotypic transition, and prions often arise at higher frequencies than genetic mutations (Brown & Lindquist, 2009). Stressful environments, then, can select for prion formation (Lancaster *et al.*, 2010).

Beyond environmental stressors, another way to increase *de novo* prion formation comes down to probability. If there are n proteins of a particular type, each of those proteins has some small probability p of transitioning to a prion on its own. Only one of those proteins needs to transition to begin the cascading process of converting other naïve proteins. The probability of having at least one protein flip, then, is one minus the probability that none of them transition because of the law of total probability.

$$P(at\ least\ one\ protein\ flips) = [1 - (1 - p)^{(n)}]$$

Thus, the probability of obtaining a prion is exponentially dependent on the number of proteins of a certain type in a cell. However, this is a rudimentary formulation, and a more complex formulation, explored later, likely models the behavior more closely. Notably, the probability of a prion forming is a monotonically increasing function of n.

This digression stands to illuminate that increasing the number of proteins in a system will proportionally increase the probability of the first prion formation. Recent work has shown that even transient overexpression of some yeast proteins is enough to create heritable phenotypes that are prion-like (Chakrabortee *et al.*, 2016).

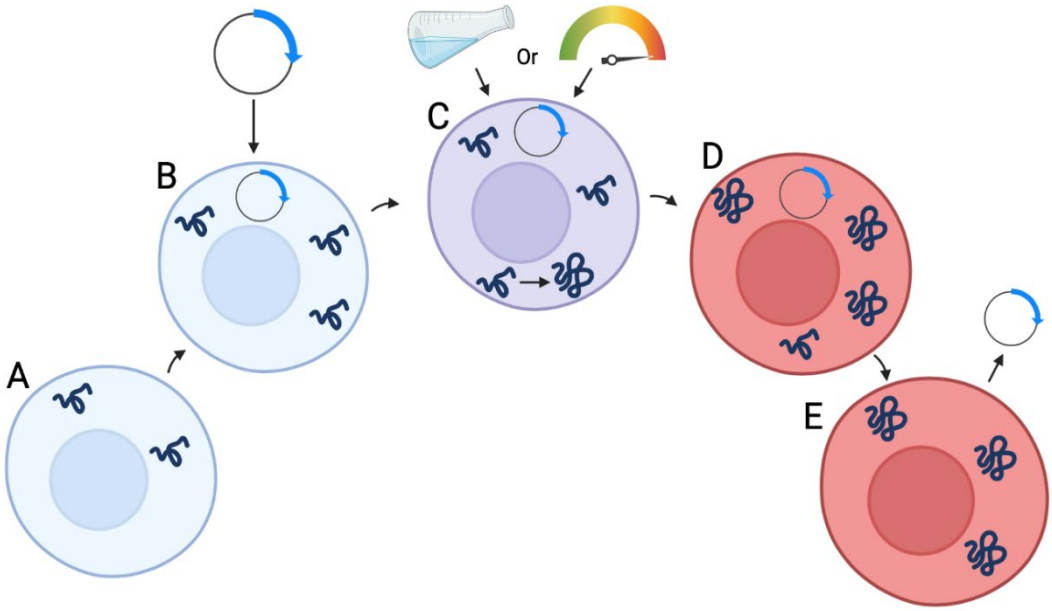

Fig 4. Method for *de novo* prion formation. (A) A baseline number of naïve proteins of a certain type exist in the cytoplasm of a cell. (B) A plasmid is transformed into the cell to overexpress, or create more of, the protein. Such plasmids are generally only active while growing on certain media to ensure transient overexpression. (C) The cell is exposed to environmental stress, whether chemical, heat, or nutrient depletion. This causes a small initial group of naïve proteins to flip to their prion form. (D) After the cell has been removed from the stress conditions, more proteins continue to be recruited and transformed to their prion state. (E) Finally, the overexpression plasmid, which has not been active for some time, is removed. The cell usually has few to no long-term phenotypic side effects from the stress and protein overexpression except for the new prions that are present in the cytoplasm. In this sense, the prions serve as a memory of the previously stressful conditions. This figure was created on BioRender.com.

Incorporating these two methods, environmental stress exposure and protein overexpression, is the basis for many of the assays and screens outlined in this work. An initial high-throughput screen for prionic phenotypes that serves as the basis for this research was conducted in precisely this manner. Seventy-two RNA-modifying

enzymes were individually overexpressed. Each of these strains was then exposed to one of 60 different stress conditions, making for roughly 4,300 different combinations. We hypothesized that several of these strains developed prions that bestow resistant, or beneficial, phenotypes when the cells are grown in acute stress. If true, this hypothesis would contribute to the growing body of research that suggests that prions are capable of creating beneficial phenotypic changes as an adaptation to acute stress, and thus are much more than simply agents of neurodegenerative disease.

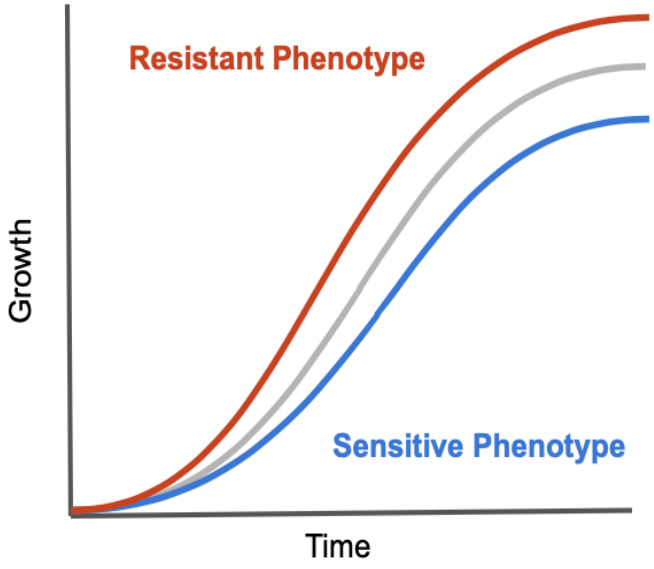

Fig 5. Typical resistant and sensitive growth phenotypes represented as growth curves compared to a control (grey curve).

RNA-modifying enzymes were of particular interest for this study for two reasons. First, they are a well-conserved class of proteins that have many human orthologs. Finding prion states in these proteins in yeast could impact our understanding of human prion biology and human health in the future. The second reason they were an ideal class of proteins is the existence of a small number of known prions in this group already (Garcia *et al.*, 2020, Suzuki *et al.*, 2012). Furthermore, developing a protein functional class-based search for prions could provide a template for investigating the

capacity for other proteins to harbor prion function, in turn widening our understanding of the evolution and function of these proteins.

With our current understanding of prion states, one way to demonstrate that the presence of prions causes a phenotype is to show that nothing else is causing it, such as a genetic mutation. Prions can be differentiated from other forms of epigenetic inheritance by their reliance on protein conformation modulators, like chaperone proteins. Additionally, their unique inheritance pattern also differentiates them from most genetic phenotypes. Assays to test these traits are used here to argue that several proteins we have identified are strong prion candidates.

Methods

Strains and Cultivation Procedures

The yeast strains used in this study are *Saccharomyces cerevisiae* from the BY strain background. Yeast strains were cultivated on YPD agar or liquid (RPI), and several were grown on amino acid dropout media where specified (Sunrise Scientific). All strains were stored as glycerol stocks (25% glycerol (Amersco) in appropriate media) at -80 °C and revived on YPD plates before testing. Yeast were grown in YPD at 30 °C unless specified. All yeast strains were sourced from the BY4741 MATa haploid knockout library (GE Dharmacon). During OD600 measurements, strains were grown in CSM (Sunrise Media), which is more translucent than YPD and allows for more accurate readings.

Data Pipeline

This research is released in conjunction with a custom program for high-throughput growth dynamics analysis, released as a Python package on Pypi called Wellcompare (https://pypi.org/project/wellcompare/). Wellcompare was created to clean, organize, and efficiently produce visual and statistical representations for large amounts of growth data represented as OD600 measurements from a BioTek Epoch2 microplate reader paired with a Biostack Microplate Stacker for automation. Unless otherwise specified, all statistical analyses and graphs were produced within the Wellcompare framework.

The OD600 readings from each experiment provide an approximate colony density every two hours for four days (96 hours). To quantitatively parameterize growth

dynamics, Wellcompare fits a logistic model over these discrete measurements, achieved with the Python SciPy package. Although alternatives to using a logistical growth model have been presented (Tsoularis & Wallace, 2002), it remains a robust model. Additionally, the interpretability of the parameters makes it ideal for this application. The precise model used was:

$$P(t) = \frac{L}{1 + Ae^{-kt}}$$

where A is the initial population [$P(0)$], t is the time since start, K is the growth rate, and L is the maximum value of the curve. Thus, once a growth curve is fit, it can be parameterized to conveniently obtain the colony growth rate and the largest size the colony reached, or y-max. Once parameterized, the growth rates and y-maxs of replicate are analyzed with an independent t-test. Additionally, the ratio of means (ROM) is used to show relative growth dynamics.

Initial Screen

All yeast strains were exposed to an environmental stressor while growth rate, carrying capacity, and lag time were measured. Strains descended from yeast that experienced previous overexpression of an RNA-modifying enzyme were compared against an identical strain exposed to the same conditions but had not had the protein overexpressed. The ratios of the growth parameters of strains with previous overexpression were normalized with the growth parameters of strains without previous overexpression as a relative measure of growth.

The initial large-scale exploratory screen of RNA-modifying enzymes (RME) and some preliminary analysis were completed before the work in this thesis. It

included overexpressing 72 different RMEs and exposing those strains to 60 different environmental stresses. The exposure to stress happened over three outgrowths, the equivalent of dozens of generations, to look at budding yeast's (*Saccharomyces cerevisiae*) long-term memory and propagation of prion states. Each strain that had one protein overexpressed and was exposed to single stress had four biological replicates to reduce the experimental noise.

To determine which strains had a potentially prion-based phenotype, the growth rates and y-maxs of the four biological replicates were averaged, and the ratios of the averages were combined into one dataset. Since the ratios are normally distributed, I

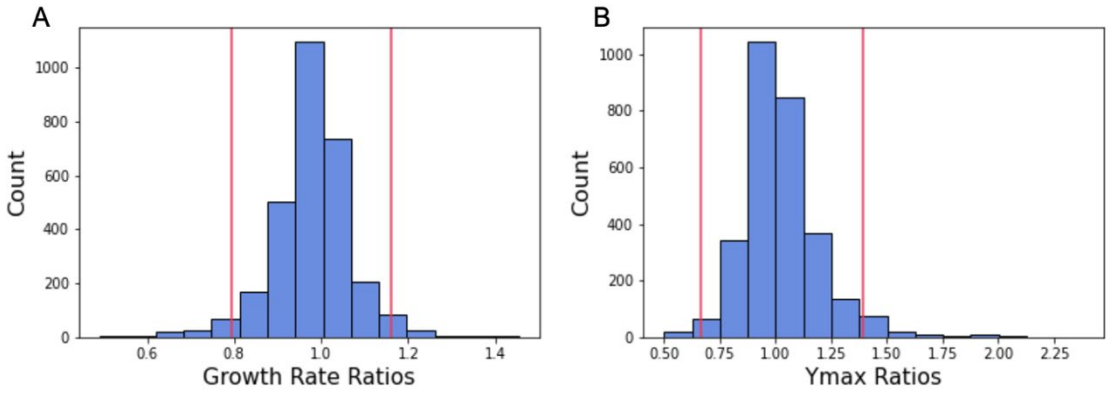

Fig 6. Growth rate and y-max ratios from the initial screen. (A) The ratio of growth rates with the second standard deviation demarcated by the vertical red lines. Only growth rates farther than two standard deviations away were considered (SD=0.0915, SEM=0.00003, n=2961). (B) The ratios of y-maxs with the second standard deviation shown with vertical red lines. Only y-maxs farther than two standard deviations away were considered (SD=0.1823, SEM=0.00006, n=2961).

looked at all of the strains at least two standard deviations from the mean and had a significant p-value ($p<0.01$). Additionally, each strain had to meet this criterion for both growth rate and y-max, and preference was given to those strains that showed a resistant phenotype (larger ratio, indicating a beneficial phenotype), rather than a sensitive

phenotype (smaller ratio, indicating a detrimental phenotype) under these conditions. The initial set of putatively prion-harboring strains is summarized in Table 1.

Repeat Screens

Once an initial set of strains was identified, all strains were tested again to ensure the results were repeatable. These smaller screens were conducted in a similar manner, where strains were exposed to a stress condition while their growth was observed. Before taking place in the repeat screens, all strains were cured of their plasmids during an outgrowth on 5-FOA media.

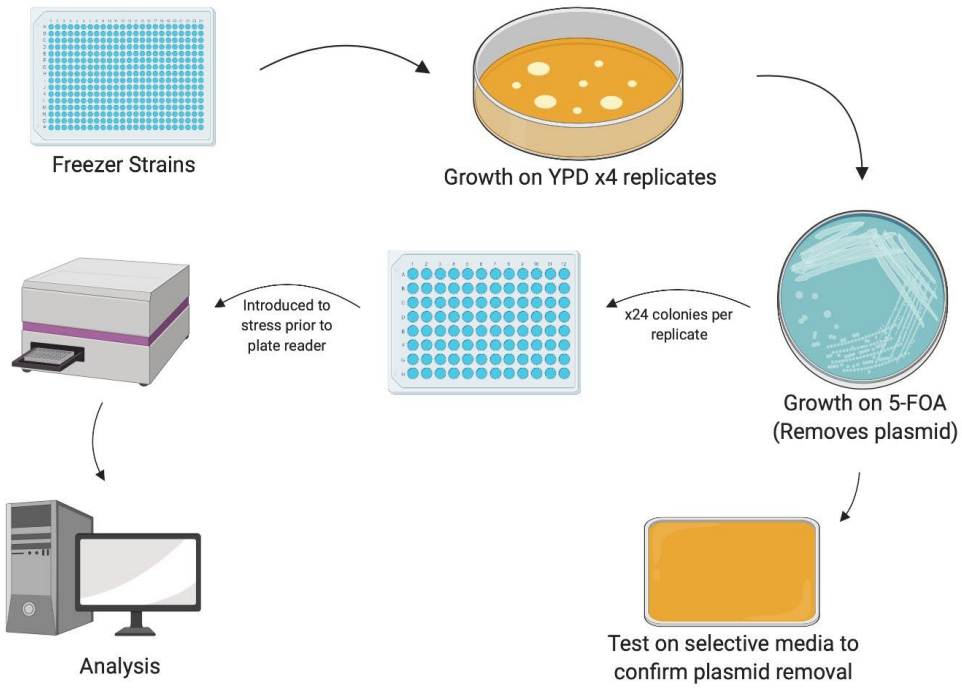

Fig 7. Yeast cultivation from frozen strains for follow-up screens. This figure was created on BioRender.com.

Each of the original four replicates of each strain was streaked out, and 24 colonies were collected from each, which greatly increased the number of replicates available for statistical analysis. To continue on to further experimentation, a strain had to have a repeatable phenotype that appeared in multiple wells derived from a single replicate. These screens eliminated several prion candidates, which could still be the focus of future work. The smaller set of strains that showed stable phenotypes were then tested in several other assays designed to determine if they had prions.

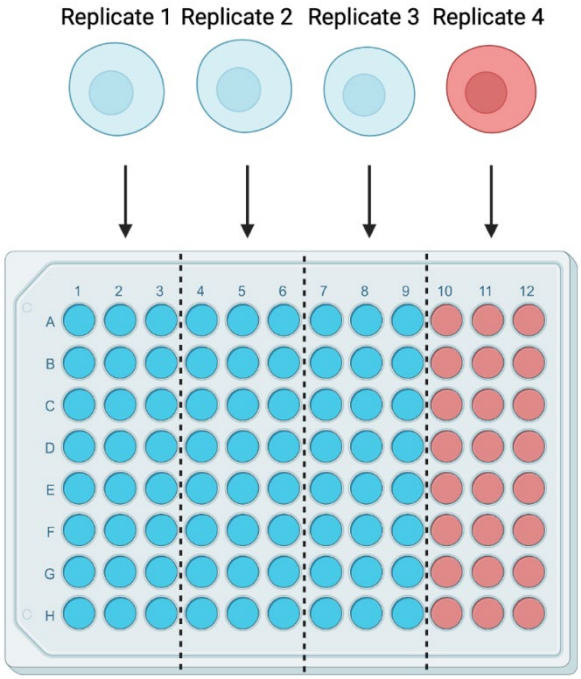

Fig 8. Phenotypic pattern expected in follow-up screens. If one of the original replicates had prions, in this case replicate four, we would expect to see numerous resistant phenotypes in the colonies derived from that replicate. This figure was created on BioRender.com

Curing the Chaperone Protein HSP70

To determine whether the phenotypic states we observed behave similarly to previously identified yeast prions (Shorter & Lindquist, 2004; Hou et al., 2011; Jarosz et al., 2014b), we tested if the inheritance of the phenotypic states would be disturbed with transient inhibition of each of three different heat-shock proteins, Hsp104, Hsp90,

and Hsp70. Of these, only Hsp70 perturbation had a significant effect on growth rate and is the only one examined in this paper. A dominant-negative variant of Hsp70 (Ssa1-K69M) was expressed on a plasmid for three outgrowths to achieve a temporary inhibition of Hsp70. Two types of plasmids expressing this variant were tested, a 2-micron plasmid and a centromeric plasmid. These plasmids generally have different levels of expression, which can affect the results of an experiment like this. The expression plasmids were then eliminated via passaging on 5-FOA media before growing the strains for another three outgrowths to restore the function of Hsp70. This protocol ensures that the elimination of Hsp70 does not have a confounding effect on growth rate while still potentially affecting the seeding of any prion agglomerations.

Once the candidate strains had their chaperone proteins transiently inhibited, they were compared back to the same strains before inhibition. In this way, they came from the same original set of cells, with the only difference in their lineage being the plasmid introduction in one of them. From here, we performed an assay to test whether the original strains grew better than the new strains, despite being nearly identical besides the temporary inhibition. Due to limitations in protein essentialness, only Abd1, Cet1, and Pus6 overexpression strains were included in this assay. We hope to test other strains in the future.

Mitotic Stability Assay

To test for mitotic stability, or how faithfully a mother cell passes a phenotype to its daughter cells, we looked at the phenotype over many outgrowths. Previous work has described these persistent phenotypes as "molecular memories" of past overexpression (Chakrabortee *et al.*, 2016). To test the strength of these molecular

memories, all of the putative prion strains were streaked to single colonies on YPD plates and grown for three days. Single colonies were picked from these plates and streaked out onto fresh plates where they were similarly grown for three days. This process was repeated ten times, with glycerol stocks taken after five and ten outgrowths as 'snapshots' of the phenotypes.

In parallel to this process, the putative prion strains were also pin replicated to liquid YPD media and grown for three-day outgrowths before being transferred to fresh liquid media. Glycerol stocks were also taken after five and ten outgrowths. Since yeast are liable to grow different on solid and in liquid media, employing both methods provided a more informative view of the inheritance. Additionally, since this protocol involves streaking the yeast to single colonies on the agar, the community dynamics are shifted, and the bottleneck effect is more likely to occur. This contrasts with the liquid outgrowth, which allowed several different yeast colonies to transfer to every outgrowth instead of just one. Finally, the number of generations that take place in a given time on agar and in liquid can vary, so it is useful to poll both.

Once the yeast had gone through five or ten outgrowths on agar or liquid media, they were put through the same screening process as the original strains. The resulting data was then run through Wellcompare, revealing whether each strain maintained its resistant phenotype over long periods.

Creation of Diploid Yeast Strains

As prions are cytoplasmic elements, they should be transmitted to the cytoplasm of a diploid during mating with a naïve strain. Diploid dominance is another prion-like

behavior that provides insight into the nature of the observed phenotype. This assay involved crossing strains with previous overexpression of an RNA-modifying enzyme with a naïve strain of the opposition mating type on YPD agar overnight. Cells were then 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 before growth was measured under stress. Due to some concern that diploid cells might react differently to various stress levels, the diploid strains were tested at 25%, 50%, 100%, 200%, and 400% of the stress concentrations used for the haploid assays.

Meiotic Inheritance of Spores

Following their creation, the 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 (a nutrient-rich media designed for robust cell growth) to grow for two additional days at 25 °C. Cells were then pelleted and resuspended in sporulation media, a nutrient-poor media. The process of quickly transferring cells between media is designed to prompt cells to undergo meiosis and form tetrads in response to changing nutrient levels. 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 with two naïve parents.

Results

Initial Screen: Transient Overexpression of RME's Creates Beneficial Traits

From the original several thousand unique strains that resulted from the Cartesian product of the 72 proteins overexpressed and 60 stresses, 13 different proteins were determined to be putative prions. One of these proteins, Pus4, had already been shown to be a yeast prion (Garcia *et al.*, 2020). The other proteins vary in function, but there are notable groupings of the other pseudouridine synthases, and Trm proteins, which are tRNA methyltransferases. In fact, of the 13 candidate prion strains, five of them are involved in methyltransferase activity.

RNA-Modifying Enzyme	**Function of Protein**	**Environmental Stresses that Induced Phenotypes**
Abd1	mRNA methyltransferase	Cycloheximide CSM-Ile-Ura Media CSM-Trp-Ura Oxaliplatin UV Irradiation
Cet1	mRNA phosphatase	Cycloheximide CSM-Ile-Ura
Dus1	tRNA-dihydrouridine synthase	Cycloheximide Cisplatin
Elp4	Elongator complex protein	Low Phosphate Media Phenanthroline

Gar1	Ribonucleoprotein complex protein	CSM-Arg-Trp-Ura Media UV Irradiation
Nop1	Histone glutamine methyltransferase	CSM-Ile-Ura UV Irradiation
Ppm2	tRNA methyltransferase	Cycloheximide CSM-Ile-Ura
Pus4	tRNA pseudouridine synthase	Radicicol Oxaliplatin
Pus5	tRNA pseudouridine synthase	Heat Shock 46C 15 min. CSM-Lys-Trp-Ura
Pus6	tRNA pseudouridine synthase	Cycloheximide CSM-Ile-Ura CSM-Trp-Ura
Trm5	tRNA methyltransferase	Cycloheximide Low phosphate media
Trm7	tRNA methyltransferase	Radicicol Manganese chloride
Trm10	tRNA methyltransferase	Cisplatin Flavopiridol

Table 1: Putative prion-harboring strains from the initial screen. All strains experienced prior protein overexpression and environmental stresses to help induce phenotypes. Cycloheximide concentration: 0.2ug/mL; radicicol concentration: 50uM; oxaliplatin concentration: 32uM; flavopiridol concentration: 50um; manganese chloride concentration: 10mM; cisplatin concentration: 0.25mM; phenanthroline: 24uM; UV irradiation strength: 70 J/m2.

After repeating the screen on this smaller subset of proteins, six protein candidates maintained a resistant phenotype under stress in a large number of their wells. Abd1, Ppm2, Pus4, Pus6, and Trm5 had a large number of colonies ($n \geq 8$) that showed significantly better growth rates in the strains that had previously had protein overexpression compared to those that did not ($p<0.05$). The final strain, Cet1, had a smaller number of wells ($n=4$) that saw significantly better growth, again defined by the parameterizations compared to control strains. In some strains, the number of significant wells per biological replicates was over 20%, which is many orders higher than the spontaneous mutation rate in *Saccharomyces cerevisiae* (Zhu *et al.,* 2014). Since each biological replicate originated from one strain, a large number of significant wells in a replicate suggest that the original replicate colony harbored a beneficial phenotypic state.

Importantly, due to constraints, several of the strains identified in the initial screen could not be retested, which means that the six identified candidate proteins identified here might not be exhaustive. Dus1, Elp4, and Trm10 did not have a representative strain tested, as chemical and facility resources were limited. Of the tested strains, some did not have all of the stress conditions tested that seemed to induce a phenotype in the original screen. Both of these areas could be the focus of future work.

Hsp70 Regulates Several of the Heritable Phenotypic States

Due to constraints, only strains that had Abd1, Pus6, and Cet1 previously overexpressed could be tested for chaperone dependency, but we hope to test the others in future projects. In the 11 isolates of Abd1 (4), Pus6 (4), and Cet1 (3) tested, over half

(6) of the isolates lost the resistant phenotype entirely and repeatably after transient inhibition of Hsp70. Of the isolates that lost their phenotype, at least one strain that harbored each of the three candidate proteins was represented. This behavior indicates some reliance on chaperone proteins for phenotype propagation, a hallmark of prion-based traits.

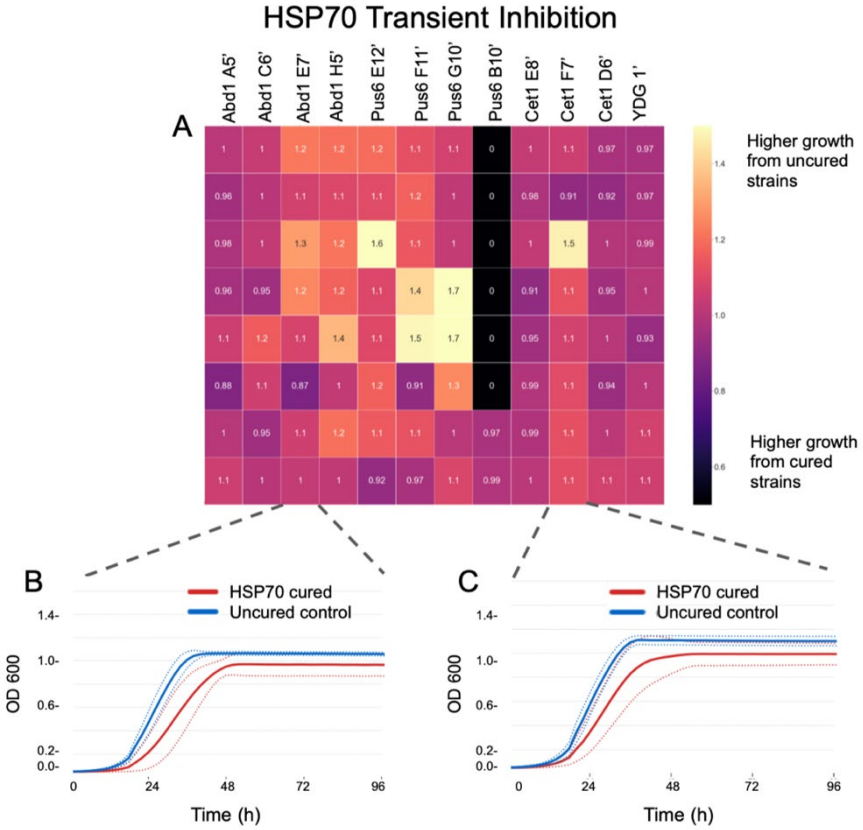

Fig 9. Growth effects of transient inhibition of the chaperone protein Hsp70 on candidate strains. (A) Heatmap of the y-max ratios of the uncured strains over the same strains that had transient inhibition of Hsp70 with a centromeric plasmid. The Pus6 B10' strain did not experience enough growth to be analyzed. (B) Average growth of the replicates that had Abd1 previously overexpressed and the same strains but with transient Hsp70 inhibition (n=8). The uncured strains reached higher growth as per the y-max ($p=0.046$), but the growth rates are not significantly different ($p=0.387$). (C) Average growth of the replicates that had Cet1 previously overexpressed and the same strains but with transient Hsp70 inhibition (n=8). The uncured strains reached higher growth as per the y-max ($p=0.022$) and growth rate ($p=0.081$), although only the y-max is significant. 95% confidence intervals are shown.

Protein-Based Inheritance Confers Mitotically Stable Traits

Next, we tested the mitotic stability of the differential growth traits exhibited when the six strains were exposed to chemical stressors over hundreds of generations. How mitotically stable a phenotype is, or how faithfully a phenotype is passed on from mother cells to daughter cells, is valuable information for understanding how it is transmitted through a population over time. Out of the candidate proteins, Abd1, Cet1, Ppm2, Pus4, and Pus6 all had representative isolates (n=6) tested for mitotic stability, with only Trm5 not being included in this assay.

Growth dynamics were measured over several outgrowths, where one outgrowth included transferring yeast to fresh media, either YPD agar plate or YPD liquid, and allowing the yeast to grow for three days. Strains grown on the YPD agar tended to maintain their stress-resistant phenotypes longer than when the same strains were grown in YPD liquid. Sixty percent (3) of the candidate proteins had strains that kept their resistant phenotypes for hundreds of generations when grown on agar plates. Abd1 (Y-max $p<0.001$), Pus6 (Growth rate $p=0.039$), and Pus4 (Growth rate $p<0.001$) had representative isolates with phenotypes that lasted until the limits of this assay over ten outgrowths on agar. In comparison, only Abd1 (Y-max $p=0.028$) had isolates that kept their phenotype for that long in liquid media. We posit this observed difference based on media format could be due to different community dynamics or a population bottleneck, although we have not tested for either of these. For example, when yeast is transferred between agar plates, a single colony is isolated and streaked out. Any colony specific idiosyncrasies are then exacerbated in later outgrowths, whereas many colonies are transferred between liquid media, so this selection does not occur. Strains with two

candidate proteins, Cet1 (Growth rate p=0.831, Y-max p=0.535) and Ppm2 (Growth rate p=0.026, Y-max p=0.122), did not have resistant phenotypes after the first outgrowth (~25 generations), raising doubts that the particular strains for these proteins tested in this assay had their prion states induced correctly. Perhaps this test for these proteins will be repeated with other candidate strains.

Beneficial Phenotypic States Are Dominant in Genetic Crosses to Naïve Strains

In the first step of determining each phenotypic states' inheritance patterns through mating, we crossed cells harboring these putative prions to naïve, isogenic controls strains, to create diploids. Next, we tested these strains in the original stress conditions at a range of concentrations. Diploid sensitivity to environmental stress varied, but most of the diploids exhibited strong phenotypes at twice the concentrations used for the haploids, although this concentration also caused increased colony death.

At double stress concentrations, five of the six candidate proteins had isolates that grew significantly better than naive diploids, with only Ppm2 not seeing a strong phenotype inherited. The diploid isolates from Abd1, Cet1, Pus4, Pus6, and Trm5 had robust resistant phenotypes. One possibility for why the resistant phenotypes were exaggerated in this assay is the more extreme stress conditions. This suggests that the resistance gained from prion states might be even more beneficial under more stress up to a point, although this was not tested here. Thus, the stress-resistant, beneficial phenotypes appear to be dominant in genetic crosses to naive strains.

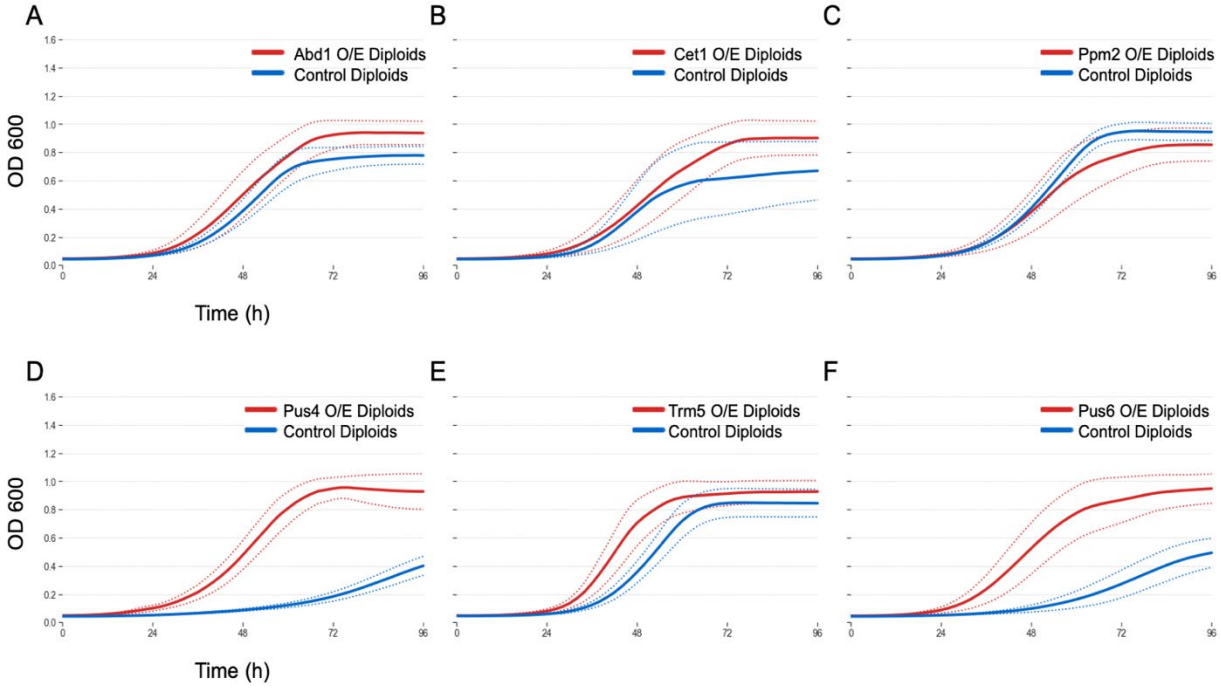

Fig 10. Dominant inheritance of resistant phenotype in diploids. (A) Abd1 overexpression diploids growing against control diploids (Growth rate p=0.0032, Y-max p=0.3674, n=18). (B) Cet1 overexpression diploids growing against control diploids (Growth rate p=0.0510, Y-max p=0.0874, n=15). (C) Ppm2 overexpression diploids growing against control diploids did not produce significant results (Growth rate p=0.7985, Y-max p=0.3797, n=18). (D) Pus4 overexpression diploids growing against control diploids (Growth rate p<0.0001, Y-max p=0.4944, n=17). (E) Trm5 overexpression diploids growing against control diploids (Growth rate p=0.0055, Y-max p=0.2466, n=12). (F) Pus6 overexpression diploids growing against control diploids (Growth rate p<0.0001, Y-max p=0.0002, n=18). 95% confidence intervals are shown.

Several Strains Show Non-Mendelian Patterns of Inheritance

Diploid inheritance is an important indicator of a dominant phenotype; however, it is not unique to prion-like traits. Observing the resulting tetrads, or progeny, though, provides information on the inheritance patterns. Non-Mendelian inheritance is a much stronger argument for a cytoplasmic element, such as prions, since genetic traits are passed on in a different, very predictable fashion. For the four progenitors, or spores, produced from a single yeast diploid, if the phenotypes observed here are DNA-based,

we would expect two spores to receive the allele and grow better. The other two spores, which received the allele from their naïve parent, would not be resistant to the stress condition and grow worse. Thus, this assay allows for classification of the phenotypic basis depending on whether all the spores grow well or whether only two of them do. Diploids are grouped by the phenotypic inheritance of their strains in Table 2.

	# Abd1 Diploids	**# Cet1 Diploids**	**# Ppm2 Diploids**	**# Pus6 Diploids**	**# Trm5 Diploids**
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**

Table 2: Phenotype inheritance in meiotic progeny. The columns represent instances of meiotic progenitors from single diploid cells, associated with the candidate prion strains with the listed proteins previously transiently overexpressed. The rows represent different inheritance patterns, where the first row, 4:0, indicates that all four of the spores had significantly better growth ($p<0.001$, $n=12$) when considering the growth rate. The red rows indicate dominant inheritance patterns that are not associated with genetic traits and consistent with prion inheritance.

This assay revealed that four of the candidate proteins had at least one, and often more, diploids that passed on their resistant phenotypes in a non-Medellin fashion. The representative strains for Ppm2 did not see this type of inheritance in any of the diploids' progeny. All representative strains from Pus4 were removed from this assay because of contamination.

Discussion

Biological Significance

Prions are a biological anomaly. Despite being the same proteins found inside of us, a change in their folding creates a molecule with entirely new behaviors. The neurological diseases some prions lead to, such as CJD in humans, are not widely understood and considered by some as "the scariest disease[s] for a neurologist" (Kitamoto, 2009). This study has shown that perhaps the reason prions are so frightening is the lack of understanding about them. From this work, we gain a glimpse of insight into putative yeast prions and find that they are much more complicated than their original designation as defunct or disease-causing elements. Instead, the proteins studied here facilitated a rapid phenotypic change under acute environmental stress that proved beneficial in many cases compared to cells lacking the phenotype. Although further experimentation will be required to confirm structural changes in the candidate proteins is occurring, the proteins can currently be organized into two categories.

Strongly Prion-like: Abd1, Cet1, Pus6, Trm5

Transient overexpression of Abd1, Cet1, Pus6, and Trm5 led to heritable, stable phenotypes that provided a beneficial resistance induced by stressful conditions. Displaying chaperone dependency, mitotic stability, and a non-Mendelian inheritance are all consistent with previously identified prion-like behavior (Chakrabortee *et al.*, 2016). Similar behavior to Pus4, a known yeast prion, is also strong evidence of prion formation from these proteins. Although Trm5 still needs to be tested for chaperone dependency and mitotic stability, the inheritance in diploids and the resulting spores is

strong evidence that the induced phenotypes are not genetic. Additionally, Cet1 not producing significant results during the mitotic stability screen is questionable, as the phenotype did not seem to be induced in the control outgrowth. These questions will also be retested in future work.

Not Prion-like: Ppm2

Despite having a resistance phenotype in the original and follow-up screens, the strains with previous overexpression of Ppm2 were not mitotically stable (although it faced a similar problem as Cet1, described above). The inheritance of the phenotype in a 2:2 manner, though, is the primary evidence that it is rooted in a genetic change, not from prions. Even though the strains were not tested for chaperone dependency, the inheritance is sufficient to claim that Ppm2 did not have a prion state induced in these experiments.

Overall, five of the six final candidate proteins showing prion-like behavior are large returns considering only 72 proteins were originally considered in this screen. Considering several promising proteins, such as Dus1 and Elp4, were not included in later assays, the lower bound for the frequency of prions is over 8% in this group and could be above 10%. If this proportion of prion-forming proteins is representative of other protein classes, then protein-based inheritance stands to be a hugely important and little-acknowledged aspect of trait inheritance and cellular memory. Considering that many of these proteins perform the same enzymatic activities and adopt similar structures in human cells, the implications at a multi-cellular level are profound. The majority of prions could be beneficial or necessary, with the disease-causing prions more of an exception rather than the rule—just a cellular mechanism that occasionally

malfunctions, similar to how many genes that control basic aspects of cell growth malfunction in cancer.

Future Work

Several areas of this work can be expanded to strengthen further the arguments presented here. Some candidate proteins were not tested in every assay due to various constraints but should be included in the future for completeness. Additionally, several promising candidate proteins (list…) were not tested in any assays but could also prove prion-like if tested with the protocols outlined here.

This research presents a list of high-throughput assays to identify putative prion proteins. As such, other classes of proteins beyond RNA-modifying proteins could be tested in the same way, which would increase our understanding of the prevalence and function of prions in yeast cells. Additionally, the human orthologs for the candidate proteins could be screened in yeast the same way. Moreover, microscopy can provide a visual confirmation of protein aggregation, a common prion behavior. Fluorescence microscopy of GFP-tagged versions of these proteins could be used to visualize the aggregation, or lack thereof, of these prion proteins. Other biochemical analysis can reveal more about whether the prionogenic conformations are structurally distinct from naïve protein.

Mathematical Models of Prion Infection

While traditional experimental screening methods can provide useful information about yeast prions' behavior and prevalence, building a model that could predict and explain the transmission and formation could greatly increase our

understanding of prions. Here, we present some possible mathematical models for prion assembly. The simplistic model for the probability of prion formation used earlier in this thesis is not particularly useful for understanding how prions infect a cell once an initial naïve protein transitions to its prion state. Having a mathematical model that describes the behavior of prion transmission through a cell could have a significant effect on our understanding of the underlying biology, however. As these transitions are discrete events over an interval of time, a Poisson distribution seems like a reasonable starting approximation for prion formation. For *n* naïve proteins and an infinitesimal probability *p* of transitioning to a prion:

$$P(X = x) = \frac{\lambda^x e^{-\lambda}}{x!}$$

Where lambda (λ) is the expected rate of the occurrence, usually defined as $\lambda = np$ (Hoffman, 2015). This value is not well defined for most yeast prions with a few exceptions (Lancaster *et al.*, 2010).

A major issue with this model, however, is that the Poisson distribution assumes independence of events. In other words, it has no memory or knowledge of how many events have previously taken place. This assumption does not hold for prion formation, as a single prion can cause other proteins to flip to prions. One way to account for this cascading effect is to generalize the Poisson distribution to include the system's history. Temporal point processes are just that: a class of processes that consider previous events. They are often employed to model self-exciting events, meaning the occurrence of an event increases the likelihood that future events happen. Earthquakes, disease spread, and cellular signaling are all areas that have seen an increased use of point

processes recently (Verma *et al.*, 2020; Fox, 2015). Prion infection through a cell could be a novel application of temporal point processes.

The Hawkes process (Hawkes, 1971) is a self-exciting process that has an intuitive understanding of its parameters in the context of prion spread:

$$\lambda(t|H_t) = \mu(t) + \sum_{t_i<t} g(t - t_i)$$

This model has two components, which in this context can be understood as the underlying rate of *de novo* protein formation and the infectiousness of the prion. The first term, μ(t), is the rate of proteins flipping to prions of their own accord at time *t* and is often a Poisson process. The second summation term is the rate at which a previous event at time t_i triggers an event at time *t*. If $t_i > t$, then, $g(t - t_i) = 0$, as future events do not affect current events in this model. We will consider this as the chance that an existing prion causes a naïve protein to transition. Due to the nature of this problem, the triggering function, $g(t - t_i)$, would most likely be exponential: $g(t - t_i) = \alpha e^{(t-ti)}$ (Fox, 2015).

The Hawkes process and similar models could be valuable tools in understanding prion biology at cellular and population scales. Self-exciting point processes seem to be an intuitive area model for positive feedback systems like prion assembly. Further work could be done on parameter estimation in order to construct accurate models.

Considering the implication prion biology has on our understanding of the central dogma of biology and how traits are passed on to future generations, there is a vast potential for future work of many kinds in this field. Our nascent understanding could be bolstered by genetic, computational, and biochemical approaches.

A greater understanding of yeast prions will undoubtedly also increase our understanding of the 'scariest disease,' human prions.

Appendix

Glossary

Biological replicates- biologically distinct samples measured together to capture random biological variation.

Carrying capacity- the maximum number of organisms an environment can support.

Chaperone proteins- proteins that assist in protein folding.

Centromeric plasmid- chunks of DNA that can be introduced into yeast and act as small independent chromosomes.

Cytoplasm- the material inside of a cell besides the nucleus.

Diploid- a cell that contains two sets of chromosomes, receiving one from each parent.

Epigenetic- heritable phenotypic changes that are not due to differences in DNA sequences.

Genetic variance- differences in phenotypes due to different alleles, or alternate forms of a gene.

Growth rate- the speed at which the number of cells or size of cells in a colony increases.

Haploid- a cell that contains one set of chromosomes.

Heritable- passed from parent to offspring.

Lag time- time delay between colony start and exponential growth phase.

Neurodegenerative- resulting in degeneration of the nervous system, especially in the brain.

Nonsense mutations- mutation in which one amino acid is changed with a terminating amino acid, resulting in the polypeptide to stop prematurely. Usually results in a nonfunctional protein.

Phenotype- observable characteristics of an individual.

Primary streak- the first streak of yeast on the plate, which usually contains the highest concentration of yeast cells.

Prion- alternatively folded protein with the ability to transmit their alternative shape to typically folded proteins.

Proteome- the entire set of proteins that are or could be expressed in an organism.

Self-templating- able to make copies of itself

Spore- a daughter cell resulting from when a yeast cell diploid splits. Four are usually produced from a single diploid.

Stop codon- a sequence in RNA that signals for the end of translation into proteins.

Transcriptome- the entire set of all messenger RNA (mRNA) molecules that are or could be expressed in an organism.

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