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Hunting for Prions: Propagating Putative Prion States in Budding Yeast

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ABSTRACT

Prions have been closely associated with fatal neurodegenerative diseases. Recent evidence, however, suggests that prions also represent 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, six induced prion-like states were found to be mitotically stable and infectious. We show that many of these putative prions are dominant and are dependent on chaperone proteins, which is consistent with a prion-based epigenetic mechanism. Prion-based inheritance is expanding on the central dogma of biology, contributing to the belief that prions work as an epigenetic mechanism for passing on heritable traits.

1. 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). Prions are alternate forms of cellular proteins that have the ability to induce normal, or naïve, proteins to take a new, altered state. In this sense, prions are a self-templating form of epigenetic regulation, which is 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 naive proteins, which in turn agglomerate and lead to neurodegeneration.

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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). Furthermore, these phenotypes are heritable in a non-Mendelian fashion. This allows species variation at the level of protein conformations, which is significant because it was previously believed that species 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, thousands of genes shared with humans, and hundreds of experiments can be run in parallel. These high-throughput growth assays allow for proteome-wide screens to be analyzed, reducing the time necessary to uncover unique phenotypes.

In order to increase the chance of a *de novo* appearance of a prion-like state in RNA-modifying enzymes, sixty of these proteins were individually expressed with centromeric plasmids. From these, six strains proliferated better during exposure to a chemical stressor than a control strain that did not experience any overexpression. Using growth assays, these traits were found to be mitotically stable, meaning the ability for the cells to grow better under adverse chemical conditions was transmitted across generations. In order to attribute these observed heritable growth traits to prion proteins, the strains needed to exhibit inheritance patterns consistent with that of established yeast prions. Namely, the growth states needed to be dominant in a diploid cell and be dependent on chaperone proteins. Here we show that many of the strains with heritable growth states, as established by previous experiments, have characteristics consistent with prion proteins, indicating that the growth states may be caused by prions.

2. LITERATURE REVIEW

Yeast prion research started with the identification of the [*PSI*$^+$] factor ψ^+, referred to as a non-Mendelian factor by Liebman *et al.* (1975), in some strains of *Saccharomyces cerevisiae*. It was initially found that the [*PSI*$^+$] factor increased the effectiveness of certain translational suppressors (Cox and Young, 1971), and 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 (1996). Furthermore, Cox and colleagues demonstrated that [*PSI*$^+$] ψ^+ was not related to any extrachromosomal DNA or RNA (1988). Similarly, Wickner found that [URE3] was a prionic form of the protein Ure2p, which is 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. (1994). Wickner also describes how overexpression of the naive Ure2p protein can lead to an increase in the frequency with which a yeast strain became [URE3] by up to 100-fold (1994).

In 1995, it was discovered that the chaperone heat-shock protein 104 (HSP104) plays a large role in the propagation of the [*PSI*$^{+}$] factor in yeast (Chernoff *et al.*). Although Hsp104 must be present in the cell for propagation of [*PSI*$^{+}$], overexpression of Hsp104 leads to the elimination of the [*PSI*$^{+}$] factor from the strain (Chernoff *et al.,*1995). Lindquist and colleagues supported a hypothesis that some chaperone proteins put proteins into a sort of transition state, from which they could either convert back to naive proteins or convert to prion form with some small spontaneous probability; however, chaperones are also responsible for breaking aggregates of prions into smaller seeds, allowing for propagation to other organisms or onto progeny (1996) (Figure 1). 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 and colleagues, 1996).

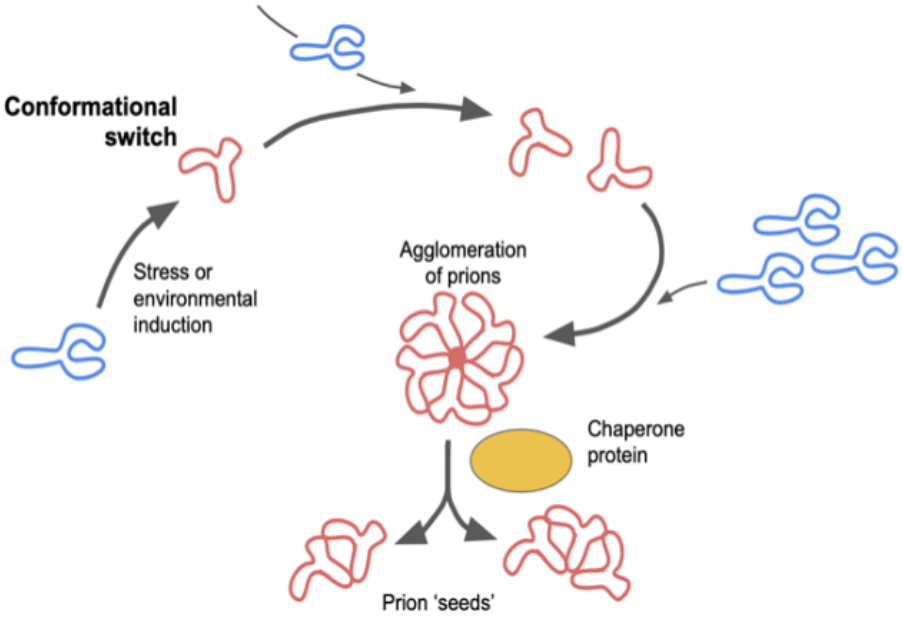

Figure 1: Model for prion formation in yeast

In addition to being chaperone-dependent, a hallmark of previously identified prion-states is their non-Mendelian inheritance (Shorter and Linquist, 2005). Since prion inheritance in yeast is not tied to chromosomal segregation, it is common to see prions passed to see most or all meiotic progeny (4:0 or 3:1 in meiotic progeny). Because of their unique inheritance mechanisms, prions also tend to be dominant in diploid cells created through genetic crosses of strains with and without the prion (Chakrabortee *et al*, 2016). Traits that arise from mutations in the genome, in contrast, are generally passed on to half of the progeny (2:2 inheritance pattern).

Prion-based information transfer has recently been found to play a much larger role in trait heritability than previously thought and provides many beneficial phenotypic changes (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.

3. RESULTS

3.1. TRANSIENT OVEREXPRESSION OF SIX RMEs CREATES BENEFICIAL HERITABLE TRAITS

To try to generate beneficial prions, we overexpressed one RNA-modifying enzyme per yeast strain and subjected the colonies to chemical stress. For a yeast colony to have a putative prion state it must have a significantly faster growth rate with protein overexpression than without ($p<0.05$). Strains were controlled to ensure that protein overexpression on its own was not a confounding variable. Of the proteins that were overexpressed, six resulted in strains that had at least a number of wells ($n\geq4$) that saw significantly faster growth than a control strain over the same number of hours. Additionally, five of those six strains, Abd1, Ppm2, Pus4, Pus6, and Trm5, had a large number of colonies ($n\geq8$). 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. See Table 1.

Table 1. RNA-modifying enzymes (RMEs) and the environmental stresses used to help induce phenotypes. Cycloheximide concentration: 0.2ug/mL; Radicicol concentration: 0.05mM.

RNA-Modifying Enzyme	Function of Protein	Environmental Stressor
Abd1	mRNA methyltransferase	Cycloheximide
Cet1	mRNA phosphatase	Cycloheximide
Ppm2	tRNA methyltransferase	Cycloheximide
Pus4	tRNA pseudouridine synthase	Radicicol
Pus6	tRNA pseudouridine synthase	Cycloheximide
Trm5	tRNA methyltransferase	Cycloheximide

3.2. SEVERAL OF THE HERITABLE PHENOTYPIC STATES ARE REGULATED BY HSP70

Prions can be differentiated from other forms of epigenetic inheritance by their reliance on protein conformation modulators. Hsp70 appears to be an extremely common chaperone protein in prion propagation in yeast (Chernoff et al., 1995; Shorter and Lindquist, 2004), as well as Hsp70 and Hsp90 (Hou et al., 2011, Brown and Lindquist, 2009; Jarosz et al., 2014b). To determine whether the phenotypic states we observed behave similarly to previously identified yeast prions, we tested if inheritance of the phenotypic states would be disturbed with transient inhibition of each of these three heat-shock proteins. Although we saw no significant changes after affecting Hsp90 and Hsp104, the three strains in which we affected Hsp70 expression, Abd1, Pus6, and Cet1, saw a virtual elimination of the phenotypic states. A dominant-negative variant of Hsp70 (Ssa1-K69M) was expressed for three outgrowths. The expression plasmid was then eliminated before growing the strains for another three outgrowths to restore the function of Hsp70. In the 11 isolates of Abd1, Pus6, and Cet1 tested, over 80% (9) of the isolates appear to have lost the phenotype entirely. We plan to test the other strains in future experiments.

3.3. 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 (Figure 2A). Strains were grown up in parallel on YPD plates and in liquid YPD for three days before being streaked out or pin replicated into fresh media. This process was repeated 10 times, with some measurements taken midway. Strains tended to keep their phenotypes longer when grown on the YPD plates over the liquid, with 60% (3) of the strains keeping at least weak phenotypes through all 10 outgrowths. Only two strains kept their phenotypes in the liquid media, and were much weaker, which we posit could be due to different community dynamics or population bottleneck, although we have not tested for either of these.

3.4. BENEFICIAL PHENOTYPIC STATES ARE DOMINANT IN GENETIC CROSSES TO NATIVE STRAINS

In the first step of determining the inheritance patterns of each of the phenotypic states, we crossed cells harboring putative prions to naive, isogenic controls to create diploids. We then tested these strains in the original stress conditions at a range of concentrations. Sensitivities varied, but the majority of the diploids exhibited strong phenotypes at twice the concentrations used for the haploids. At this double concentration, 80% (57) of the isolates grew significantly better than naive diploids (Figure 2B). Thus, the heritable phenotypes appear to be dominant in genetic crosses to naive strains and continue to be beneficial under stressful conditions.

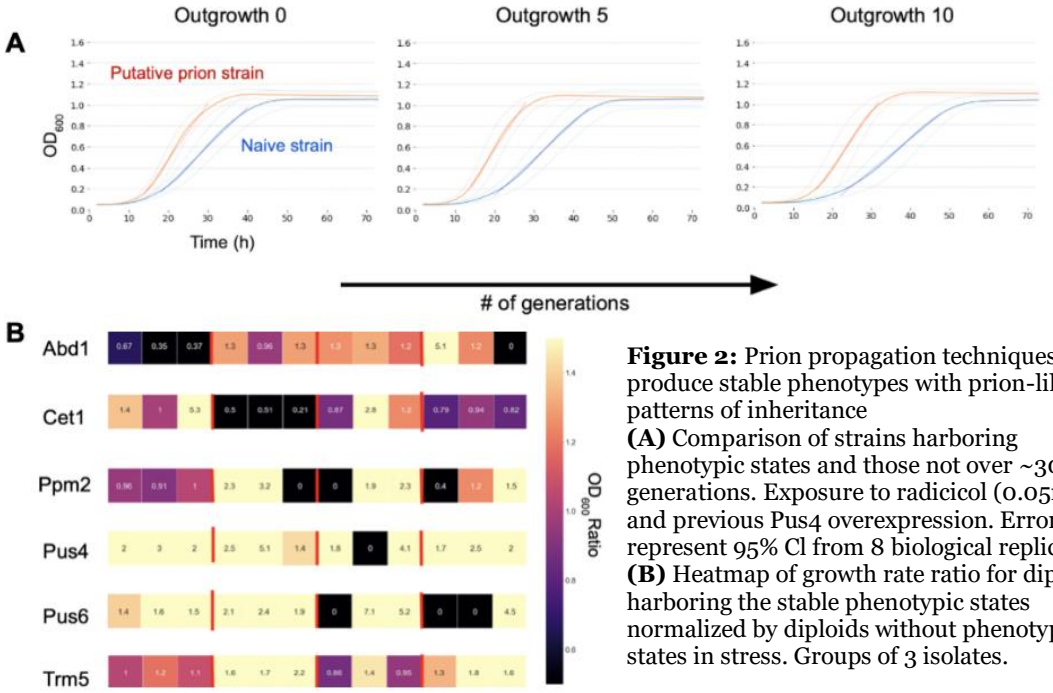

Figure 2: Prion propagation techniques produce stable phenotypes with prion-like patterns of inheritance
(A) Comparison of strains harboring phenotypic states and those not over ~300 generations. Exposure to radicicol (0.05mM) and previous Pus4 overexpression. Error bars represent 95% Cl from 8 biological replicates. **(B)** Heatmap of growth rate ratio for diploids harboring the stable phenotypic states normalized by diploids without phenotypic states in stress. Groups of 3 isolates.

4. DISCUSSION

Each of the six heritable phenotypic states, created through the transient overexpression of RNA-modifying enzymes, exhibit characteristics consistent with prion proteins. The data presented here show that the heritable phenotypic states are mitotically stable, are regulated by the chaperone protein Hsp70, and are dominant. These characteristics are in keeping with that of established prion proteins and suggest that a prion protein is present in the cell. This finding supports the idea that prions serve as an epigenetic mechanism in budding yeast to transmit heritable information. However, further experiments are necessary to attribute these six heritable phenotypic states to prion proteins. Namely, using a tetrad sporulation and dissection protocol, we will investigate the meiotic inheritance of the heritable phenotypic states to determine if they follow the non-Mendelian patterns of inheritance consistent with known prion proteins. Additionally, we will investigate the molecular mechanisms by which the putative prions listed here affect the ability of yeast cells to proliferate in adverse chemical conditions. Understanding how prions affect key biological processes can help uncover the role epigenetic mechanisms play in a cell's response to stress.

5. METHODS

5.1. 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).

5.2. QUANTITATIVE TESTS FOR *DE NOVO* APPEARANCE OF BENEFICIAL PHENOTYPES

All yeast strains were exposed to an environmental stressor and growth rate, carrying capacity, and lag time were all measured. Strains descended from yeast that experienced overexpression of an RNA-modifying enzyme were compared against an identical strain that had been exposed to the same conditions but had not had the protein overexpressed. Growth rates were extrapolated from lines fit with the SciPy library for Python. The ratios of the growth rates between strains with overexpression and strains without overexpression to normalize was used as a qualitative measure of growth dynamics. In order to attribute the heritable growth traits seen here to prion proteins, genetic crosses and chaperone protein curing protocols were paired with growth assays to determine if these heritable growth states exhibit the same inheritance patterns seen in strains with known prion proteins.

5.3. CREATION OF DIPLOID YEAST 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.

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