[GAR$^+$]: A Novel Type of Prion Involved in Glucose Signaling and Environmental Sensing in *S. cerevisiae*

by

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Abstract

Several well-characterized fungal proteins act as prions, proteins capable of multiple conformations, each with different activities, at least one of which is self-propagating. We report a protein-based heritable element that confers resistance to glucosamine, [GAR$^+$]. Genetically it resembles other yeast prions: it appears spontaneously at a rate higher than mutations and is transmissible by non-Mendelian, cytoplasmic inheritance. However, [GAR$^+$] is in other ways profoundly different from known prions. [GAR$^+$] propagation involves Pma1, the plasma membrane protein pump, and [GAR$^+$] formation is induced by Std1, a member of the Snf3/Rgt2 glucose signaling pathway. Also, [GAR$^+$] does not appear to involve the formation of an amyloid template and the prion state represents only a fraction of the Pma1 protein in the cell, consistent with the prion form constituting a complex between Pma1 and Std1, a much lower abundance protein. [GAR$^+$] propagation is subject to a strong species barrier, as substitution of *PMA1* from other *Saccharomyces* species blocks propagation to *S. cerevisiae PMA1*. Direct competition between [gar$^-$] and [GAR$^+$] cells indicate that cells carrying [GAR$^+$] have an advantage under certain environmental conditions. [GAR$^+$] appears spontaneously in a yeast isolated from a variety of sources and can be induced by co-culturing yeast and a number of *Staphylococcus* species. Overall, [GAR$^+$] expands the conceptual framework for self-propagating protein-based elements of inheritance to include non-amyloid, potentially multicomponent systems such as transmembrane proteins and signal transducers.

Summary

Several well-characterized fungal proteins are capable of acting as prions: proteins capable of multiple self-propagating conformations, each with different activities. The different prion conformers in cells with identical genotypes exhibit multiple different phenotypes. The most thoroughly characterized phenotypes are [PSI^+], the prion form of the translation termination factor Sup35, and [URE3], the prion form of the nitrogen catabolite repressor Ure2. Both are well studied in *S. cerevisiae* but are conserved in diverse fungi, including *K. lactis* and *C. albicans*. The Sup35 and Ure2 proteins enter into an aggregated, amyloid-like conformation in the [$PRION^+$] state. Whether many proteins can form prions and whether all prions involve an amyloid-like state are points of considerable debate.

Here I present evidence that the previously unexplained non-Mendelian element, [GAR^+], is a novel type of prion that does not aggregate or form an amyloid. [GAR^+] (glucosamine resistant) was isolated in a screen for resistance to the non-metabolizable glucose analog D-(+)-glucosamine. It showed non-Mendelian inheritance patterns and could not be explained by contemporary knowledge (Ball et al., 1976; Kunz and Ball, 1977). I found that the genetic attributes of [GAR^+] overlap with those of fungal prions: it appears spontaneously at a high frequency (~5 in 10^4 cells), and segregates in a non-Mendelian 4 [GAR^+] to 0 [gar^-] pattern following meiosis. [GAR^+] can be inherited by cytoplasmic transfer without nuclear exchange (cytoduction). Also, [GAR^+] can be converted to [gar^-] by altering levels of molecular chaperones (i.e. “cured”).

I found that [GAR^+] results from the association of Pma1, the plasma membrane proton pump, and Std1, a member of the Snf3/Rgt2 glucose signaling pathway. Transient

overexpression of *STD1* strongly induces [GAR$^+$] but *STD1* was not required for [GAR$^+$] propagation. Instead, *PMA1*, and possibly *STD1*, are involved in [GAR$^+$] propagation. [GAR$^+$] thus presents a marked distinction from known prions in having separable induction and propagation elements. [GAR$^+$] further differs from other yeast prions in that it does not cause aggregation of the prion determining protein. Instead, Pma1 is still located at the plasma membrane in [GAR$^+$] but associates with Std1 rather than its ortholog, Mth1, which Pma1 associates with in the [gar$^-$] state. Mutations in phosphorylation sites in Pma1 alter the frequency of [GAR$^+$] within a population and show defects in signaling down the Snf3/Rgt2 pathway, suggesting that Pma1 is involved in glucose signaling.

Finally, I address the question of whether [GAR$^+$] has any role in wild yeasts. I found that the average rate of appearance of [GAR$^+$] is 20-fold higher in strains isolated from fruit than in clinical isolates. Using quantitative trait locus (QTL) analysis, I found that the rate of [GAR$^+$] is influenced by regions on chromosome VIII and chromosome XIV. [GAR$^+$] has an increased growth rate compared to [gar$^-$] when grown in a mixture of glucose and other carbon sources, suggesting that it might have a competitive advantage under particular conditions as well. Thus, I conclude that [GAR$^+$] is a protein-based heritable element that can be induced in non-lab yeast and might confer a competitive advantage on its host.

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Chapter One:

Introduction

Inheritance of biological information across generations is essential for life. The primary example is the temporal heritability of nucleic acids, which carry the information necessary for the production of a new organism. Information can also be passed spatially, such as signaling between types of tissue in a multicellular organism and in the quorum sensing phenomenon in bacteria and fungi. However, this type of information is not generally heritable.

Cross-generational inheritance can be either Mendelian or non-Mendelian. Mendelian inheritance involves the chromosomal-based inheritance patterns described by Mendel's theories of segregation. It is the predominant mechanism for information transfer between generations and is essential for biological replication. "Mendelian" was historically used to describe only inheritance patterns that follow the simple phenotypic segregation predictions of Mendel's monoallelic traits but was eventually expanded, through the work of Bateson and Punnett, to include multigenic, chromosomal traits. Chromosomal traits are largely encoded by the sequence of nucleic acid basepairs.

Epigenetic inheritance is caused by chemical changes to the nucleic acids that do not modify the sequence, such as DNA methylation, that changes the way information is used but not the basic information recorded. This can occur on a cellular/organismal level or a generational level. Cellular differentiation, which prevents cells from returning to a pluripotent state, is the result of epigenetic modification that inactivates factors involved in pluripotency (Reik et al., 2001). On a generational level, parental imprinting can alter the expression of particular regions of chromosomes depending on whether the

chromosome originated in sperm or egg (Nafee et al., 2008). Imprinting can also affect the penetrance of alleles. For example, a particular allele in sheep gives rise to its associated phenotype only when the allele is paternally inherited (Cockett et al., 1996).

Non-Mendelian inheritance refers to traits whose segregation patterns do not follow those of chromosomes. The most salient examples are extranuclear and infectious. Following the reemergence of Mendelism in the late 19th century, a number of investigations tested inheritance patterns of easily observable plant phenotypes; some of these led to the accidental observation of extranuclear traits. Carl Correns and Erwin Baur, who simultaneously studied leaf color variegation in different plants, showed that *Mirabilis jalapa* and *Pelargonium zonale* leaf color violates Mendel's laws; this was the first cytoplasmically inherited trait described. The *Mirabilis* leaf color variegation trait was eventually identified as a chloroplast mutation by Ruth Sager (Goldschmidt, 1950). In addition to organelle-based traits, virus- and plasmid-based inheritance was eventually categorized as extranuclear (infectious) and non-Mendelian. This allowed the term "non-Mendelian" to cover an extremely wide variety of mechanisms of inheritance.

Scrapie, Prions, and the "slow virus"

The work on the "transforming principle," first Frederick Griffith (1928) and then Avery, McCarty, and MacLeod (1944) established that DNA and not protein is the heritable macromolecule. Therefore, explanations for a group of slow-acting central nervous system diseases, now known to be caused by protein, initially centered around the "slow virus" hypothesis (Sigurdsson, 1954). These included scrapie, a disease of sheep and goat; kuru, which infected the Fore tribe in New Guinea; and human prion diseases such

as Creutzfeldt-Jacob (CJD, which can be either familial or sporadic). Connections between scrapie and CJD and kuru and scrapie were suggested based on infectivity and the pathology of the diseases (Hadlow, 1959; Klatzo et al., 1959).

Early studies of scrapie showed that it was caused by a biological factor that was capable of reproducing itself. An infectious agent found in brain homogenate could still cause disease following seven serial-dilution passages through sheep, which the authors calculated must have diluted the original by agent 10^{-18} (Stamp et al., 1959). However, the infectious agent was still active after eight hours at 100°C, treatment with acetylethyleneimide, treatment with formalin (Stamp et al., 1959), or exposure to UV radiation (Alper et al., 1967), all of which had been shown to inactivate viruses. Proposed explanations included self-replicating polysaccharides (Field, 1966), a "true" virus combined with a heat-resistant agent (Stamp et al., 1959), a completely novel macromolecule (Alper et al., 1967), or a protein (Pattison and Jones, 1967).

Another unusual aspect of diseases such as scrapie and CJD is that patients did not mount an immune response, as would be expected in a viral infection (Prusiner, 1998). This lack of immune response was one reason why investigators hypothesized that these diseases might be caused by a non-viral agent. However, the conceptual framework for an infectious element that did not involve nucleic acid did not exist in the 1950s.

Initial resistance to the idea that scrapie might be caused by a protein-based agent was based on two factors: DNA was known to be the hereditary material and a mechanism for self-replicated proteins was not known. There was, as described by J.W. Griffith, "fear that the existence of a protein agent would cause the whole theoretical

structure of molecular biology to come tumbling down." Griffith then proposed several mechanisms by which a protein could cause a heritable phenotype. One of these depended on contemporary ignorance concerning the function of the adaptive immune system. The other two, though, are quite impressive. One involved the scrapie-causing protein acting as an inducer for the gene that encodes it. The other is a kinetic model in which the scrapie agent is a multi-subunit oligomer for which dimer formation is highly energetically unfavorable but addition of further subunits is very energetically favorable (Griffith, 1967).

Contemporary proponents of the Central Dogma, including Francis Crick, argued that Griffith's models violated the Central Dogma (Crick, 1970). However, the contemporary discovery of reverse transcriptase established precedent for expanding the Central Dogma, albeit only for nucleic acids. The protein agent model received a further boost when work from Stanley Prusiner's lab demonstrated partial purification of the infectious scrapie agent. The agent was termed "prion" for "proteinaceous infectious particle," and no detectable nucleic acids were found in the purification (Prusiner et al., 1981). A nearly homogenous purification (Bolton et al., 1982) identified one particular protein, which was named PrP, for prion protein.

The discovery that PrP was a normal protein in the mammalian brain provided a stunning change in the prion concept (Prusiner, 1998). It was no longer necessary to postulate that an exogenous protein had replicative capability. Rather, the infectious agent merely had to change the conformation of a protein that was already present in or to replicate infectivity. Indeed, PrP protein present in normal brain is rich in α-helices and the form found in infectious material is β-sheet rich (Harris and True, 2006).

PrP, is a small (~27kDa) GPI-linked plasma membrane protein. Initial evidence in favor of PrP being the scrapie agent was mostly correlative. The PrP proteins of patients with familial forms of prion disease carry mutations that are genetically linked to the disease (Hsiao et al., 1989; Gabizon et al., 1993). Mice in which the PrP gene has been knocked out do not acquire scrapie when infected with scrapie brain homogenate (Weissmann et al., 1994) and mice overexpressing PrP show higher rates of spontaneous scrapie disease (Westaway et al., 1994). The transmissible disease-causing scrapie form of PrP is termed "PrP^{Sc}," named after scrapie disease; the non-infectious form of PrP is termed "PrP^{C}," for the normal "cellular" form.

A few scientists still object to the protein-only theory of scrapie. One argument is that trace amounts of nucleic acids could remain in PrP purifications (Manuelidis et al., 1995). Some propose that PrP could be a receptor for a virus that causes scrapie, which would explain why homozygous PrP null mice are resistant to scrapie (Chesebro and Caughey, 1993). The best way to resolve this debate would be to induce scrapie infectivity following injection of a purified, recombinant, scrapie conformer of PrP into wildtype animals. Researchers have come close, but the acid test has yet to be fully realized. Recombinant PrP in a β-sheet rich conformation was injected into mice and caused disease. However, this has only been seen with very large innocula and in mice overexpressing PrP at levels just below those which cause spontaneous disease (Legname et al., 2004). Thus it has been argued that this injection only hastens the natural disease process. Among the most convincing pieces of evidence in favor of the protein-only hypothesis is that PrP^{Sc} can convert large amounts of PrP^{C} to PrP^{Sc} *in vitro* when PrP^{C} is in extreme excess. The newly converted PrP^{Sc} was serially diluted and the conversion of

PrP^{C} to PrP^{Sc} continued, even after the starting material was so dilute that it could no longer be detected (>10^{3}-fold dilution) (Saborio et al., 2001). Indeed, *in vitro* conversion of PrP^{C} to PrP^{Sc} can be detected even after the starting PrP^{Sc} material has been diluted up to 10^{-55}. Furthermore, *in vitro* converted PrP^{Sc} material has been shown to be infectious in a mouse model after 10^{-20} dilution of the starting material (Castilla et al., 2005). This provides powerful evidence that protein conformational conversion alone is sufficient for prion disease.

Non-Mendelian inheritance and fungal prions

Protein-based elements with genetic properties that are normally reserved for nucleic acids ("prions") are also found in fungi, where they are both infectious and heritable. In contrast, mammalian prions are only infectious. (Uptain and Lindquist, 2002; Chien et al., 2004; Wickner et al., 2004; Shorter and Lindquist, 2005). The first genetic elements later realized to be prions found in *S. cerevisiae* were [URE3] (Lacroute, 1971) (caused by the protein Ure2 (Wickner, 1994)) and [PSI^{+}] (Cox, 1965) (caused by the protein Sup35 (Chernoff et al., 1993; Ter-Avanesyan et al., 1993; Doel et al., 1994; Ter-Avanesyan et al., 1994; Patino et al., 1996)). Another prion, [Het-s], was genetically characterized in the filamentous fungus in *Podospora anserina* (Rizet, 1952) and is now known to be caused by the protein Het-s (Coustou et al., 1997). Later prions identified include [RNQ^{+}] (also called [PIN^{+}]) (Derkatch et al., 2000; Sondheimer and Lindquist, 2000; Derkatch et al., 2001) and [SWI^{+}] (Du et al., 2008) in *S. cerevisiae* and [Cin] in *S. pombe* (Collin et al., 2004).

Fungal prions also exhibit diverse phenotypes. [URE3] is involved in the uptake of nitrogen sources (Lacroute, 1971) and [PSI^+] showed increased read-through at stop codons (Cox, 1965). [Het-s] causes heterokaryon incompatibility, in which hyphae from genetically diverse *P. anserina* mycelia die after cell-cell fusion (Rizet, 1952). However, all the fungal prions had distinct and similar genetic attributes.

Early work on fungal prions described their non-Mendelian patterns of inheritance. Naturally this led to comparisons with known mechanisms of infectious or non-Mendelian inheritance, such as fungal viruses (mycoviruses) and organellelar traits. These prions, however, showed their own distinct inheritance patterns (figure 1.1) that did not match the genetic characteristics of any of the other mechanisms of non-Mendelian inheritance (table 1.1). Eventually Reed Wickner postulated that that [URE3], and by extension [PSI^+], were analogous to mammalian prions in that they were caused by a protein-based heritable element (Wickner, 1994). Still, because of study of fungal prions began with non-Mendelian inheritance and progressed to protein-based heritable elements (prions), the former will be discussed first.

Non-Mendelian Inheritance in S. cerevisiae

Prions are one of many mechanisms of non-Mendelian inheritance in *S.* cerevisiae. These include mitochondrial traits, dsRNA viruses, ssRNA viruses, plasmids, retroviral transposons, and retro-transposing mitochondrial introns. Most of these modes of inheritance are infectious, meaning that cytoplasmic transfer is sufficient for inheritance. The genetic characteristics of these non-Mendelian mechanisms overlap to some extent with those of yeast prions. Distinguishing attributes include *de novo*

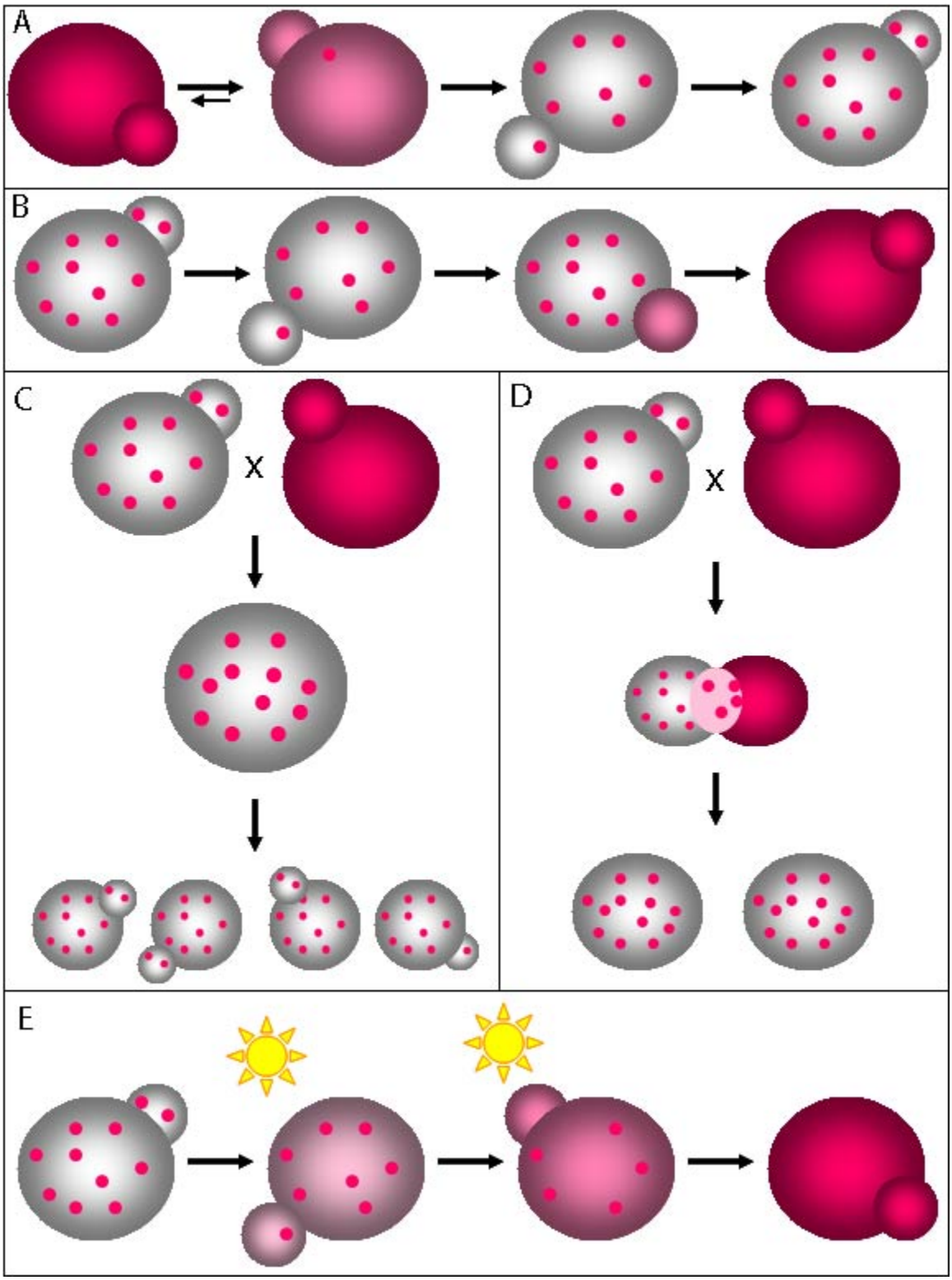
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Figure 1.1: Genetic attributes of yeast prions based on self-propagating amyloid templates a) Yeast prions appear and disappear spontaneously ("metastable"). Molecules of protein occasionally assemble into self-templating aggregated conformations. These can then be passed from mother to daughter during mitosis. The [PRION$^+$] element is stably inherited because the daughter cell's protein is efficiently templated by aggregates inherited from its mother. b) Aggregates are occasionally not passed to daughters, resulting in heritable loss of the prion. c) Prions are dominant and inherited in a non-Mendelian manner. When an aggregate-carrying [PRION$^+$] cell is mated to a [prion$^-$] cell, the resultant diploid contains heritable [PRION$^+$] aggregates that can seed [prion$^-$] protein into the [PRION$^+$] conformation. The [PRION$^+$] element is therefore dominant. [PRION$^+$] aggregates are passed to daughter cells during meiosis, resulting in non-Mendelian 4 [PRION$^+$] : 0 [prion$^-$] segregation. d) Prions are passed to mating partners by cytoplasmic mixing. When a mating is performed between a [PRION$^+$] and a [prion$^-$] cell, one of which carries a *kar1* mutation, cells fuse and the cytoplasm mixes but nuclei do not fuse and nuclear material is not exchanged. Instead the binucleate cell produces a monokaryotic bud, which contains mixed cytoplasm but only one parental nucleus ("cytoductant") (Conde and Fink, 1976). The [PRION$^+$] element is transmitted in these "cytoduced" cells that contain cytoplasm from the [PRION$^+$] parent but a nucleus from the [prion$^-$] parent. Prion elements can therefore be inherited independently of the nucleus. e) Changes in levels of chaperones, which are proteins involved in the folding and unfolding of other proteins, can prevent [PRION$^+$] aggregate inheritance. When chaperones levels increase, such as in response to heat or chemical stress, protein aggregates are no longer efficiently passed down to daughter cells. This converts cells from [PRION$^+$] to [prion$^-$]. Chaperones can block aggregate inheritance by changing prion folds or other mechanisms. Chaperones can also promote prion function by shearing aggregates. In this case loss of shearing results in loss of the prion.

acquisition rates, whether these traits show chaperone-dependent reversible curing, and whether the phenotype of interest can be induced by protein-only transformation (table 1.1).

Chaperones are proteins that assist in the folding of other proteins. Perturbations in chaperone levels can "cure" prions by preventing the inheritance of [PRION^{+}] protein aggregates by daughter cells. This curing and reappearance of a phenotype is specific to fungal prions and is an important feature in distinguishing prions from viruses (Shorter and Lindquist, 2005) and other forms of infectious inheritance.

The [PRION^{+}] form of a protein is induced by transient overexpression of the prion-determining protein. This attribute was fundamental to Wickner's original argument that [URE3] represented an altered state of the Ure2 protein (Wickner, 1994). The particularly surprising aspect of this prion attribute is that a temporary change in protein levels is sufficient to induce a permanent change in the cell's phenotype (Uptain and Lindquist, 2002; Wickner et al., 2004). This ability is not observed in any other mechanism of non-Mendelian inheritance. Furthermore, it has been successfully exploited many times to aid in the identification of new prions (Derkatch et al., 2001) and prion-determining proteins (Chernoff et al., 1993; Ter-Avanesyan et al., 1993).

A defining attribute of prions is protein-only transformation. As prions are heritable proteins, the ultimate proof of protein-based inheritance is inducing a prion

	spontaneous, frequent appearance	dominant	non-Mendelian inheritance	infectious (cytoplasmic) inheritance	chaperone-dependent curing	reversible chaperone-dependent curing
Prion	+	+	+	+	+	+
organelle trait	+	sometimes	+	+	-	-
mycovirus	-	+	+	+	-*	-
plasmid	-	+	+	+	-	-
transposon	+	sometimes	-	-	-	-
retro-transposing intron	+	sometimes	-	-	-	-

*correct folding of the KIL+ toxin requires ER lumen chaperones

Table 1.1: Non-Mendelian inheritance in yeast

phenotype by transformation of prion conformers produced *in vitro*; this was accomplished in 2002 for [Het-s] and in 2004 for [PSI^+] (Maddelein et al., 2002; King and Diaz-Avalos, 2004; Tanaka et al., 2004). As the Het-s and Sup35 protein had been purified from *E. coli* and the prion-inducing fibers created *in vitro*, these data firmly establish that protein fibers are sufficient to induce a prion. This method has since been used to indisputably demonstrate protein-only inheritance of [URE2] (Brachmann et al., 2005), and [RNQ^+] (Patel and Liebman, 2007). Protein-only transformation is always sufficient to demonstrate that a phenotype is based on a prion. The transformation procedure necessitates that the infectious protein conformation can be produced *in vitro*. However, this technique only works if the prion-determining protein can be purified from *E. coli* and if it can be converted into the stable prion conformation *in vitro*. Because it is a relatively new technique and was not used historically to distinguish prions from other mechanisms of infectious inheritance and because it is virtually impossible to perform if the prion conformation of the protein cannot be stably induced *in vitro*, protein transformation is not discussed in detail in the following section.

Yeast prions and organellar traits have a number of genetic attributes in common. Yeast has long been used as a model organism to study organelle inheritance, which was the first mechanism of extranuclear non-Mendelian inheritance discovered (Goldschmidt, 1950). The first mitochondrial mutation, *petite* (ρ^0), was isolated in *S. cerevisiae* (Ephrussi et al., 1949). However, the first mitochondrial mutations to be identified as such were isolated in *Neurospora* (Mitchell and Mitchell, 1952). Organelle inheritance violates Mendel's laws of segregation and independent assortment because organelles are passed on to all progeny, either bi- or uni-parentally, following meiosis.

Mitochondrial inheritance

Yeast mitochondria are inherited cytoplasmically in a polarized, actin-dependent manner that is linked to cell cycle control (Boldogh et al., 2001). Unlike mammalian mitochondrial inheritance, yeast mitochondria show biparental inheritance. Indeed, the diploid colony (i.e. several generations downstream of the mating event) formed by mating two haploids is highly heterogeneous: markers from both parental cells are present in the colony and both or just one are present in individual cells. Within 20 generations each diploid cell will contain only a single mitochondrial marker (Dujon et al., 1974). There is some stochasticity in the process, as either marker could be present in the end cell tested, but the yeast cells eventually become mitochondrially "pure."

Mitochondrial traits superficially resemble yeast prions in that they show 4:0 segregation and infectious inheritance. Furthermore, the heterogeneity typical of mitochondrial crosses superficially resembles chaperone-dependent curing of prions in that the phenotype of interest is lost over the course of generations. However, "curing" of mitochondrial traits is not reversible, as the chaperone-mediated curing of yeast prions is, nor does it depend predominantly on chaperones, the protein folding machinery of the cell. Also, once a mitochondrial trait has reached purity, it is no longer be curable. The distinguishing characteristics between yeast prions and mitochondrial traits is thus reversible curing.

Mycoviruses

It is also important to compare mycoviruses to fungal prions because the inheritance patterns of the two are quite similar and can be somewhat difficult to

distinguish. *S. cerevisiae* contains both dsRNA and ssRNA viruses. dsRNA viruses in yeast belong to two major families, L-A and L-BC, or a subfamily, M. L-A and L-BC each contain two ORFs, one for a coat protein (Gag) and one for an RNA polymerase (Pol). L-A and L-BC are sufficiently widespread that a majority of wild yeast isolates contain a member of one or both families (Wickner, 1996a). These viruses are spread horizontally by cell-cell fusion (primarily by mating but sometimes by heterokaryon formation) and vertically by mitosis and meiosis; they show 4:0 meiotic segregation. Yeast viruses do not have a known mechanism of extracellular spread (i.e. cell lysis), presumably due to the difficulty of crossing the yeast cell wall. They are therefore occasionally referred to as "virus-like particles" (VLPs) for historical reasons (Schmitt and Breinig, 2006).

L-A family of viruses are frequently found in conjunction with a member of the smaller M family of dsRNA viruses, which together cause a "killer" phenotype. Yeast that are KIL^+ secrete a peptide toxic to kil^- yeast. The M family has three known members, M_1, M_2, and M_{28}. Each contains a single open reading frame encoding a preprotoxin (pptox; K1, K2, and K28, respectively). M viruses confer both the ability to produce the killer toxin and immunity to it. As M viruses contain neither *gag* nor *pol* genes, they require the presence of L-A viruses to replicate (Schmitt and Breinig, 2006). The presence of particular chromosomal alleles is also necessary for the maintenance of dsRNA virus (Wickner, 1980). Therefore, the phenotype exhibited by these viruses, KIL^+, requires at least two viruses and one chromosomal genetic element for exhibition. The complexity of the KIL^+ phenotype has resulted in occasional speculation that it

might on some level involve a heritable protein element (Uptain and Lindquist, 2002). For this reason KIL^+ is discussed in depth here.

The "killer" phenotype requires the synthesis and secretion of one of the three toxic peptides coded by the M viruses. These peptides consist of five regions and an HDEL-retention sequence (Schmitt and Breinig, 2006). These regions, N-terminius to C-terminus, are the secretion signal ("pre"), a middle region ("pro"), α, γ, and β. Through interaction with cytosolic and luminal chaperones and protein modification enzymes, the toxin precursor protein is processed in the ER and golgi, then secreted into the culture medium (Martinac et al., 1990).

Because the processing of killer toxin involves chaperones, KIL^+ shows inheritance patterns similar to yeast prions. However, since killer toxin is secreted, it can be assayed for in the yeast growth medium. One can therefore distinguish between yeast prions and KIL^+ by testing whether phenotypes of interest can be conveyed independently of cells.

S. cerevisiae also contains ssRNA viruses (genus: narnavirus (Van Regenmortel et al., 2000)) that are spread by cytoplasmic exchange (mating) and show 4:0 non-Mendelian segregation. 20S (Kadowaki and Halvorson, 1971), 23S ssRNAs, and replicative intermediates W and T have so far been characterized (Wickner, 1996b). 20S and 23S ssRNA code only for an RNA polymerase and lack capsid proteins. Without capsid proteins, 20S ssRNA (and presumably 23S ssRNA) is protected from degregation by interaction with its RNA pol proteins (Fujimura and Esteban, 2007). 20S and 23S are highly contagious and found in most yeast laboratory strains (Wickner, 1996b) but their phenotypic consequences have not been well characterized. Neither 20S nor 23S

narnaviruses have been shown to be curable (Solorzano et al., 2000). Therefore, although ssRNA viruses show non-Mendelian segregation, they would not resemble prions because they would not be susceptible to chaperones, would not be induced by transient overexpression of causal genes, and could not be transmitted by transformation with protein.

Overall, the genetic characteristics of fungal viruses very closely resemble those of fungal prions. Segregation is 4:0 following meiosis and phenotypes are inherited independently of the nucleus, as shown by cytoduction. Some phenotypes caused by viruses, such as KIL^+, are sensitive to chaperones. However, KIL^+ and viruses in general do not reappear in yeast cells from which they have been "cured" due to chaperone effects (reversible curing).

Plasmids

Inheritance of plasmids and other "selfish" genetic elements is by definition extrachromosomal and shows non-Mendelian segregation (Beggs, 1978). Nonetheless, plasmids are not technically infectious, as they do not show efficient cytoplasmic inheritance (cytoduction). 2μ plasmids are found in the majority of yeast strains but they are intra-nuclear. Mutations that alter chromosome segregation affect plasmid partitioning and plasmids missegregate in tandem with chromosomes (Velmurugan et al., 2000). More recent work showed that the cohesin complex, which is involved in sister chromatid segregation and is necessary for timely and proper segregation of chromosomes, is also found on 2μ plasmids (Mehta et al., 2002). A 2μ plasmid-

dependent phenotype, albeit showing non-Mendelian segregation, would be "cured" by mutants that affect chromosome segregation and not by chaperones.

Mechanisms of spontaneous phenotypic acquisition: Ty elements and infectious introns

Another mechanism for the spontaneous acquisition of new phenotypes is Ty transposition. The *Saccharomyces cerevisiae* nuclear genome contains five families of retrotransposable elements (Ty1-5) that are capable of causing phenotypic change (Lesage and Todeschini, 2005). Ty elements consist of two long terminal repeats (LTR) flanking two open reading frames that code for enzymes needed for replication. Ty elements are fairly common in the yeast genome (3.1% of S288C sequence is predicted to consist of Ty elements; (Kim et al., 1998)).

There are three possible ways that Ty factor integration can induce change: altered expression from an adjacent gene; novel regulation of an adjacent gene, or genomic rearrangement due to recombination between Ty elements. When Ty1 is inserted in a 5' upstream region in the same orientation as the downstream gene it results in decreased expression from the adjacent gene because the Ty1 element replaced the regulatory elements of the gene. Ty1 has also been shown to result in novel regulation when inserted 5' and within 175bp of an ATG in the opposite orientation of a gene (Lesage and Todeschini, 2005) because it contains binding sites for a number of transposons (Gray and Fassler, 1993; Baur et al., 1997; Madhani and Fink, 1997).

Another mechanism of non-Mendelian inheritance in *S. cerevisiae* is that of self-splicing "infectious" introns. They are found predominantly in the mitochondria but can also be nuclear. Transposition can be either site-specific (group I) (Lambowitz and

Belfort, 1993) or random (group II) (Mueller et al., 1993). Intron insertion can change the phenotype of a cell by either altering expression from a gene of by genomic rearrangements via recombination between intron copies (Mueller et al., 1993).

Overall, retrotransposable elements and infectious introns do not greatly resemble yeast prions genetically. They phenomena are similar to prions, however, in that they allow for spontaneous acquisition of new phenotypes. These novel phenotypes tend to appear at rates similar to genetic mutation but sometimes appear at higher rates (Taguchi et al., 1984). However, once the Ty- or intron-induced changes are inserted into the genome, the resultant phenotypes show Mendelian segregation. Therefore, they easily be differentiated from fungal prions by analysis of segregation patterns.

Fungal prions

Prion-dependent phenotypes in fungi were identified in a manner similar to mammalian prions: researchers found phenotypes that could not be explained by contemporary knowledge of molecular biology. The earliest of these was [Het-s], a cytoplasmic element in *P. anserina* involved in mating-type incompatibility (Rizet, 1952). Two later-identified, albeit better-studied, phenomena in *Saccharomyces cerevisiae*, [URE3] (involved in uptake of nitrogen sources) (Lacroute, 1971) and [PSI^+] (which showed stop codon read-through) (Cox, 1965) were described as being extrachromosomal, inherited by infectious cytoplasmic exchange (cytoduction), non-viral, non-mitochondrial, and not due to a known plasmid (Tuite et al., 1982). All of these early prions were defined by their genetic characteristics (see figure 1.1), which are unique to prions (Wickner, 1994).

In 1994, Reed Wickner suggested that [URE3], and by extension [PSI^{+}], represent an *S. cerevisiae* phenomenon similar to mammalian prions: that a phenotype was caused by an alternative conformation of a protein (Wickner, 1994). There was, however, one key difference: fungal prions are heritable and infectious, whereas mammalian prions are only infectious. The heritability of the fungal prion phenotype allowed for the elucidation of a distinct set of genetic characteristics, outlined by Wickner, that formed the basis for identifying additional fungal prions (figure 1.1) (Derkatch et al., 2000; Sondheimer and Lindquist, 2000; Derkatch et al., 2001; Collin et al., 2004; Du et al., 2008).

[URE3] showed non-Mendelian segregation and cytoplasmic inheritance (Lacroute, 1971), attributes shared by mycoviruses, the non-Mendelian elements that most closely resemble prions (table 1.1). However, what surprised Wickner was that the gene *URE2* was required for [URE3] maintenance but that a *ure2* knockout mutant, Δ*ure2*, exhibited the same phenotype as [URE3] (Aigle and Lacroute, 1975; Wickner, 1994). This was shocking; Wickner pointed out that one could not expect a heritable element to have the same phenotype as a loss-of-function mutation in a gene that is required for the maintenance of that heritable element.

With mycoviruses, the knockout phenotypes of genes required for production of L-A and/or M viruses (e.g. *TOP1*, *MAK3*, *MAK10*) are different from the phenotype caused by the element they control, the viruses themselves. The knockout phenotypes of Δ*top1*, Δ*mak3*, and Δ*mak10* include sensitivity to DNA-damaging agents, elongated telomeres, and a growth defect on non-fermentable carbon sources, respectively. The

viral phenotype is the ability to kill yeast cells that do not carry L-A and M viruses ("killer" phenotype) (Wickner, 1996a).

Wickner's second observation that [URE3] is inconsistent with a mycovirus was that transient overexpression of the *URE2* gene product increases the rate of appearance of the [URE3] prion. Fungal viruses have no known mechanism of extracellular spread, so in contrast to mammalian prions, the argument cannot be made that Ure2 represents a receptor for the [URE3] virus. However, if [URE3] was an altered, prion, form of Ure2, an increase in the amount of Ure2 protein by overexpression of the gene product would increase the probability of the protein entering the [URE3] form (Wickner, 1994). It was also intriguing that the overexpression phenotype of *URE2* was the same as [URE3] and, as mentioned previously, Δ*ure2* (Aigle and Lacroute, 1975). None of these would be expected for virus-based heritable elements (Wickner, 1996a, b).

The final puzzling attribute of [URE3] was that it showed reversible curing. Treatment with guanidinium hydrochloride (guanidine or GdHCl) (Wickner, 1994), converts [URE3] to [ure3] with almost 100% efficiency. Wickner was readily able to reisolate [URE3] derivatives from cured cells. The reappearance of [URE3] following curing implies that the [URE3] "replicon" is not removed during curing. When mycoviruses are cured, however, the virus phenotype cannot spontaneously reappear in the cured strain. The combination of curing data with data linking the *URE2* gene product with propagation and induction of [URE3] suggest that [URE3] is an alternative state of the Ure2 protein (Cox, 1994b; Wickner, 1994).

Wickner's original yeast prion paper is very interesting and important for what it did argue but it is also worth noting what it did not argue. Primarily, Wickner suggested

that [URE3] might be the result of a conformational changes but he was not adamant about a precise mechanism. The definition and the argument were entirely genetic and allow for a wide range of physical models. Possibilities include a purely conformational difference between [prion$^-$] and [PRION$^+$] macromolecules, self-propagating covalent modification such as glycosylation or phosphorylation causing an "altered" state that creates a new phenotype but also causes the proteins to perpetuate the modification, auto-activating enzymes, or self-maintaining signaling cascades. Contemporary reviews focused on conformational change or auto-activating enzymes as mechanisms (Cox, 1994a; Wickner et al., 1995). Notably, the mammalian prion field also did not initially make any claim about conformation, modification, structure, etc., and focused solely on whether the heritable agent of the infectious scrapie/CJD/kuru pathogen is proteinaceous. In any case, proteins in multiple kingdoms of life can perform functions traditionally thought to be reserved to nucleic acids (figure 1.1).

[PSI$^+$] and conformational change

[PSI$^+$] was identified in 1965 as an permanent, non-Mendelian enhancer of the *SUQ5* stop-codon suppressor (Cox, 1965). [PSI$^+$] was originally thought to be caused by a plasmid or some unknown nucleic acid determinant, although that was controversial (Cox et al., 1988). The plasmid theory was based on mutagenesis studies showed that [PSI$^+$] could be converted to [psi$^-$] by exposure to UV and that this conversion to [psi$^-$] could by reduced by induction of DNA repair enzymes. (Later it was shown that Hsp104, the protein conformation remodeling factor that cures [PSI$^+$], is induced by many stresses, including UV (Sanchez et al., 1992)). The kinetics were similar to those of

mitochondrial mutations, implying a nucleic acid determinant (Tuite and Cox, 1980). Claims were made that transformation of DNA from a [PSI$^+$] strain was capable of converting [psi$^-$] and that this activity co-purified with a 3μ circle (Dai et al., 1986), but this was not reproduced. (Indeed, since that time it has been shown that aging yeast cells characteristically generate episomes of ribosomal DNA and this was likely the origin of the circles (Sinclair and Guarente, 1997)).

Work relating [PSI$^+$] to a change in protein folding started because the protein chaperone Hsp104 was shown to cure [PSI$^+$] to [psi$^-$] (Chernoff et al., 1995). Chaperones, which are necessary for the proper folding of proteins, had not been shown to act on nucleic acids, thus implying that [PSI$^+$] might be caused by a heritable protein fold. Wickner had presciently suggested in his seminal [URE3] paper that [PSI$^+$] might be propagated by a similar protein-based mechanism (1994).

[PSI$^+$] had been linked to the *SUP35* gene prior to Wickner's publication. Specifically, overexpression of either full-length or the N-terminus of *SUP35* increased the percentage of read-through of stop codons (Ter-Avanesyan et al., 1993) and induced [PSI$^+$] (Chernoff et al., 1993). In fact, the N-terminal region of *SUP35* is required for [PSI$^+$] maintenance (Ter-Avanesyan et al., 1994). A dominant point mutant that prevented [PSI$^+$] propagation was mapped to the N-terminal region of *SUP35* (Doel et al., 1994).

Direct support for the proteinaceous nature came when it was discovered that the Sup35 protein in the cell forms large, insoluble aggregates in the [PSI$^+$] form but remains soluble in [psi$^-$] (Patino et al., 1996; Paushkin et al., 1996). Like PrP^{Sc}, Sup35 from [PSI$^+$] cells is partially resistant to proteinase K digestion. Finally, changes to the state of

Sup35 correlated with the switch between [psi$^-$] and [PSI$^+$]. Sup35 was fused to GFP and its localization observed; in [psi$^-$], Sup35-GFP showed diffuse, cytosolic localization. In [PSI$^+$], Sup35-GFP rapidly formed aggregates (Patino et al., 1996). Curing of [PSI$^+$] to [psi$^-$] correlated with the return of Sup35 to a soluble state (Chernoff et al., 1993; Patino et al., 1996). Combined, these data suggested that [PSI$^+$], and by extension [URE3], represented an altered aggregated form of the Sup35 (and Ure2) proteins that had an infectious character: the ability to recruit newly-made protein to the same aggregated conformation.

[Het-s]

[Het-s] was the first fungal prion to be described (Rizet, 1952) but one of the more recent to be identified as a prion. This is because the [Het-s] phenotype, which causes heterokaryon incompatibility in the [PRION$^+$] form, is among the more complicated prion-based phenotypes. For starters, *Podospora anserina* carries multiple *het-s* alleles, one of which is capable of forming a prion and one of which is not. This results in three possible "genetic" states: HET-S (protein coded by allele that cannot form prion), [Het-s] (prion form of protein), and [Het-s*] (non-prion state of prion-forming allele). Second, the prion state, [Het-s], only results in a phenotype when its organism forms heterokaryons with a *Podospora* strain containing the *het-S* allele, which cannot form a prion. When a [Het-s] *Podospora* hypha fuses with a het-S hypha to form a heterokaryon, apoptosis is triggered and the heterokaryon dies.

[Het-s] fulfils the genetic criteria of prions: reversible curing, induction of the [Het-s] phenotype with overexpression of the *het-s* gene, and a *het-s* knockout strain (*het-*

s^0) cannot propagate the [Het-s] prion phenotype (Coustou et al., 1997). However, *het-*s^0 does not phenocopy [Het-s]. Het-s protein isolated from a [Het-s] colony is resistant to protease-K digestion, whereas Het-s protein from [Het-s*] colony is not protease-K resistant. The Het-s protein itself therefore shows a conformational change, very strongly suggesting a prion-based mechanism of inheritance rather than a plasmid (Coustou et al., 1997). PrP^{Sc} shows a similar protease resistance when compared to PrP^{C} (Bolton et al., 1984). With the advent of protein transformation, [Het-s] was shown to be induced by the transformation of Het-s protein fibers; [Het-s] is therefore a prion.

Mechanism of prion inheritance

All yeast prions identified to date operate by a similar mechanism but involve unrelated proteins and result in different phenotypes. In [PSI^+] and [URE3], the prion determining protein (Sup35, a translation termination factor, and Ure2, a nitrogen catabolite repressor, respectively) is soluble in the [$prion^-$] state and either cytosolic or nuclear, respectively. When in the [$PRION^+$] form, the majority of the Sup35 and Ure2 protein aggregates in the cytoplasm. This titrates away soluble protein, resulting in a [$PRION^+$] phenotype that phenocopies either a partial ([PSI^+]) or complete loss-of-function ([URE3]) phenotype of the determining protein. [PSI^+] thus results in increased readthrough at stop codons and [URE3] causes a defect in nitrogen regulation that results in uptake of poor nitrogen sources (ureidosuccinate) in the presence of good nitrogen sources (ammonia) (Shorter and Lindquist, 2005). The phenotypic consequences of [PSI^+] and [URE3] are thus very different.

[Het-s] has a different mechanism of action. Specifically, while the het-s protein is aggregated in the [Het-s] form and soluble in the non-prion, [Het-s*] form (Coustou-Linares et al., 2001), the [Het-s] phenotype does not phenocopy a loss-of-function of the *het-s* genetic locus. Loss of *het-s* has no known phenotype (Coustou et al., 1997). [Het-s] resembles PrP in this property, as the PrP^{C} protein has no known phenotype other than an inability to propagate prion disease for many years (Steele et al., 2007). These data imply that the [$PRION^{+}$] form represents a gain-of-function phenotype. Still, the het-s protein aggregates in [Het-s], and thus all the early fungal prions involved some state of heritable aggregation.

Similarities between mammalian and fungal prions: "species barrier," "strains," and amyloids

Fungal and mammalian prions have three major overlapping attributes: the species barrier phenomenon; the ability to exist in distinct, self-propagating prion strains; and a difference in protein structure associated with the [$PRION^{+}$] state. Prion strains and the species barrier were particularly puzzling for mammalian prion researchers. Viruses also involve "strains" with varying phenotypes and a species barrier to infectivity. The finding that fungal prions also exhibit a species barrier and can propagate as distinct prion strains strengthens the connection between seemingly disparate phenomenon.

Prions and amyloids

Perhaps the most important conserved characteristic between PrP and the known fungal prions is the ability of these proteins to assume very different structures in [PRION$^+$] and [prion$^-$] forms. The [PRION$^+$] form of all known fungal prion proteins is amyloid. Amyloid is an extremely stable, β-sheet rich protein fold that is also found in several late-onset neurodegenerative diseases, including Parkinson's, Alzheimer's, and Huntington's diseases (Chiti and Dobson, 2006). Amyloids do not denature in 1% SDS; cause fluorescence or birefringence of the dyes thioflavin T or Congo Red, respectively; and are often self-templating *in vitro*. It is thought that most proteins can form amyloid under some condition, based on the observation that a number of non-aggregating proteins unrelated to amyloid diseases spontaneously form amyloid *in vitro* (Guijarro et al., 1998; Litvinovich et al., 1998; Serio et al., 2000). These were later shown to be a generic property of many polypeptides under semi-denaturing conditions (Chiti et al., 1999). However, fungal prion-determining proteins are among the few genetically tractable amyloids and have been extensively studied for that reason.

Amyloid and pre-amyloid structures (fibrils, oligomers, etc.) can be heritable and infectious because the ends of amyloid fibrils self-template. This recruits protein of the non-prion form into the amyloid deposits (Shorter, 2008). The rate of fragmentation of the amyloid fibers is thought to contribute to whether an amyloid-forming protein is heritable (Tanaka et al., 2006). Fragmentation forms oligomeric seeds; if seeds do not form at a sufficient rate to be passed on to daughter cells, the amyloid state is not

inherited. Why oligomers are toxic in mammalian cells but heritable in fungi is poorly understood but a major point of investigation (Douglas et al., 2008).

Prion Strains

The potential structural diversity of prion-forming proteins results in another unusual characteristic of prions: the strain phenomenon. Different infectious lines, still presumably caused by PrP^{Sc}, resulted in different incubation times and caused spongiform pathology in different regions of the brain. Incubation times and infected brain regions were, however, stable within a line (Aguzzi et al., 2007). These differences can be propagated through serial rodent infections, so the difference in phenotype is stable and transmissible (Dickinson and Fraser, 1977). These data were initially interpreted as strong evidence against the protein-only hypothesis because "strains" are characteristic of viruses. Strains were hypothesized to result from mutations within a virus, whereas a mechanism by which a protein could accomplish such stable infectious specificity was difficult to imagine. However, it was shown that PrP^{Sc} from different prion strains showed different digestion patterns by proteinase K (Bessen and Marsh, 1994). The strain-associated differences of PrP^{Sc} transmit to and convert PrP^{sen} into the different PrP^{res} strains *in vitro* (Bessen et al., 1995).

Fungal prions also exhibit "strains" that correlate with [$PRION^{+}$] protein structure. Fungal prion strains tend to be either "strong" or "weak" version of the [$PRION^{+}$]-associated phenotype (e.g. strong [PSI^{+}] shows more read-through of stop codons than weak [PSI^{+}]). These strains are stably propagated and in a non-Mendelian manner (Derkatch et al., 1996). The amount of aggregated protein differs between

strains, which presumably is what causes differences in strength of the prion phenotype (Bradley et al., 2002). Variances between yeast prion strains appear to be entirely structural, as formation of infectious Sup35 fibers at different temperatures is sufficient to result in different strains without any difference in sequence or change other than temperature of the assembly reaction (Tanaka et al., 2004). Furthermore, the regions of the Sup35 protein forming the "core" of the prion-causing fiber differ between [PSI^{+}] strains (Krishnan and Lindquist, 2005), as do the amino acid contacts formed during the polymerization (Tessier and Lindquist, 2007). These data combine to show that, analogously to PrP, the proteins that cause fungal prions are capable of entering into several different possible prion-causing structures and that structural differences of the infectious form of the prion result in strain differences.

Overall, the prion strain phenomenon is thought to result from the inherent flexibility of the protein causal agent (Morales et al., 2007). Because the causal protein is inherently unstructured, it is capable of entering into a number of related folds. Some of these are self-propagating, which creates prions. Slight differences in the self-propagating prion fold results in different prion strains. This structural flexibility of proteins capable of forming prions implies that a large number of different strains are possible. In mammals the self-propagating strains lead to different diseases, many with different clinical symptoms. Understanding the structure of PrP^{Sc} and how it differs between species and strains is therefore an important epidemiologic question.

The species barrier prevents cross-species infectivity

The "species barrier" refers to the inefficiency of transmission of infectious prion material from one species to another. Inter-species infection occurs at much lower frequencies and requires longer incubation times than the infection within a species (Moore et al., 2005). The species barrier can be "crossed" by transmitting the disease to a different species than the originating one, but only at low frequency and with long incubation times (Zlotnik and Rennie, 1965). When the species barrier is crossed the strain in question then propagates in the new species with increased efficiency that eventually stabilizes (Kimberlin and Walker, 1977).

This species barrier was shown to be attributable to the prion protein itself. Transgenic mice carrying copies of the hamster PrP gene acquire prion disease more efficiently when infected with prion inoculum from hamsters and disease was then transmitted efficiently to hamsters (Scott et al., 1989) but not to mice (Prusiner et al., 1990). The neuropathology resembled the originating species rather than the infected species (Scott et al., 1989; Prusiner et al., 1990).

Differences in the primary sequence of PrP from different species are thought to contribute to structure differences within the PrP protein that cause the species barrier phenomenon (Morales et al., 2007). For example, the amino acid residues implicated in the mouse/hamster prion species barrier are 138 (Priola and Chesebro, 1995) and 154 (Priola et al., 2001). Replacement of one residue with the other prevents the conversion of PrP^{C} to PrP^{Sc} in a cell-based assay by PrP^{Sc} from the origin species (e.g. hamster PrP^{Sc} cannot convert hamster PrP^{c} that carries the mouse amino acid at position 138 or 154) (Priola et al., 1994).

Residue 129 in human PrP contributes to susceptibility to the infectious prion diseases vCJD and kuru. Residue 129 is either methionine (M) or valine (V) in all populations tested, with approximately 51% of the UK population being M/M homozygous (Collinge et al., 1991). Kuru patients show an over-representation of M/M homozygotes (Lee et al., 2001) and all but one of the 130+ vCJD cases are M/M homozygotes (Peden et al., 2004). Sporatic (Palmer et al., 1991) and iatrogenic (Brandel et al., 2003) CJD patient populations are enriched for homozygotes at 129 (either M/M or V/V). M/V heterozygotes appear to be protected from kuru (Lee et al., 2001; Mead et al., 2003). There has also been selection for heterozygosity at the PrP allele (Mead et al., 2003), possibly because variation within an individual's native PrP allele is thought to prevent PrP protein polymerization and thus PrP^{Sc} formation (Palmer et al., 1991). A very controversial interpretation of this data is that the selection for heterozygosity of PrP is indicative of widespread cannibalism in early human populations (Mead et al., 2003).

Fungal prions also exhibit a species barrier both *in vivo* and *in vitro* (Santoso et al., 2000). The *SUP35* genes from fungi such as *Candida albicans* and *Pichia methanolica* have been engineered into *S. cerevisiae* and can act as prions in that yeast. However, the foreign Sup35 protein does not coaggregate with *S. cerevisiae* Sup35 protein or induce *S. cerevisiae* Sup35 to form [PSI^+] (Chernoff et al., 2000; Santoso et al., 2000). Similar results were obtained with *SUP35* alleles from *sensu stricto Saccharomyces* species, which can mate with *S. cerevisiae* but do not produce viable offspring. These proteins show between 77% and 94% amino acid identity with the prion-causing region of *S. cerevisiae* Sup35. However, they still cannot induce *S. cerevisiae* Sup35 into the [PSI^+] form, despite coaggregation of the different Sup35

proteins within the same cell (Chen et al., 2007). These data suggest that alterations to the primary sequence alone are not sufficient to explain the species barrier and that environment, and thus presumably structure, plays some part.

Sup35 protein from *Candida albicans* that was converted *in vitro* into self-polymerizing fibers cannot induce fiber formation of Sup35 from *S. cerevisiae,* nor vice versa (Chien and Weissman, 2001). This fiber formation is an attribute of most prion-causing proteins and has been used extensively to study mechanisms of self-propagation, the species barrier phenomenon, and protein infectivity. A chimeric species that contains the N-terminal *S. cerevisiae* PrDs and C-terminal *C. albicans* PrD shows seeding of soluble *S. cerevisiae* Sup35 protein under some conditions but *C. albicans* Sup35 under others. The specific residues involved in the nucleation of polymerization have been mapped, differ between *C. albicans* and *S. cerevisiae*, and found to contribute to the species barrier (Tessier and Lindquist, 2007).

Are all prions amyloid?

Some fungal prion proteins, including Sup35 and Ure2, form amyloids in the prion-associated forms. Although amyloids can be prions and prions can be amyloid, an amyloid or amyloid-like fold is not required in order to be a prion. Mammalian prions were defined by a protein-based heritable element; the analogous fungal phenomenon was defined by genetic attributes. Any protein-based mechanism that fits the genetic criteria could therefore theoretically fit under the definition of "prion."

Along these lines, Roberts et al. recently proposed that a self-activating vacuolar protease, [β], can be a prion (2003). This claim has been criticized because [β] is only

auto-activating under artificial conditions. However, it represents an important proof-of-principle experiment because it is this first example in fungi of a non-amyloid self-templating protein element.

The active form of protease B (PrB), is formed when the protease B precursor protein (proPrB) is cleaved, first by protease A (PrA, coded by the *PEP4* gene) then by mature protease B (PrB or [β]). PrB is capable of self-cleavage in the absence of PrA, which results in the [β] (self-processed) form of PrB. This self-processed form can be called a prion because it is auto-activating. [β] can be cytoduced to [β-o] ([prion$^-$]) cells, and [β] can be cured by extended growth under conditions that repress proPrB transcription (Roberts and Wickner, 2003). However, PrB is only self-activating under artificial conditions of a Δ*pep4* background. Another aspect of [β] is that its self-activation requires a covalent modification rather than a change in protein conformation. The overall case in favor of [β] is thus a very nuanced argument, as the PrB/[β] phenotype is present in wildtype yeast but PrB is only the non-Mendelian element [β] in a Δ*pep4* mutant background. However, [β] does demonstrate that non-amyloid prions could exist.

Another putative prion in *Podospora anserina*, *C* (crippled growth), is proposed to result from a self-activating MAP kinase cascade (Kicka and Silar, 2004; Kicka et al., 2006). The MAP kinase changes location (cytosolic to nuclear) in concert with the appearance of the *C* phenotype and the *C* phenotype requires MAP kinase kinase and MAP kinase. Overexpression of the MAP kinase also increases the appearance of *C*. The authors have shown that C is infectious but they know little about its mechanism of inheritance (Kicka et al., 2006).

Overall, both mammalian and some fungal prions enter into an amyloid form but amyloids are not necessary and sufficient for prions. Any type of self-propagating protein structure, theoretically including signaling cascades and self-activating enzymes, or even an RNA molecule with a self-templated change in folding, could fulfill the genetic criteria for being fungal prions. Because [PSI^+] and [URE3] are so well studied, they historically provided a yardstick by which to measure putative prions. This criterion biases the discovery of new prions in favor of those such as [RNQ^+], which is a self-templating amyloid that is mechanistically similar to [PSI^+] and [URE3].

Prions and chaperones

Reversible curing is one of the key distinguishing features of prion-based phenotypes. Curability both establishes that the heritable element is not the result of a genetic alteration, which would only rarely be "cured" without mutagenesis under nonselective conditions, and demonstrates that the heritable element is not a virus, as viruses would not reappear *de novo* after curing. It was eventually determined that methods for curing prions acted through chaperones (Jones and Tuite, 2005; Shorter and Lindquist, 2005). The study of prion/chaperone interactions has provided rich insight into prion formation, phenotypic consequences, and even mechanisms of inheritance.

Molecular chaperones are proteins that assist in the proper folding, translocation, subunit assembly, and unfolding of the majority of proteins in a cell. As protein folding in a cell takes place under crowded conditions, a small amount of "misfolding" is not unusual (Luby-Phelps, 1994). Several chaperones act constitutively to prevent aggregation or unfold misfolded states. Stress conditions increase the basal misfolding

rate. Many chaperones are induced by cellular stress such as heat shock and thus are also termed "heat shock proteins" (Hsps). This induction is necessary for survival of stressful conditions. Pretreatment under moderate stress conditions (e.g. 37°C for *S. cerevisiae*, a mild heat shock) increases chaperone levels and increases survival under more severe stress (e.g. 50°C) (Sanchez and Lindquist, 1990).

Chaperones are divided into families by function and molecular weight: Hsp40s (DnaJ in prokaryotes), Hsp70s (DnaK in prokaryotes), Hsp100s, small Hsps (sHsps), Hsp90s, and Hsp60s/chaperonins (GroE in prokaryotes). Hsp40s, Hsp70s, and Hsp100s are the subclasses predominantly shown to date to be involved in prion formation and inheritance. Hsp90s have only recently been implicated in prion inheritance, and even then by an unknown and possibly indirect mechanism (Fan et al., 2007). Chaperonins have not been shown to be involved in prion formation and propagation and so will not be discussed.

Various eukaryotic chaperones have *de novo* folding activity and together have the abilities to unfold and prevent aggregation. The predominant chaperones of the eukaryotic cytosol are Hsp40s and Hsp70s. These two combined have "holding" activity to prevent aggregation (Hsp40) and ATP-dependent release mechanism that allows folding (Hsp70). Interaction between Hsp70 and substrate-bound Hsp40 transfers the unfolded substrate to Hsp70 and stimulates hydrolysis of ATP by Hsp70, which increases the affinity of Hsp70 for the substrate. Nucleotide exchange factor (NEF) binds to Hsp70, releasing ADP (Liberek et al., 1991). Hsp70 and Hsp40 can prevent protein aggregation by binding to and "holding" unfolded peptides, which keep them from

aggregating. Hsp70 acts with Hsp90 to complete folding of certain substrates following Hsp40 and NEF disassociation (Walter and Buchner, 2002).

The chaperone critical to prion inheritance is Hsp104, a member of the Hsp100/ClpB family (Shorter, 2008). Hsp104 was first identified as a protein that confers tolerance to extreme stresses (Sanchez et al., 1992; Lindquist and Kim, 1996). It increases survival by as much as 10,000 fold and does so by disaggregating aggregated proteins. This ability of Hsp104 to resolve aggregated protein requires the Hsp70 Ssa1 and the Hsp40 Ydj1 *in vitro*. In the case of prion amyloids, Hsp104 activity also increases prion protein seeds by fragmenting them, which are necessary for heritability (Shorter and Lindquist, 2004). Without Hsp104, seeds are not created and a prion is not passed on to the daughter cell. Too much Hsp104 fragments [PSI^+] seeds, destroying them and curing the prion. Hsp104 therefore cures prions by either deletion or overexpression (Jones and Tuite, 2005; Shorter, 2008).

The finding that aggregated proteins could be renatured was shocking to the chaperone community because aggregated proteins were thought to be dead (reviewed in Bösl 2006). Prior to this work, the most likely mechanisms for thermotolerance had been thought to be either prevention of aggregation (Hsp70-like) (Sanchez et al., 1993) or proteolysis of aggregated proteins (ClpB-like). Hsp104 acts particularly efficiently on amyloid forms of proteins (Shorter and Lindquist, 2006), including Sup35 in the [PSI^+] form, Ure2 in the [URE3] form, and some proteins associated with neurodegenerative diseases (Shorter and Lindquist, 2004; Vacher et al., 2005; Lo Bianco et al., 2008).

Small heat shock proteins (sHsps) are, like Hsp104, involved in rescuing proteins from aggregation. *S. cerevisiae* contains two sHsps, Hsp26 (Petko and Lindquist, 1986)

and Hsp42 (Haslbeck et al., 2004), and analogous proteins are found in bacteria. sHsps bind unfolded proteins (Cashikar et al., 2005; Haslbeck et al., 2005). sHsps are function in protein disaggregation by rendering refolding by Hsp104 more efficient (Cashikar et al., 2005). sHsps, however, apparently are not involved in prion propagation and inheritance.

The disaggregating activity of Hsp104 is critical to the formation of the prion seeds that are passed from mother to daughter cell during mitosis or meiosis (Cox et al., 2003). Deletion of *HSP104* cures all *S. cerevisiae* prions and decreases the fidelity of [Het-s] propagation. The interplay between Sup35/[PSI^+] and Hsp104 is particularly tight, since [PSI^+] is "cured" to [psi^-] by either overexpression or deletion of Hsp104. Hsp104 aids prion propagation by promoting formation of critical prion oligomers. It does this by creating [PSI^+] "seeds" via interaction with prion protein fibers and fragmenting seeds into heritable oligomers (propagons) (Shorter and Lindquist, 2004). These propagons are passed from mother to daughter in a cytoskeleton-dependent fashion (Ganusova et al., 2006). A daughter cell that does not inherit a sufficient number of propagons becomes [$prion^-$]. Inhibiting the activity of Hsp104 decreases the number of propagons and thus cures [PSI^+] (Ness et al., 2002; Kryndushkin et al., 2003). Instead, the prion-determining protein forms extremely large cytoplasmic aggregates that are not passed on to the daughter. Overexpression of Hsp104 fragments amyloid fibers formed by Sup35, possibly fragmenting them into oligomers (or monomers) smaller than the minimum propagon size. This would cure because few, if any, seeds would be in existence to be passed down to the daughter cell (Cox et al., 2003).

Hsp70s and Hsp40s are also involved in prion formation and propagation, change in their expression has less drastic effects than change in Hsp104. Overexpression or deletion of various Hsp70s cures [URE3] and [PSI^+] (Ness et al., 2002; Shorter and Lindquist, 2004; Kryndushkin and Wickner, 2007). As Hsp40s and Hsp70s act in concert with Hsp104 in *in vitro* disaggregation assays (Glover and Lindquist, 1998), this is expected. However, different Hsp70 isoforms have different affects on the same prion. Overexpression of the *SSA* family of cytosolic Hsp70s increases conversion to [PSI^+] but overexpression of the *SSB* family of cytosolic Hsp70s prevents [PSI^+] formation (Allen et al., 2005). [URE3] also responds to different Hsp70s: overexpression of *SSA* family member *SSA1* cures [URE3] (Schwimmer and Masison, 2002).

The dependence of all the early fungal prions on aggregation and chaperones that act on aggregated proteins raises the questions of whether all aggregating, amyloid-forming proteins are prions and whether all prions must aggregate and/or form amyloid. Modeling of disease-causing proteins from humans in yeast show that such proteins aggregate and are toxic (Lindquist et al., 2001; Meriin et al., 2002; Outeiro and Lindquist, 2003) but there is no evidence that these aggregates are heritable. Yeast can therefore distinguish between heritable and non-heritable aggregates. There is growing evidence that the heritability of [PSI^+]-like aggregates depends on association with the cytoskeleton(Ganusova et al., 2006) and might be linked to an aggresome-like structure in yeast (Tyedmers and Lindquist, unpublished).

Amyloid-based prions oligomerize, aggregate, and then interact with chaperones to form prion seeds. Would prions that do not aggregate show a similar dependence on chaperones? Since all proteins must fold to function, there would presumably be a basic

dependence on protein folding. Prions that involved an alteration in protein conformation would probably depend on chaperones, since changing the efficiency of protein folding could cause switching between [prion$^-$] and [PRION$^+$]. Whether self-activating, self-propagating enzymes would require chaperones is more difficult to predict.

Identification of additional fungal prions

[PSI$^+$], [URE3], and [Het-s] are phenotypically different but mechanistically similar. How common, then, are fungal prions and what sorts of phenotypes might they cause? Two primary methods were used to identify further prions: finding prions with similar sequences or finding phenotypes that act like prions genetically.

Prion-determining regions (PrDs) of the Sup35 and Ure2 prion-determining proteins have several identifying characteristics: extreme amino acid bias and modularity. Sup35 and Ure2 PrDs are strongly enriched in Q/N residues (Harrison and Gerstein, 2003). This bias is conserved among hemiascomyces (Ure2) and even out to basidiomyces and euascomyces, which are estimated to have diverged from the hemiascomyces ~1 billion years ago (Harrison et al., 2007). PrDs are also modular and transferable. When the PrD of *SUP35* (termed "NM" for N-terminus and Middle region) is fused to the sequence of glucacortacoid receptor (GR) it is sufficient to confer prion properties to GR. NM-GR can switch between [prion$^-$] and [PRION$^+$] forms and is cured by chaperones (Li and Lindquist, 2000). Sup35 PrD contains six imperfect oligopeptide repeats that are involved in [PSI$^+$] induction (Liu and Lindquist, 1999) and maintenance (Osherovich et al., 2004). Repeats are also found in the mammalian prion protein but not the Ure2 PrD (Tuite, 1994).

Several groups attempted to identify additional prions by sequence (Michelitsch and Weissman, 2000; Sondheimer and Lindquist, 2000; Harrison and Gerstein, 2003). The *RNQ1* gene was found by searching for regions similar to the Sup35 PrD that showed approximately constant expression (less than two-fold change) between logarithmic and stationary phases (Sondheimer and Lindquist, 2000). Expression levels of candidate genes were considered because [PSI^+] and [URE3] are maintained over many generations, which would require constant protein levels. However, neither Δ*rnq1* nor [RNQ^+] had a discernible phenotype, so experiments had to be performed on Rnq1-GFP and other such non-native fusion proteins. [RNQ^+] exhibits all the genetic attributes of a prion, including chaperone-dependent curing, cytoplasmic inheritance, and non-Mendelian inheritance (Sondheimer and Lindquist, 2000).

A second protein capable of forming a prion, [NU^+], was also discovered by searches for Q/N-rich genes (Michelitsch and Weissman, 2000; Osherovich and Weissman, 2001). This search identified the *NEW1* and *PAN1* genes as prion-forming candidates (Osherovich and Weissman, 2001). New1 showed all the genetic characteristics of prions but Pan1 did not. Although Pan1 aggregates, these aggregates are not heritable (Michelitsch and Weissman, 2000). The distinguishing feature between New1 and Pan1 are imperfect repeats in the putative PrD of New1, which resemble those in the Sup35 PrD (Michelitsch and Weissman, 2000). These repeats were later shown to be involved in [NU^+] maintenance and prion stability (Osherovich 2004). Like [RNQ^+], [NU^+] did not have a phenotype when first discovered and thus had to be studied as a fusion protein (Osherovich and Weissman, 2001).

Recent work on the *S. cerevisiae* chromatin remodeling factor Swi1 suggests that Swi1 is capable of acting as a prion, [SWI$^+$] (Du et al., 2008). Swi1 was identified by searching for glutamine and asparagine rich proteins. A number of members of the Swi/Snf complex, including Snf5, also show N- and Q-rich regions but do not act as prions. However, [SWI$^+$] is dominant and shows infectious, cytoplasmic inheritance. [SWI$^+$] acts independently of Snf5, as Swi1 but not Snf5 aggregates in [SWI$^+$].

Although searches for Q/N-rich proteins have identified interesting prions, they have several limitations. Neither PrP nor Het-s are Q/N-rich so Q/N predominance is not deterministic of prion-forming ability. Proteins identified in sequence-based searches are biased to aggregate but aggregation is not sufficient for heritability. Some *SUP35* mutants aggregate but do not propagate [PSI$^+$] (Osherovich et al., 2004) and polyQ aggregates in yeast but is not heritable (Meriin et al., 2002). Furthermore, a protein does not have to aggregate or form an amyloid to be a prion (Wickner et al., 2007). While all known fungal prions aggregate, there were only three known fungal prions at the time of these studies. Various genetic mechanisms, such as feedback loops or self-activating enzymes, might result in the genetic attributes of fungal prions. Thus searching for prions by sequence, while informative, limits the type of prions that one finds.

Attempts to identify additional prions based on phenotypes do not bias the outcome, as sequence-based attempts do, but require an appropriate starting phenotype. One such candidate, [PIN$^+$] (PSI-inducible), was based on the observation that the induction of [PSI$^+$] by overexpression of *SUP35* requires a non-Mendelian, Hsp104-dependent element (Derkatch et al., 1997). [PIN$^+$] arises frequently from previously cured ([pin$^-$]) yeast and is necessary for [PSI$^+$] induction but not maintenance.

Researchers thus had an intriguing phenotype but no causal agent for [PIN$^+$], a frustrating state that lasted until 2001. A genomic screen for chromosomal regions that induced [PIN$^+$] when overexpressed identified 11 candidates, including the prion-causing genes *URE2*, *RNQ1*, and *NEW1* (Derkatch et al., 2001). The authors then demonstrated that [URE3], [RNQ$^+$], and [NU$^+$] could act as [PIN$^+$]. This established a phenotype for [RNQ$^+$] and [NU$^+$], showed that prions can interact with each other, and proposed eight other putative prions and a phenotype to test them under. Identifying new prions by phenotype can thus be rewarding but is limited by candidate phenotypes.

The *S. pombe* phenotype [Cin] allows cells to survive in the absence of the essential chaperone calnexin (Collin et al., 2004). To perform mutation analysis of calnexin, the authors transformed cells deleted for the genomic copy of the essential calnexin gene with plasmids carrying wildtype and mutant calnexin genes. These were scored for wildtype or mutant plasmids after six days of growth. The authors observed that cells carrying a mutant form of calnexin from which the highly conserved domain (hcd) had been removed showed loss of both wildtype and mutant plasmids. Assuming that the calnexin gene had simply recombined into the genome, they performed Southerns, Westerns, and Northerns but could not detect any evidence of calnexin. This state was named [Cin] (calnexin independent). [Cin] is dominant in a mating experiment and shows non-Mendelian inheritance by random spore analysis. The authors transformed [Cin] cell extracts into [cin$^-$] *S. pombe* to induce [Cin]. These extracts were not sensitive to DNase, RNase, or UV treatment but could not induce [Cin] when treated with UV. [Cin] is thus probably caused by a proteinaceous factor. However, this factor

has yet to be identified. The difficulty of identifying factors from such studies is the primary difficulty when investigating prions based on phenotype.

Phenotypic consequences and biologic importance of fungal prions

An ongoing controversy in the field of prion biology is the relevance of prions to the biology of their fungal hosts. Prion-determining regions in Sup35 and Ure2 are conserved to euascomycota (~1 billion years) and hemiascomyces (same order as *S. cerevisiae*; ~700 million years (Hedges et al., 2004)), respectively (Harrison et al., 2007). The PrDs of Rnq1 and New1 are less well conserved. Whether the prion domains are conserved for prion-determining properties or other reasons is a point of controversy (Wickner et al., 2007). Sup35 from *C. albicans* and *K. lactis* have been tested for prion-forming ability. Both can form [PSI^+] in *S. cerevisiae* (Nakayashiki et al., 2005; Tanaka et al., 2005) but only *K. lactis* Sup35 has been tested in its native organism, where it can also form a prion (Nakayashiki et al., 2001).

One proposal in favor of the relevance of fungal prions is that the read-through of stop codons caused by [PSI^+] results in new phenotypes. [PSI^+] results in growth differences compared to [psi^-] under a variety of conditions that vary with genetic background (True and Lindquist, 2000). Over 50 different culture conditions were tested in seven genetic backgrounds; [PSI^+] and [psi^-] showed growth differences approximately 50% of the time. In 25% of those cases [PSI^+] had an overall growth advantage compared to [psi^-]. Whether a condition was advantageous differed with genetic background and what might be an advantageous condition for [PSI^+] in one genetic

background was not necessarily advantageous in another. For example, [psi$^-$] grew better than [PSI$^+$] at pH 6.0 in one genetic background (D1142) but worse in another (5V-H19).

[PSI$^+$]-dependent traits are genetically complex and have not yet been mapped to causal loci. [PSI$^+$]-dependent traits are also polyallelic, as outcrossed phenotypes never segregated 2:2 (True et al., 2004). Phenotypes also were easily fixed, as outcross progeny sometimes maintained the [PSI$^+$]-dependent phenotype following curing (True 2004). This latter point is especially interesting because it provides a mechanism for fixation if a [PSI$^+$]-dependent phenotype is advantageous. That way the host cell does not need to maintain detrimental read-through for long. These studies suggest that [PSI$^+$] might allow for the gain and loss of read-through dependent traits that vary with genetic background and thus acts as an agent of phenotypic plasticity.

The counterargument to the idea that [PSI$^+$] can provide an advantage under specific growth conditions is that [PSI$^+$] appears to be disadvantageous because it has never been found in a wild *S. cerevisiae* strain (Nakayashiki et al., 2005). This theory criticizes many authors for studying [PSI$^+$] only in laboratory strains. Nakayashiki et al. (2005) also argue that True and Lindquist (2000) show that a deletion of the *SUP35* PrD, Δ*nm*, results in a phenotypic change compared to either [PSI$^+$] or [psi$^-$] in some genetic backgrounds and environmental conditions (True and Lindquist, 2000). Δ*nm* is obligatorily [psi$^-$] (Ter-Avanesyan et al., 1994), so the claim that Δ*nm* showed a phenotype that differed from [psi$^-$] raises the possibility that the PrD of Sup35 could have a function other than prion maintenance.

Nakayashiki et al. (2005) extended the argument against the possible utility of yeast prions by testing for [PSI$^+$], [URE3], and [RNQ$^+$] in a variety of "wild" yeast that

had never been cultivated in the laboratory. The logic behind these experiments is that, as prions are transferred horizontally like viruses and can arise spontaneously in ~1 in 10^5 to 10^7 cells, they should be widespread unless selected against. The authors found neither [PSI^+] nor [URE3] in any of the 70 yeast strains tested but did detect [RNQ^+] in approximately 16% (11 out of 70 samples). For comparison, they also tested for 2μ plasmids, RNA viruses, and ssRNA replicons. 2μ plasmids, which are mildly detrimental to the yeast host cell (Futcher 1983; Mead 1986), were found in 54% of the yeast strains tested. The two RNA viruses and two ssRNA replicons tested varied in frequency from 1-20% (Nakayashiki et al., 2005). As 2μ plasmids were more common than [RNQ^+], the authors concluded that [RNQ^+] is more detrimental to the host than the plasmid and that [PSI^+] and [URE3], which were not found at all, are more toxic than [RNQ^+]. Finally, Nakayashiki et al. (2005) suggested that a growth advantage for [PSI^+] is perhaps less relevant than a survival advantage, since no one knows the conditions experienced by yeast in the wild. True and Lindquist had also originally proposed that [PSI^+] is usually detrimental but very rarely advantageous (2000) and the data from Nakayashiki and colleagues (2005) are consistent with this hypothesis.

Overall, while *S. cerevisiae* prions other than [RNQ^+] have not been found in wild yeast, it is difficult to discount prions function when prion-determining regions are so well conserved (Harrison et al., 2007). Even wild and industrial yeasts that are not natively [PSI^+] carry *SUP35* alleles capable of forming [PSI^+] (Chernoff et al., 2000), as do *C. albicans* clinical isolates (Handwerger and Lindquist, unpublished). Recent work shows that the rate of [PSI^+] appearance increases under a variety of strong stress conditions (up to 90% lethality) regardless of whether [PSI^+] is advantageous under that

condition (Tyedmers et al., unpublished). [PSI^+] induction could therefore be a general response to stress which is only occasional advantageous, and thus would not be expected to be observed in wild yeasts.

[Het-s], the prion from the filamentous fungus *Podospora anserina*, is involved in vegetative heterokaryon incompatability and meiotic drive. The ability of [Het-s] to induce apoptosis in the mycelia of *P. anserina* carrying the non-prion forming *het-S* allele is well documented (Rizet, 1952). The prion-forming *het-s* allele is present in a majority of *P. anserina* isolates (60% of 102 strains) (Dalstra et al., 2003); a majority of these carry the prion form, [Het-s], (51%) instead of the non-prion form, [Het-s*]. The [Het-s] prion is therefore thought to provide some benefit to its host, possibly because [Het-s]-induced apoptosis would prevent the horizontal spread of fungal viruses to unexposed populations (Wickner et al., 2007). This heterokaryon incompatibility takes place during the vegetative cycle of the organism. [Het-s] was recently shown to be involved in spore killing and acts as a meiotic drive element to favor its own inheritance (Dalstra et al., 2003).

Several non-infectious (and thus non-prion) amyloids have been shown to have a beneficial phenotype that requires the amyloid fold. Secreted proteins from several different bacterial species form amyloids, including *E. coli* (Chapman et al., 2002) and *Streptomyces coelicolor* (Claessen et al., 2003). These amyloids are involved in biofilm formation or formation of hydrophobic surfaces, respectively. *Neurospora crassa* has a similar amyloid-like hydrophobin (Mackay et al., 2001) and an amyloid-like protein in the silkworm eggshell protects from environmental damage (Iconomidou 2000). Intracellular amyloids can also perform novel functions; a prion-like amyloid-forming

protein (Si et al., 2003b) in the neurons of the sea slug *Aplysia californica* is involved in mRNA binding and the maintenance of synapses (Si et al., 2003a). "Functional" amyloids are also found in the melanophore organelle *Homo sapiens* melanocyte and retinal pigment epithelial cells, where the protein Pmel17 forms amyloid fibrils following protease cleavage (Berson et al., 2003; Fowler et al., 2006). These amyloids provide a scaffold for the formation of melanin pigment (Fowler et al., 2006).

Overall, amyloids have been shown to provide unique functions to the cells that carry them and prions could well do the same (Chiti and Dobson, 2006). Furthermore, prions potentially provide a new level of cellular regulation, with a single protein giving rise to multiple phenotypes. As prions appear at a rate similar to or higher than genetic mutation (True and Lindquist, 2000), prion-dependent traits potentially provide a phenotypic flexibility not possible through standard genetic changes. Thus identification of new prions and characterization of their functions could provide important information on how microorganisms survive and adjust to changing conditions.

[GAR$^+$]: a non-Mendelian phenotype conferring resistance to D-(+)-glucosamine

The ultimate goal of my thesis work was to identify new prions in *Saccharomyces cerevisiae* and determine the phenotypic consequences of the [PRION$^+$] form on the host organism. I have attempted to address the two basic questions of interest to the field: whether prions are common mechanisms of regulation and whether prions could be beneficial for their hosts. I took a phenotype-based approach to identifying additional prions, which did not bias these studies towards identifying prions that necessarily resemble the well-characterized prions in sequence and/or mechanism.

Given the tenuous nature of identifying potential prions computationally, searching for phenotypes with similar genetic attributes to known prions could be a more promising approach. One could start by investigating dominant phenotypes that segregate in a non-Mendelian pattern or permanent phenotypic changes induced by transient overexpression of an ORF. One of the most promising of these a the non-Mendelian phenotype that confers resistance to D-(+)-glucosamine ([GAR^+]), which was characterized as independent of mitochondria, cytoplasmically inherited, and described as possibly "allelic to [PSI^+] and [URE3]" (Kunz and Ball, 1977).

D-(+)-glucosamine (glucosamine) is a non-metabolizable glucose mimetic (Woodward and Hudson, 1953) that is sufficient to maintain glucose repression in *Saccharomyces* (Hockney and Freeman, 1980). When glucose is present, *S. cerevisiae* and other yeasts activate genes involved in glucose metabolism and growth and repress genes involved in the processing of secondary carbon sources. Therefore, when both glucose and secondary carbon sources are present, many fungi will utilize the available glucose before processing the secondary carbon sources such as glycerol or galactose (Santangelo, 2006). Glucosamine, like glucose, blocks growth on alternative carbon sources such as galactose and glycerol, probably by activating glucose signaling and/or repression pathways (Hockney and Freeman, 1980; Nevado and Heredia, 1996). Resistance to glucosamine therefore implies a lack of repression of non-glucose carbon pathways and thus confers the ability to process alternative carbon sources in the presence of glucose. This ability might occassionally confer a growth advantage in the wild, since under some conditions it could be advantageous to switch rapidly between glucose and alternative carbon source utilization (Verstrepen et al., 2004; Santangelo,

2006). Glucosamine can serve as a competitive inhibitor of hexokinase (McGoldrick and Wheals, 1989) and is a precursor to N-glucosamine, a component of yeast cell walls (Bulik et al., 2003).

The goal of this work was to identify new prions, specifically by focusing on the non-Mendelian phenotype [GAR^+]. [GAR^+] was selected because it showed cytoplasmic, non-Mendelian inheritance and was not mitochondrial (Ball et al., 1976; Kunz and Ball, 1977). Furthermore, [GAR^+] conveys an interesting phenotype, glucosamine resistance, that one could imagine might have some importance for yeast under non-lab conditions. When I began my graduate studies only five fungal prions had been identified. All aggregated in the [$PRION^+$] form, involve an infectious amyloid species, and all but [Het-s] are rich in glutamines and asparagines. While every graduate student has some hopes for an unusual project, I did not expect [GAR^+] to be quite as surprising as it turned out to be. While [GAR^+] shows non-Mendelian, cytoplasmically infectious inheritance, the causal agents do not form an amyloid, do not aggregate, are not N/Q-rich, act independently of Hsp104, and overall represent a novel type of prion. [GAR^+] shows a growth advantage over [gar^-] in particular environmental conditions and its frequency varies with the ecological niche of its host. Overall, [GAR^+] is a novel type of prion that shows environmental sensitivity.

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Chapter Two:

[GAR$^+$], a non-amyloid protein-based inheritance system involved in glucose signaling

Introduction

The stable inheritance of biological information and phenotype across generations is a fundamental property of living systems. Prions, self-perpetuating protein conformations that cause multiple phenotypes, represent an unusual mechanism of information transfer that occurs via protein instead of nucleic acid (Wickner, 1994). Prion proteins can assume multiple conformations and each conformation alters protein functionality, resulting in different phenotypes (Wickner et al., 2004; Shorter and Lindquist, 2005). Because these conformational switches are self-templating, prion proteins acquire characteristics normally restricted to nucleic acids. The first prion protein identified, the mammalian protein PrP, can behave as a transmissible pathogen and causes a neurodegenerative disease in its prion form (PrP^{Sc}) (Prusiner, 1998). The prion proteins described in fungi, which are unrelated to PrP and to each other, act as non-Mendelian elements of inheritance by switching to the self-perpetuating, cytoplasmically transmissible prion conformation (Wickner, 1994).

Four prions have been identified to date in fungi: [PSI$^+$], [URE3], [Het-s], and [RNQ$^+$]. [PSI$^+$] (Cox, 1965) is caused by a change in conformation of the translation termination factor Sup35 (Stansfield et al., 1995; Patino et al., 1996; Paushkin et al.,

1996) that results in increased read-through at stop codons. [URE3] (Lacroute, 1971) is an altered form (Wickner, 1994) of the nitrogen catabolite repressor Ure2 that alters transcription (Courchesne and Magasanik, 1988). [RNQ^{+}] controls the ability of a cell to induce the [PSI^{+}] prion, and its causal protein, Rnq1, has no known function other than the ability to form a prion and, thereby, influence the rate of appearance of other prions (Derkatch et al., 2000; Sondheimer and Lindquist, 2000; Derkatch et al., 2001). [Het-s], found in the filamentous fungus *Podospora anserina*, causes heterokaryon incompatibility and cell death with certain mating partners (Rizet, 1952; Coustou et al., 1997). This is thought to serve as a mating isolation system to prevent the spread of viruses (Wickner et al., 2007). All four proteins form a highly insoluble, self-templating conformation in the [PRION^{+}] form (Chien et al., 2004).

The four fungal prions share distinct and unusual genetic characteristics despite their disparate functions (Wickner 1994). They characteristically appear spontaneously at a frequency higher than the frequency of genetic mutation. All show dominant inheritance, non-Mendelian 4 [PRION^{+}] to 0 [prion^{-}] (or sometimes 3:1) segregation following meiosis, and are transmissible by cytoduction (cytoplasmic transfer). Further, their inheritance is linked to the activities of chaperones, proteins that mediate conformational changes in other proteins (Uptain and Lindquist, 2002). The yeast prions also share a distinctive feature of mammalian prions, a species barrier for transmission. Due to differences in amino acid sequence, proteins from one species exhibit defects in converting the homologous protein from another species to the [PRION^{+}] form, even though the homologous protein is itself capable of forming a prion (Aguzzi et al., 2007).

Moreover, all four prions share a common mechanism for conformational change and propagating that change. The infectious conformation is a self-templating amyloid fiber. A striking feature of these prions is that transient overexpression of the prion protein is sufficient to cause a permanent change in phenotype. It does so by nucleating formation of the amyloid fiber template (Patino et al., 1996; King et al., 1997; Sondheimer and Lindquist, 2000; Speransky et al., 2001; Kimura et al., 2003; Bagriantsev and Liebman, 2004). *In vitro* these amyloids template soluble protein into the amyloid state (Glover et al., 1997; Paushkin et al., 1997; Taylor et al., 1999). These amyloids are the sole determinant needed for prion formation because amyloid fibers are sufficient to convert [prion$^-$] cells to [PRION$^+$] cells (Maddelein et al., 2002; Tanaka et al., 2004; Brachmann et al., 2005; Patel and Liebman, 2007). The self-templating conformation and the [PRION$^+$] phenotype that results from the change in conformation are thus stably inherited by daughter cells following mitosis or meiosis (Uptain and Lindquist, 2002). Another striking feature of prions is that transient changes in chaperone levels are sufficient to permanently eliminate (or "cure") cells of the prions by altering conformational states of the protein and their transmission to daughter cells.

The simple and robust character of self-templating amyloids provides a compelling framework for protein-based inheritance. Amyloid structure is therefore commonly held to be a critical feature of all naturally occurring systems for protein-based inheritance (Chien et al., 2004). However, Prusiner had initially defined "prion" as "a small proteinaceous infectious particle…resistant to inactivation by most procedures that modify nucleic acids" but made no restriction as to mechanism (Prusiner, 1982). Wickner extended this to proteins other than PrP by outlining genetic criteria for prions in

fungi (Wickner, 1994). These include reversible curing, overexpression of the prion protein increasing the frequency of [PRION^{+}] formation, and a phenotypic link between the prion-dependent phenotype and a mutation in the causal protein. But he too did not restrict possible mechanisms (Wickner et al., 1999). Indeed, both Wickner and Cox proposed multiple possible modes by which proteins could create heritable phenotypes, including self-modifying enzymes or altered conformations that convert the normal conformation (Cohen et al., 1994; Cox, 1994; Wickner, 1994).

Taking an unbiased approach to identifying new prions, we searched the literature for phenotypes in *S. cerevisiae* that had prion-like characteristics of inheritance. An unusual heritable phenotype was described many years ago in a screen for mutants resistant to the non-metabolizable glucose mimetic D-(+)-glucosamine (Ball et al., 1976). The basis of the screen was the extreme preference of yeast cells for glucose as a carbon source. If glucose is present they will repress the cellular machinery necessary to process other carbon sources such as glycerol (Santangelo, 2006). Glucosamine also mediates this repression but it cannot be used to produce energy. Yeast cells, therefore, cannot grow on glycerol in the presence of glucosamine (Hockney and Freeman, 1980; Nevado and Heredia, 1996). Some cells spontaneously acquire the ability to use glycerol in the presence of glucosamine, presumably due to defects in glucose signaling and/or the response pathways that mediate glucose repression. Ball and colleagues demonstrated that in some cases cells that can grown on glycerol in the presence of glucosamine show non-Mendelian inheritance of the phenotype. Further, the phenotype is not the result of a mitochondrial mutation or a plasmid (Kunz and Ball, 1977). Puzzled, the authors

described the factor responsible for this non-Mendelian element of inheritance as possibly similar to [PSI$^+$].

We show here that this factor, [GAR$^+$], exhibits all of the genetic characteristics of a yeast prion but is physically of a very different type. [GAR$^+$] involves at least two factors: the glucose signaling protein Std1 and Pma1, stably associated in an oligomeric complex (Schmidt et al., 1999). Pma1 is a P-type H^+-ATPase and is the predominant protein at the plasma membrane. It is a large (100kDa) essential protein with ten transmembrane domains. Its cytoplasmic N- and C-termini are predicted to be unstructured or α-helical (Morsomme et al., 2000) and are thought to be important for regulation of both its oligomerization and ATPase function (Morsomme et al., 2000; Kuhlbrandt et al., 2002). Pma1 is the major controller of plasma membrane potential and cytoplasmic pH (Morsomme et al., 2000) and undergoes a glucose-dependent conformation shift (Lecchi et al., 2005) that increases its ATPase activity 10-fold (Serrano, 1983).

Our data indicate that Pma1 acts with Std1 to form [GAR$^+$] by a novel mechanism that does not appear to involve protein aggregation or the formation of amyloid. Instead, Pma1 associates with Std1 in [GAR$^+$] cells and Mth1, the homolog of Std1, in [gar$^-$] cells; these changes alter signaling through the Rgt2/Snf3 glucose signaling pathway. [GAR$^+$] appears spontaneously at a high frequency in many strain backgrounds and shows chaperone-dependent non-Mendelian inheritance. It also exhibits a strong species barrier within sibling species. Replacing the *S. cerevisiae* Pma1 protein with that of the sibling species is sufficient to prevent the propagation of [GAR$^+$]. Because [GAR$^+$] involves proteins that are membrane-associated, not cytoplasmic or amyloid-forming, our

work suggests that self-perpetuating protein-based elements of inheritance can operate outside of the scope of an amyloid template and may be a much broader phenomenon in nature than previously surmised.

Results

[GAR$^+$] shows non-Mendelian, infectious inheritance

We obtained glucosamine-resistant cells of the type described by Ball and colleagues (Ball et al., 1976; Kunz and Ball, 1977) by selecting for cells that could grow with 2% glycerol as a carbon source in the presence of 0.05% glucosamine. Glucosamine resistant colonies appeared at a rate of approximately 5 in 10^4 cells in the W303 genetic background (figure S2.01). Because some loss-of-function mutations can give rise to recessive glucosamine resistance (Ball et al., 1976; see table S2.1 table) and the novel phenotypes described by Ball and colleagues were dominant, we first crossed glucosamine-resistant colonies to wildtype. All glucosamine-resistant colonies showed semi-dominant resistance (figure 2.1a). Specifically, a cross of glucosamine-resistant to glucosamine-sensitive cells invariably yielded a mixed population of diploids, some of which showed "strong" (large) glucosamine-resistant colonies and others "weak" resistance (small colonies). Weak glucosamine-resistant colonies invariably converted to strong over approximately 25 generations. "Strong" and "weak" strains are characteristic of mammalian and fungal prions.

In yeast, chromosomally inherited traits show 2:2 segregation following meiosis, tetrad dissection, and spore analysis. All of our dominant glucosamine-resistant variants

exhibited non-Mendelian inheritance, segregating 4 resistant spores to 0 sensitive spores in each tetrad after meiosis (figure 2.1b). Both strong resistant colonies and weak resistant colonies exhibited 4:0 segregation. The spores produced by weak diploids generally had strong [GAR$^+$] phenotypes (the meiotic products of fig2.1b are from the diploids of fig2.1a). (Interestingly, when cells with a weak [GAR$^+$] phenotype did produce weak [GAR$^+$] spores after meiosis, this phenotype was observed in all four meiotic progeny (figure 2.1b, bottom)). We named this genetic element [GAR$^+$] for glucosamine resistant, with capitol letters indicating dominance and brackets its non-Mendelian character.

To determine whether [GAR$^+$] is "infectious," we used a mutant defective in nuclear fusion (*kar1-1*). During mating *kar1* cells fuse (with cytoplasmic mixing) but nuclei do not (Conde and Fink, 1976). Selecting for a nucleus and cytoplasm of interest after mating effects cytoplasmic exchange without the transfer of nuclear material. We mated a *Ura*$^+$ *His*$^-$ [GAR$^+$] strain to a *kar1-1 Ura*$^-$ *His*$^+$ [gar$^-$] strain, then selected for the nucleus originally associated with [gar$^-$] (*Ura*$^-$ *His*$^+$) and against the nucleus originally associated with [GAR$^+$] (*Ura*$^+$ *His*$^-$). All ten of the resultant strains tested positive for [GAR$^+$] (figure 2.1c), demonstrating the "infectious" character of [GAR$^+$] inheritance.

[GAR$^+$] appears at high frequency in a variety of genetic backgrounds

We next examined the frequency of [GAR$^+$] appearance in different genetic backgrounds. In the BY background, [GAR$^+$] appeared at a rate of ~9 in 10^5 cells. The rate was ~1 in 10^4 cells in 74D, ~5 in 10^4 cells in W303, and ~7 in 10^4 cells in Sigma. In the SK1 background, [GAR$^+$] appeared at the astonishingly high rate of ~4 in 10^3 cells

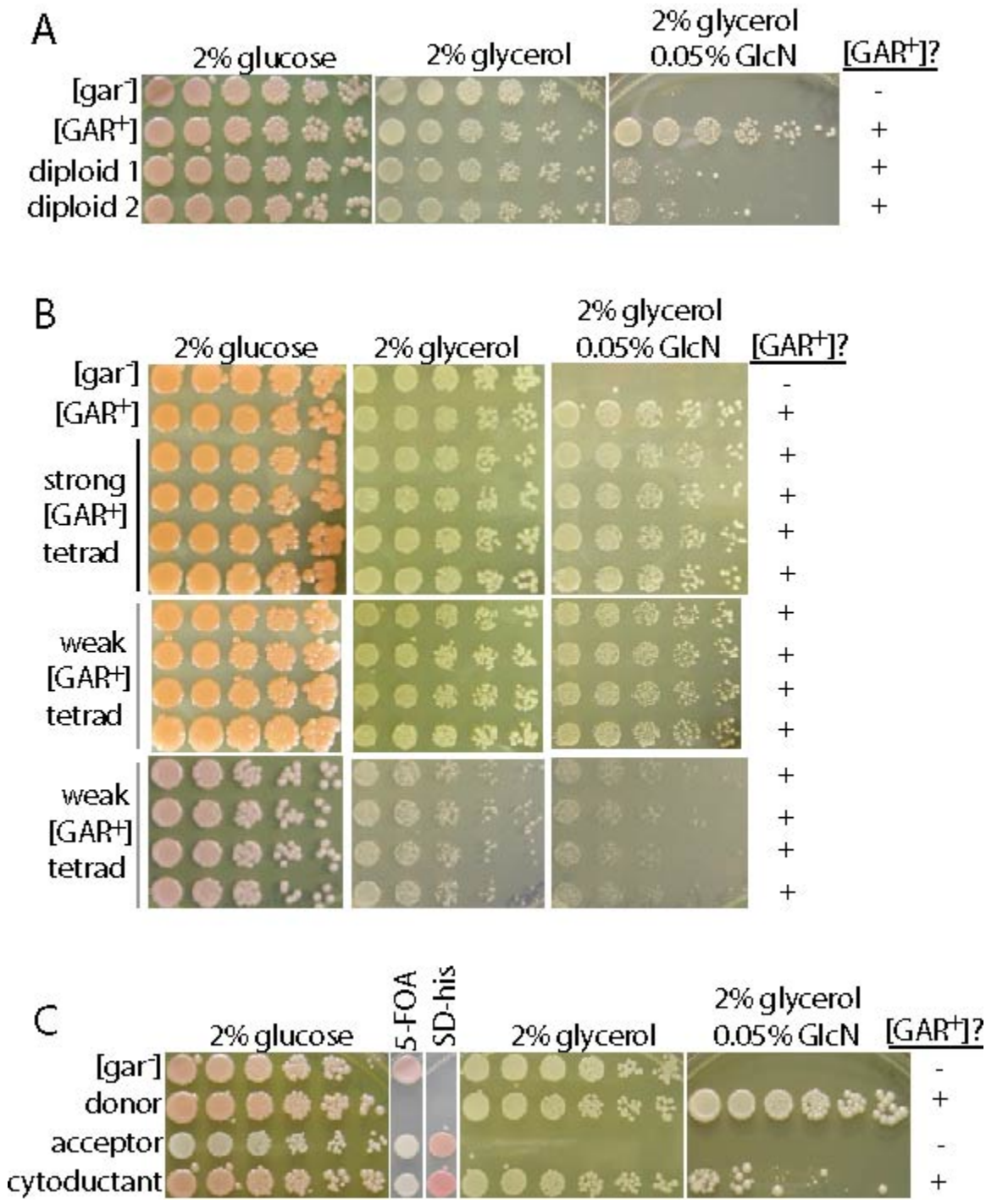

Figure 2.1: [GAR$^+$] shares the genetic characteristics of yeast prions

Figure 2.1: [GAR$^+$] shares the genetic characteristics of yeast prions

a) Mating of [gar$^-$] MATa to [GAR$^+$] MATα in the W303 background. Resultant diploids show semi-dominant [GAR$^+$] with a mixed population of large colonies ("strong") and small colonies ("weak"). All spot tests shown are five-fold dilutions. b) Tetrad spores from the "strong" [GAR$^+$] (top) and "weak" [GAR$^+$] (middle, bottom) diploids in part A show non-Mendelian segregation of [GAR$^+$]. "Weak" commonly reverts to "strong" during meiosis (middle) but occasionally stays "weak" (bottom). c). Cytoduction shows cytoplasmic inheritance of [GAR$^+$]. The [GAR$^+$] donor is 10B Ura$^+$ His$^-$ ρ^+ *kar1-1* and the acceptor is W303 Ura$^-$ His$^+$ ρ^0 *KAR1*. d). [GAR$^+$] frequency in various lab strains. Data are shown as mean +/- standard deviation (n=6). e). Tetrad spores from a [GAR$^+$] diploid with the genotype *hsp104::LEU2/HSP104*. Δ*hsp104* spores are still [GAR$^+$]. f). Tetrad spores from a [GAR$^+$] diploid with the genotype *ssa1::HIS3/SSA1 ssa2::LEU2/SSA2*. Δ*ssa1*Δ*ssa2* spores are no longer [GAR$^+$].

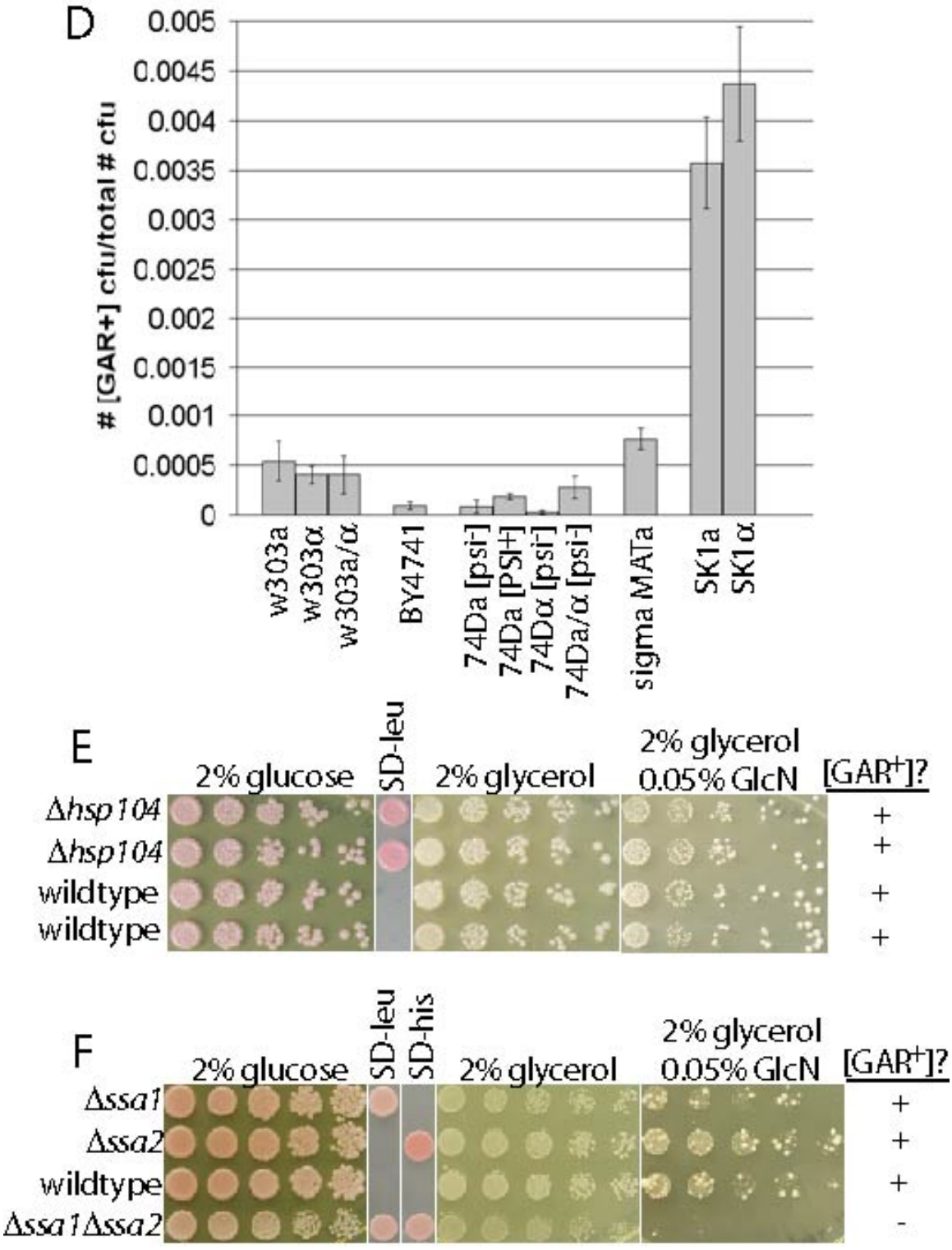

Figure 2.1, continued.

(figure 2.1d). Unlike Ball and colleagues, we did not observe a single incidence of Mendelian segregation of the glucosamine-resistant phenotype. This is presumably because, in contrast to that study, we did not start with mutagenized cells.

[GAR$^+$] is curable by transient changes in chaperone protein levels

To test the influence of the major chaperone proteins on the inheritance of [GAR$^+$], we crossed [GAR$^+$] cells to cells carrying mutations in *hsp104* or the Hsp70 proteins *ssa1* and *ssa2* (Werner-Washburne et al., 1987). Hsp104 is required for the propagation of [PSI$^+$], [URE3], and [RNQ$^+$], but was not required for [GAR$^+$] inheritance, as Δ*hsp104* spores were still [GAR$^+$] (figure 2.1e). However, when Δ*ssa1*Δ*ssa2* cells were crossed to [GAR$^+$] cells, all the meiotic products that were Δ*ssa1*Δ*ssa2* lost the glucosamine-resistant phenotype (figure 2.1f).

The ability of transient changes in chaperone expression to establish a heritable change in phenotype, by curing cells of the self-perpetuating protein conformation, is a hallmark of prion-based inheritance. We tested whether the loss of the [GAR$^+$] phenotype was due to an actual curing of the [GAR$^+$] genetic element or whether it was retained in Δ*ssa1*Δ*ssa2* cells in a cryptic state by crossing them back to wildtype [gar$^-$] (figure S2.02a). Restoration of *SSA1* and *SSA2* function did not cause reappearance of the [GAR$^+$] phenotype. A transient change in chaperone protein levels was sufficient to cure cells of [GAR$^+$]. The [GAR$^+$] phenotype readily reappeared upon selection for glucosamine-resistance (figure S2.02b). Thus, this curing was reversible, another hallmark of prion biology (Wickner, 1994). [GAR$^+$] therefore exhibits the distinguishing

characteristics of yeast prions that would indicate it is due to the propagation of an altered protein conformation.

[GAR$^+$] is regulated by the Rgt2/Snf3 glucose signaling pathway

We performed microarray analysis of [gar$^-$] and [GAR$^+$] to identify transcriptional consequences of the [GAR$^+$] phenotype. The results were surprising: only one gene showed a detectable difference between [gar$^-$] cells and [GAR$^+$] cells, and that gene was very strongly affected. Hexose Transporter 3 (*HXT3*) was approximately 36-fold down-regulated in [GAR$^+$] cells compared to [gar$^-$] cells (figure S2.03). No other transcript exhibited more than a two-fold change. To investigate further, we examined the levels of an Hxt3-GFP fusion protein under the control of the endogenous *HXT3* promoter. Hxt3-GFP was readily visible at the plasma membrane in [gar$^-$] cells prior to diauxic shift but no signal was detected in [GAR$^+$] cells (figure 2.2a). This result led us to hypothesize that a negative regulator of *HXT3* expression is the causal agent of [GAR$^+$].

Work by many labs has established the pathways that regulate *HXT3* expression (Santangelo, 2006). *HXT3* is predominantly silenced by the Snf3/Rgt2 pathway. Hence, to investigate factors underlying the [GAR$^+$] phenotype we first focused on this pathway (Kim et al., 2003). When glucose is present, transmembrane glucose sensors Snf3 and Rgt2 transmit a signal to Yck1 and Yck2, which consequently phosphorylate Mth1 and Std1, marking them for degradation (figure 2.2b). When glucose is not present, Mth1 and Std1 accumulate and are free to interact with Rgt1. The Rgt1/Std1/Mth1 complex then binds to and represses the upstream region of *HXT3*. Std1 and Mth1 are both necessary for the binding of Rgt1 to DNA (Lakshmanan et al., 2003).

To discover the protein responsible for the [GAR$^+$] state, we took advantage of the fact that transient overexpression of the prion protein dramatically increases the appearance of the prion, because increased protein concentrations increase the likelihood of conformational change (Patino et al., 1996). We tested each member of the Snf3/Rgt2 regulatory pathway for induction of the [GAR$^+$] phenotype when overexpressed from a plasmid with a strong constitutive promoter, GPD (table 2.I). The *STD1* plasmid caused in extraordinary increase in [GAR$^+$] frequency in every strain tested. In W303, for example, this increase in [GAR$^+$] frequency was ~900 fold over that obtained with the empty vector; more than one in ten cells in these cultures converted to [GAR$^+$]. No other gene in this pathway induced [GAR$^+$]. Overexpression of the *STD1* paralog *MTH1* blocked the spontaneous appearance of [GAR$^+$], reaffirming the importance of this pathway.

We also screened the *S. cerevisiae* haploid deletion library for mutants that are incapable of inducing [GAR$^+$] (table S2.2), show a high rate of appearance of [GAR$^+$] (table S3), or glucosamine-resistance (table S2.1). Four of the eight members of the Snf3/Rgt2 pathway were found in this screen ($p = 8x10^{-6}$; Fisher's exact test). We also did not find many members of other glucose signaling pathways in this screen, reaffirming our decision to focus on the Rgt2/Snf3 pathway. We also screened a library of ~5000 ORFs to identify genes that induce [GAR$^+$] following overexpression. and did not find any of the genes identified in the deletion library screen (see chapter three, figure S3.1).

Because overexpression of *STD1* strongly induced [GAR$^+$], *STD1* was identified by three different experiments as involved in [GAR$^+$] (microarray, overexpression screen,

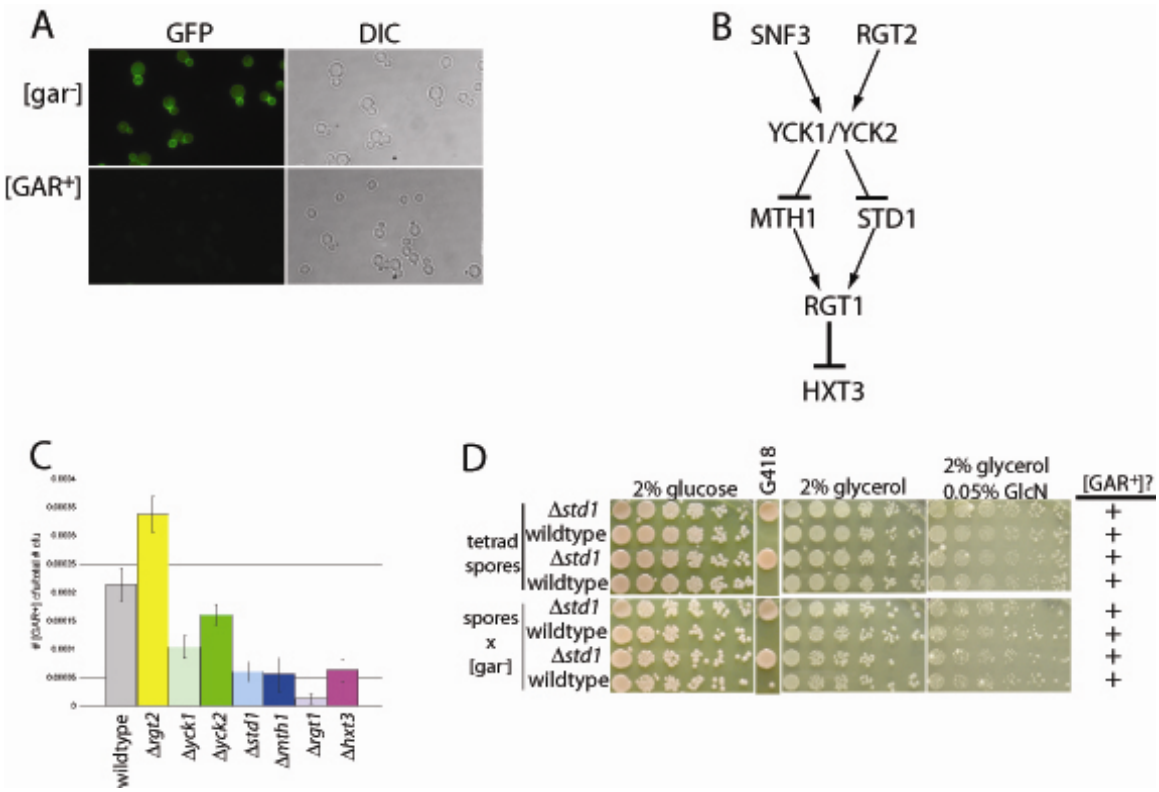

Figure 2.2: The Snf3/Rgt2 glucose signaling pathway affects [GAR$^+$]

a) Hxt3-GFP signal in [gar$^-$] and [GAR$^+$] cells by fluorescence microscopy. Further microarray data are deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi ?token=btyhxgeiaauwyji&acc=GSE12479. b) The Snf3/Rgt2 glucose signaling pathway (adapted from (Moriya and Johnston, 2004). c) [GAR$^+$] frequency of knockouts in the Snf3/Rgt2 glucose signaling pathway. Δ*snf3* is completely resistant to glucosamine and therefore [GAR$^+$] frequency could not be measured. This pathway is enriched for genes that alter [GAR$^+$] frequency when knocked out relative to the library of nonessential genes ($p = 8 \times 10^{-6}$, Fisher's exact test). d) Top: tetrad spores from a [GAR$^+$] diploid with the genotype *std1::kanMX/STD1*. Bottom: spores from top crossed to a [gar$^-$] strain with a wildtype *STD1* allele. e) Std1, Mth1, and Rgt1 are required [GAR$^+$]-dependent decrease in Hxt3-GFP levels.

gene	[GAR+] frequency relative to vector
vector	1
SNF3	0.7±0.3
RGT2	1.5±0.5
YCK1	1.9±0.6
YCK2	1.7±0.5
STD1	877±100
MTH1	0.03±0.02
RGT1	1.3±0.4
HXT3	1.2±0.3

Table 2.1: [GAR^+] induction following transient overexpression of Snf3/Rgt2 pathway members

knockout library screen) we hypothesized that Std1 was the [GAR$^+$] prion protein. To test this hypothesis, we asked whether *STD1* is required for the maintenance of [GAR$^+$] by performing a propagation assay. This involves measuring whether [GAR$^+$] could be transferred ("propagated") through a strain that does not express *STD1*, a Δ*std1* knockout mutant. If *STD1* were required for [GAR$^+$] propagation we would observe a ratio 2 [gar$^-$]: 2 [GAR$^+$] following mating of spores to [gar$^-$] (see figure S2.02a for a diagram of this cross) instead of the 4 [GAR$^+$] to 0 [gar$^-$] segregation we normally observed. A [GAR$^+$] diploid heterozygous for an *STD1* deletion was sporulated and dissected. Surprisingly, all the Δ*std1* progent were glucosamine-resistant. To determine whether Δ*std1* simply masked the phenotype of [GAR$^+$] or actually carried the heritable [GAR$^+$] element, we back-mated them to wildtype [gar$^-$] cells. Surprisingly, [GAR$^+$] was present in all of the diploids. [GAR$^+$] inheritance therefore does not require *STD1* (figure 2.2c). This makes [GAR$^+$] unique among yeast prions in appearing to have separable inducing and propagating agents.

We examined all other members of the Rgt2/Snf3 pathway for their ability to propagate [GAR$^+$]. None of the other members behaved as would be expected for the [GAR$^+$] casual agent. Knockouts were all capable of propagating [GAR$^+$] (figure S2.04). The *rgt1* knockout itself did not exhibit glucosamine resistance. However, *RGT1* is not the [GAR$^+$] heritable element because [GAR$^+$] was retained in the Δ*rgt1* cells in a "cryptic" form: the [GAR$^+$] phenotype reappeared when Δ*rgt1* cells were crossed back to [gar$^-$] *RGT1* cells.

Pma1 associates with Std1 and is a component of [GAR$^+$]

Since *STD1* acts as an inducing agent for [GAR$^+$], we hypothesized that it would physically interact with the unknown propagating agent. We therefore immunoprecipitated HA-tagged Std1p from [gar$^-$] and [GAR$^+$] cells. A high molecular weight band was found in [GAR$^+$] protein lysates but not in [gar$^-$] lysates (figure 2.3a). Mass spectrometry analysis identified the protein as Pma1. To ensure that this association was specific to Std1, we also preformed the IP in Δ*std1* cells. Our band of interest was not detected in this lane.

Pma1, an essential P-type ATPase with ten transmembrane domains, contains cytoplasmic N- and C-termini. The N-terminal domain of Pma1 is predicted to be unstructured (a characteristic of yeast prion proteins) while the C-terminal regions is predicted to be α-helical (Morsomme et al., 2000). To test whether Pma1 affects [GAR$^+$], we asked if the frequency at which cells were converted to [GAR$^+$] increased following transient *PMA1* overexpression. Pma1 is the most abundant plasma membrane protein in yeast (Morsomme et al., 2000) and overexpression is not well tolerated (Eraso et al., 1987). We were, however, able to obtain a three-fold transient increase in Pma1 protein levels and a corresponding increase in [GAR$^+$] frequency (figure 2.3b).

When an N-terminally truncated (Δ40) mutant of *PMA1* was transiently overexpressed, no increase in [GAR$^+$] appearance was observed despite increases in Pma1 protein levels (figure 2.3b). When a C-terminally truncated *PMA1* was overexpressed it did not accumulate as strongly as the wildtype protein but still caused a 2.2 fold increase in conversion to [GAR$^+$]. These data indicate that the N-terminus of Pma1 contributes to the formation of [GAR$^+$]. To ensure that this was due to overexpression of the *PMA1*

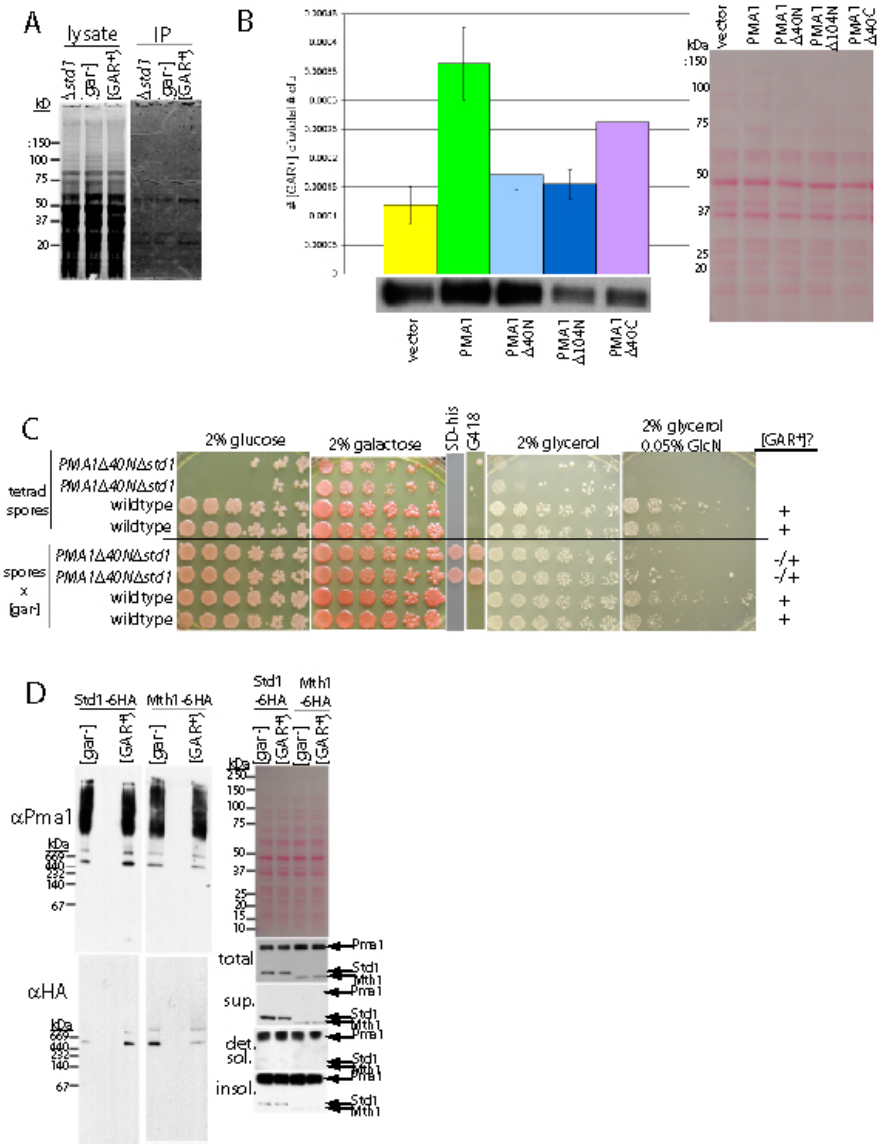

Figure 2.3: Pma1 is involved in [GAR$^+$] propagation

Figure 2.3: Pma1 is involved in [GAR$^+$] propagation

a) Immunoprecipitation of Std1-6HA from Δ*std1*, [gar$^-$], and [GAR$^+$] strains. One band was found in [GAR$^+$] but not [gar$^-$] or Δ*std1*. This was analyzed by mass spectrometry and found to be Pma1. Coverage was >25% of the protein. b) [GAR$^+$] induction by transient overexpression of *PMA1* in a wildtype background. Data is shown as the mean of [GAR$^+$] frequency +/- standard deviation (n=6). Western is total protein probed with αPma1 antibody and quantified using Scion Image. c) Propagation of [GAR$^+$] is impaired in *PMA1Δ40N Δstd1* double mutants. Top: tetrad spores from a [GAR$^+$] diploid with the genotype *GAL-PMA1Δ40N/PMA1 std1::kanMX/STD1*. Bottom: spores from top crossed to a [gar$^-$] strain with wildtype *PMA1* and *STD1* alleles. *PMA1Δ40N* Δ*std1* spores cannot propagate [GAR$^+$] to wildtype [gar$^-$] yeast. d) Native gel of Pma1, Std1, and Mth1 in [gar$^-$] and [GAR$^+$]. Either Std1 (left) or Mth1 (right) was tagged with six tandem HA tags and samples were processed as described from [gar$^-$] and [GAR$^+$] strains of each background. Total, supernatant (sup.), digitonin soluble (det. sol.), and digitonin insoluble (insol.) fractions were run on SDS gels and probed for Pma1 and Std1 or Mth1 (lower right). Blots of the total fraction were stained with Ponceau Red to confirm equal amounts of starting material (top right).

protein, we introduced a stop codon at position 23 or 59 on the N-terminal region. Neither plasmid increased [GAR$^+$] frequency relative to vector alone (figure S2.05).

We could not test the effect of Pma1 deletions on [GAR$^+$] propagation because *PMA1* is essential. Indeed, even deletions of the N-terminal domain is lethal (Portillo et al., 1989). Mutations of up to 40aa from the N-terminus are tolerated but only when expressed from a very strong promoter such as *GAL1* (Liu et al., 2006). [GAR$^+$] was propagated through *PMA1*Δ40N cells (figure S2.06). Strikingly, however, it was not propagated through a *PMA1*Δ40N Δ*std1* double mutant (figure 2.3c), suggesting that Std1 and the N-terminus of Pma1 are together involved in [GAR$^+$] propagation but that each can maintain [GAR$^+$] in the absence of the other.

Pma1 functions as an oligomeric complex at the plasma membrane (Kuhlbrandt et al., 2002). Because transient overexpression of *STD1* induces [GAR$^+$] and *MTH1* inhibits it we asked if there were heritable changes in association of these proteins with Pma1 in [gar$^-$] and [GAR$^+$] cells. In both [gar$^-$] and [GAR$^+$], Pma1 formed unresolved high molecular weight (HMW) oligomers and two lower molecular weight (LMW) oligomers when visualized by Blue Native gel analysis (figure 2.3d). Std1 was more strongly associated with the LMW oligomers in [GAR$^+$] than in [gar$^-$]. Mth1, the homolog of Std1, showed the reverse: stronger association with LMW oligomers in [gar$^-$] than in [GAR$^+$]. Pma1 showed a minor but statistically significant change in protease sensitivity between [gar$^-$] and [GAR$^+$]: total Pma1 was digested slightly more rapidly by trypsin when vesicles are isolated from [GAR$^+$] cells than from [gar$^-$] (figure S2.07). Small differences in protease sensitivity could be consistent with the small amounts of Pma1 associated with Std1 in [GAR$^+$].

Pma1 does not change SDS solubility between [gar$^-$] and [GAR$^+$]

Other yeast prions exhibit changes in localization and solubility when they enter into the prion state (Uptain and Lindquist, 2002). Neither Pma1 nor Std1 formed an SDS-resistant species in [GAR$^+$] (figure S2.08). The lack of an SDS-resistant Pma1 species and targeting to the same cellular location in [gar$^-$] and [GAR$^+$] show that Pma1 does not form amyloid in [GAR$^+$].

Pma1 is a determinant of [GAR$^+$]

In order to strengthen the connection between Pma1 and [GAR$^+$], we investigated whether mutations in genes involved in Pma1 oligomerization and trafficking to the plasma membrane alter [GAR$^+$] frequency. We hypothesized that genes involved in Pma1 trafficking and oligomerization, as shown by previous studies and Blue Native gels would exhibit a change in [GAR$^+$] frequency when knocked out (fatty acid synthase *SUR4* (Lee et al., 2002) and COPII coat protein *LST1* (Roberg et al., 1999)). Mutants that do not affect Pma1 oligomerization (sphingolipid synthesis genes *LCB3, LCB4, DPL1*) (Lee et al., 2002) or affect trafficking of mutant Pma1 but not wildtype (*ATG19*) (Mazon et al., 2007) should have the same [GAR$^+$] frequency. This was indeed the case: mutants that showed changed Pma1 oligomer patterns by Blue Native gel (figure 2.3d) also showed decreased [GAR$^+$] frequency (figure 2.3e). Overall, these data demonstrate that alterations in Pma1 oligomers are correlated with alterations in [GAR$^+$] frequency.

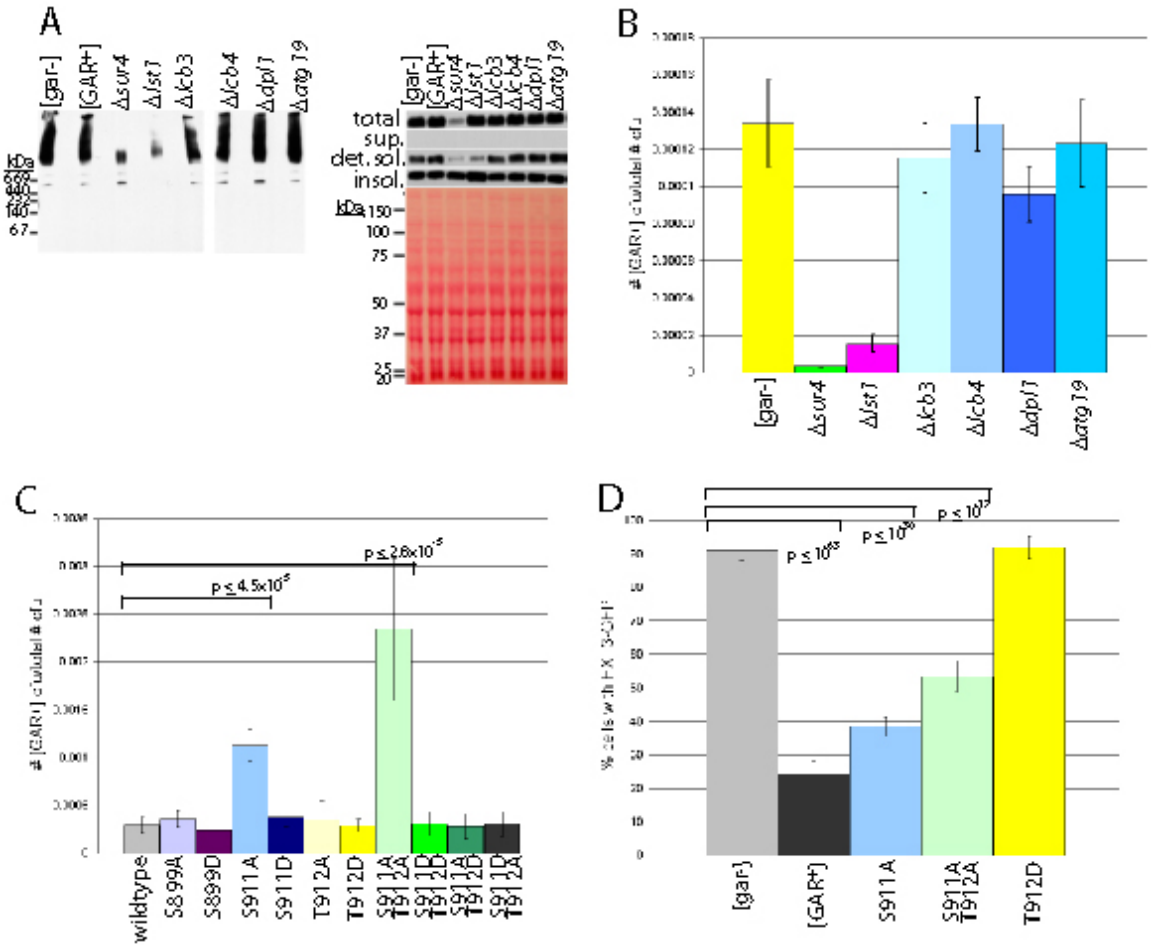

Figure 2.4: Alterations to Pma1 affect [GAR$^+$]

Figure 2.4: Alterations to Pma1 affect [GAR$^+$]

a) Native gel blotted for Pma1 from knockout mutants of genes previously shown to affect (Δ*sur4*, Δ*lst1*) (Eisenkolb et al. 2002) (Roberg et al. 1999) or not affect (Δ*lcb3*, Δ*lcb4*, Δ*dpl1*) (Gaigg et al. 2005) attributes of wildtype Pma1 (left). SDS gels of total, supernatant (sup.), digitonin soluble (det. sol.), and digitonin insoluble (insol.) fractions were probed with αPma1 antibody following blotting (right). The "total" blot was also stained with Ponceau Red to confirm equal amounts of starting material (bottom right). b) Measurement of [GAR$^+$] frequency in mutants from part a. c) Mutants in phosphorylation sites at the C-terminus of Pma1 affect [GAR$^+$] frequency. Starting strain is haploid, [gar$^-$], genotype *pma1::kanMX* with p316-PMA1. p314-PMA1 carrying wildtype *PMA1* or mutants of interest were transformed in and then p316-PMA1 plasmid selected against by growth on 5-FOA. Graph represents the mean +/- standard deviation (n=6). P-values are the binomial distribution of the mean. d) Pma1 mutants that increase [GAR$^+$] frequency show decreased levels of Hxt3-GFP. Graph represents the mean +/- standard deviation ($n \geq 6$) and p-values were determined using the chi-squared test. Strain background is a hybrid of W303 and S288C.

Furthermore Δ*lst1* and Δ*sur4* reduced the frequency of [GAR$^+$] appearance they were not required for its propagation (figure S2.09).

Pma1 activity is regulated by both glucose and other environmental conditions. The addition of glucose to carbon-free medium increases the ATPase activity of the enzyme (Serrano, 1983) and results in a conformational shift (Miranda et al., 2002). Glucose-dependent phosphorylation contributes to this phenomenon (Lecchi et al., 2005). Residues in the C-terminal cytoplasmic tail of the protein, S899 (Eraso et al., 2006), S911, and T912 (Lecchi et al., 2007) are phosphorylated in response to carbon source conditions and are thought to contribute to the conformational shift in Pma1. There is also evidence that the N-terminal region of Pma1 is phosphorylated but particular sites have not been identified (Lecchi et al., 2007). This is unfortunate, as our previous data implicate the N-terminal region in [GAR$^+$] induction (figure 2.3b). However, we hypothesized that if Pma1 causes [GAR$^+$], changing the activity and conformation of Pma1 would affect [GAR$^+$] frequency, even if the mutations made were not located in the putative prion-determining domain.

Pma1 has been shown to be responsive to glucose (Serrano, 1983) but has not been linked to a particular signaling pathway. Our observation that Pma1 associates with Std1, a member of the Rgt2/Snf3 glucose signaling pathway, implies a connection. To test this, we mutated the well-characterized regulatory sites S899, S911, and T912 to alanine, which cannot be phosphorylated, or aspartic acid, which mimics constitutive phosphorylation. We then measured whether these mutants affect [GAR$^+$] frequency or *HXT3* expression. The latter serves as a downstream readout of the Rgt2/Snf3 pathway and, as demonstrated above, *HXT3* is turned off in [GAR$^+$] (figure 2.2a).

Two mutations, S911A and S911A/T912A, increased [GAR$^+$] frequency (figure 2.4c). These same two mutants also had reduced Hxt3-GFP signal (figure 2.4d). No other mutant showed a significant change, either increased or decreased, although T912D, which showed no significant change in [GAR$^+$] frequency or Hxt3-GFP signal compared to wildtype, was included as an additional control. This demonstrates a connection between Pma1 and the Rgt2/Snf3 glucose signaling pathway. Further, the fact that mutants in Pma1 affect the frequency of [GAR$^+$] appearance by as much as 10 fold supports the role of Pma1 in determining the prion state.

[GAR$^+$] is sensitive to a Pma1-dependent "species barrier"

To test the relation between Pma1, Std1, and [GAR$^+$] more definitively, we performed a classic "species barrier" experiment. Small differences in amino acid sequence cause prions that originate in one species to fail in transmission to another species. Prions cannot even propagate through their originating organism if the prion-determining gene from the host has been replaced with the prion-determining gene from another species. This has been observed for mammalian prions (Prusiner, 1998) and for [PSI$^+$] (Santoso et al., 2000; Bagriantsev and Liebman, 2004; Chen et al., 2007).

We chose two species, *S. bayanus* and *S. paradoxus*, which can acquire spontaneous glucosamine-resistance (figure 2.5a). The sequence differences in Pma1 between the species are slight (figure S2.10): *S. paradoxus* Pma1 differs from *S. cerevisiae* Pma1 only at four amino acids, three of them in the N-terminus, and *S. bayanus* Pma1 differs from *S. cerevisiae* in fewer than a dozen amino acids, most in the N-terminus. In a [GAR$^+$] background, we replaced *S. cerevisiae PMA1* with that from

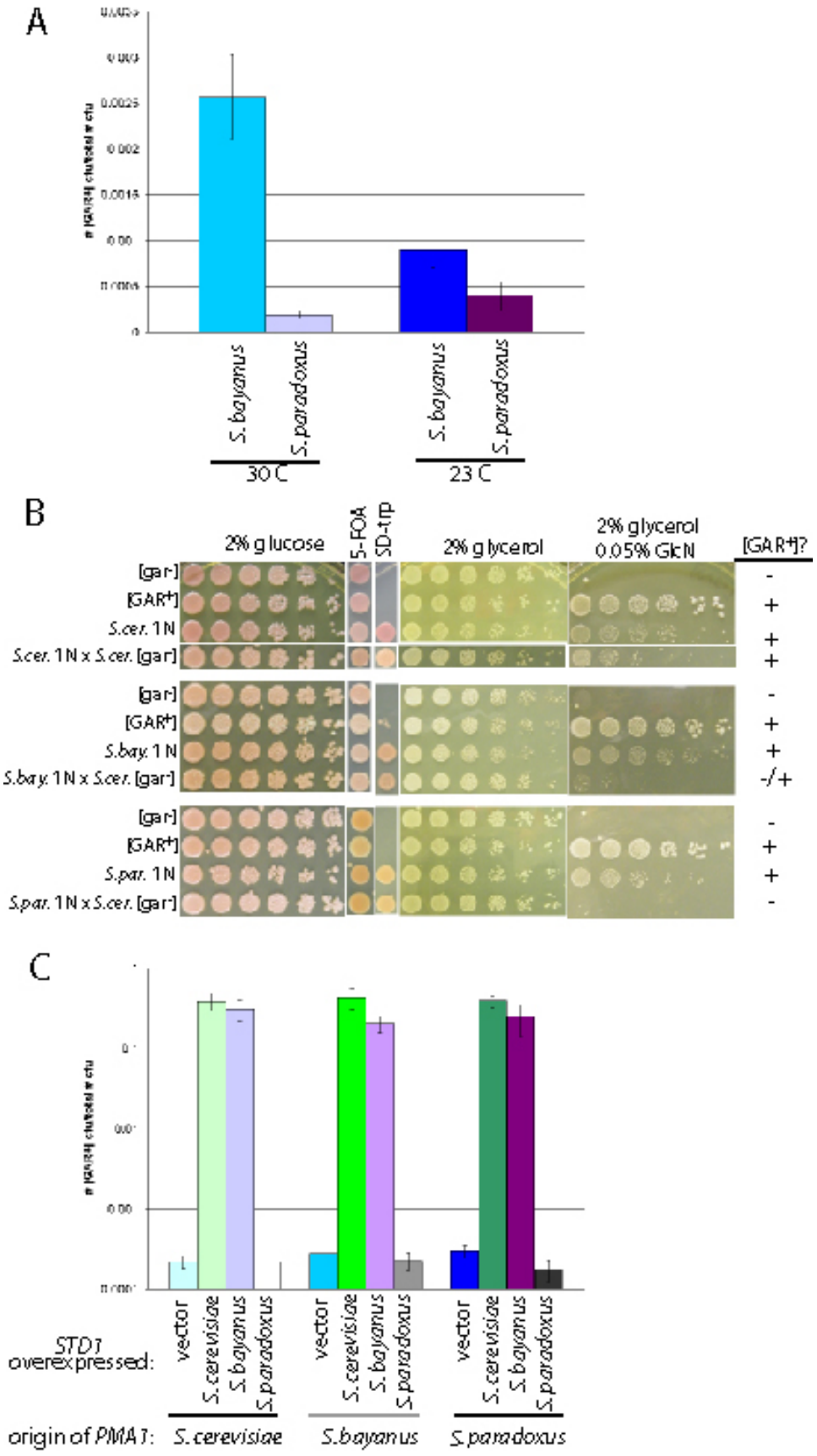
A
S. bayanus
S. paradoxus
S. bayanus
S. paradoxus
30 C
23 C
B
2% glucose
5-FOA
SD-trp
2% glycerol
2% glycerol
0.05% GlcN
[GAR+]?
[gar-]
[GAR+]
S.cer. 1N
S.cer. 1N x S.cer. [gar-]
S.bay. 1N
S.bay. 1N x S.cer. [gar-]
S.par. 1N
S.par. 1N x S.cer. [gar-]
C
vector
S. cerevisiae
S. bayanus
S. paradoxus
STD1 overexpressed:
origin of PMA1:
S. cerevisiae
S. bayanus
S. paradoxus

Figure 2.5: [GAR$^+$] exhibits a Pma1-dependent species barrier

a) The frequency of [GAR$^+$] cells in populations of *S. bayanus* and *S. paradoxus* were measured at 30°C, the optimal growth temperature of *S. paradoxus*, and 23°C, a preferred growth temperature of *S. bayanus*. b) Substitution of *PMA1* from *S. cerevisiae* with *PMA1* from *S. bayanus* or *S. paradoxus* prevents [GAR$^+$] propagation. Starting strain is haploid, [GAR$^+$], genotype *pma1::kanMX* with p316-PMA1 *S. cerevisiae* as a covering plasmid. p314-PMA1 carrying *PMA1* from *S. cerevisiae* (*S.c.*, top), *S. bayanus* (*S.bay.*, middle), or *S. paradoxus* (*S.par.*, bottom) was transformed in and p316-PMA1 *S.c.* selected against by replica plating to 5-FOA (*S.c.* 1N, *S.b.* 1N, or *S.p.* 1N). These haploids were mated to a wildtype *S. cerevisiae* [gar$^-$] background, restreaked two times, and tested for [GAR$^+$]. Representative data from three independent experiments is shown.

the *sensu stricto* species *S. bayanus* and *S. paradoxus* via plasmid shuffle (see plasmids table, S2.4). The *S. paradoxus* or *S. bayanus* transgenics were then mated to wildtype [gar$^-$] *S. cerevisiae* to test the ability of the Pma1 protein from *S. paradoxus* or *S. bayanus* to both hold and propagate *S. cerevisiae* [GAR$^+$] (figure 2.5b). In a background where the entire genome otherwise remains the same, *S. paradoxus PMA1* did not propagate *S. cerevisiae* [GAR$^+$]. *S. bayanus PMA1* propagated *S. cerevisiae* [GAR$^+$] very weakly.

PMA1 demonstrated a strong species barrier preventing [GAR$^+$] propagation. Does [GAR$^+$] induction also exhibit a species barrier? The spontaneous appearance of glucosamine-resistant colonies is much higher in *S. cerevisiae* and *S. bayanus* than in *S. paradoxus*, despite *S. paradoxus* Pma1 being closer to *S. cerevisiae* in sequence than *S. bayanus* Pma1 is (figure S2.11). However, the sequence of Std1, the [GAR$^+$] induction factor, is much closer in *S. cerevisiae* and *S. bayanus*. We therefore tested whether induction of [GAR$^+$] by Std1 exhibits a species barrier. *STD1* from the various species was transiently overexpressed in cells carrying Pma1 from the different species (figure 2.5c). *STD1* from *S. cerevisiae* and *S. bayanus* acted as general inducers: in a background where the entire genome otherwise remains the same, both *STD1* alleles induced [GAR$^+$] almost 1000 fold in strains carrying *PMA1* from any of the three sibling species. In contrast, *STD1* from *S. paradoxus* did not induce [GAR$^+$] in strains carrying any *PMA1*. Std1 from *S. cerevisiae* and *S. bayanus* therefore acts as an inducer of [GAR$^+$] but *S. paradoxus* Std1 does not. Std1, like Pma1, contributes to the strong species barrier observed in [GAR$^+$] induction and propagation.

Discussion

The ability of cells to sense and adapt to their nutritional circumstances is crucial to survival in the highly competitive and rapidly fluctuating environment. Here we describe [GAR$^+$], a prion-based heritable element involved in glucose sensing and signaling. We demonstrate that [GAR$^+$] involves the plasma membrane proton pump Pma1 and the glucose signaling factor Std1. [GAR$^+$] fulfills the genetic criteria of prions: it shows non-Mendelian inheritance, can by transferred via cytoplasmic exchange, is metastable, and is semi-dominant. However, Pma1 and Std1 do not aggregate or form an SDS-resistant species (and thus amyloid) in [GAR$^+$]. Instead, Pma1 associates with Std1 in [GAR$^+$] but the homolog of Std1, Mth, in [gar$^-$], all while remaining at the plasma membrane. Pma1, certainly, and possibly Pma1 and Std1 together, are involved in [GAR$^+$] propagation, thus possibly making [GAR$^+$] a prion with multiple determining factors. Mutations in glucose-sensitive phosphorylation sites affect silencing of *HXT3*, demonstrating that Pma1 is involved in glucose signaling. These same phosphorylation site mutants affect [GAR$^+$] frequency, suggesting a method of regulation of [GAR$^+$]. Overall, the non-Mendelian mechanism that best describes [GAR$^+$] is prion.

[GAR$^+$] is a non-Mendelian element that functions through a prion-based non-amyloid mechanism

[GAR$^+$] fulfills all the genetic characteristics of yeast prions but differs in that it does not involve a heritable amyloid conformation. This likely explains why [GAR$^+$] does not require Hsp104, the canonical prion chaperone, for propagation. Prusiner's original definition of "prion" was simply "proteinaceous infectious particle that lacks

nucleic acid" (Prusiner 1998). This neither proposes a mechanism nor requires that a prion-causing protein undergoes a conformational shift. In fungi, a self-activating protease, [β], can act a prion (Roberts and Wickner, 2003). [β] fulfills all the genetic characteristics (Wickner, 1994) of an infectious proteinaceous element: it catalyzes its own activity by cleaving the inactive form, thus converting it to the active form, and the presence of the active form is therefore self-propagating. However, [β] does not involve a conformational change in the causal protein but instead an enzymic self-catalyzing cleavage (?that is only self-activating in the absence of another protease) (Roberts and Wickner, 2003). The use of the term "prion" in this context has been criticized because it to date only applies to an artificial situation, not one that occurs spontaneously or is found in nature.

Another non-amyloid prion, *Crippled growth* (C), found in the filamentous fungus *Podospora anserina*, is thought to result from a self-activating MAP kinase cascade (Kicka et al., 2006) but bears some resemblance to conventional prions because one *C* causal protein contains a poly-glutamine region and several of the involved proteins show a change in localization between *C* and normal growth (Kicka and Silar, 2004). [GAR^+] also fulfills all the genetic criteria of an infectious proteinaceous element and does not form an amyloid. However, [GAR^+] is even more unusual than *C* because it does not cause a change in localization of involved proteins nor includes a glutamine- or asparagine-rich protein. Overall, we propose that [GAR^+] acts as a prion regardless of whether its infectivity involves a conformational change in Pma1. Instead, we support the idea that "prions" can result from a variety of infective mechanisms, including self-

activating enzymes and self-sustaining signaling cascades, as long as they are capable of acting as heritable genetic elements.

Pma1 is involved in [GAR$^+$] propagation

Overall, [GAR$^+$] exhibits a strong species barrier for propagation that depends on Pma1 (figure 2.5b). [GAR$^+$] also exhibits a species barrier for induction that involves Std1 (figure 2.5c). This conclusively demonstrates the importance of Pma1 in [GAR$^+$] propagation. However, these results might not be explained by sequence alone. It is possible that the swap of *PMA1* from different *Saccharomyces* species cures [GAR$^+$] because it disrupts interactions between Pma1 and other proteins such as Std1. Also, we have shown that a double mutations in *std1* and the N-terminal 40aa of *pma1* is sufficient to cure [GAR$^+$] (figure 2.3c) even though neither single mutant alone is sufficient (figure 2.2d, S2.04). Therefore, it is possible that Std1 plays a role in the propagation of [GAR$^+$] and not just the induction, perhaps by stabilizing the [GAR$^+$]-forming Pma1 complex.

Are elements in addition to Pma1 and potentially Std1 involved in [GAR$^+$] propagation? Pma1 is the predominant protein at the plasma membrane; quantity alone suggests that it has the opportunity to interact with a wide variety of other proteins. It is possible that other elements are involved in the [GAR$^+$] heritable structure in addition to Std1 and Pma1. Furthermore, these proteins are involved in signaling cascades and are thus likely to participate in numerous transient associations. Our work to identify additional factors has not yet but successful but this is an interesting area for future exploration.

Does [GAR$^+$] cause a new signaling complex?

[GAR$^+$] exhibits some unusual and intriguing characteristics for yeast prions, particularly that it does not involve formation of an amyloid-like conformation. Instead, [GAR$^+$] involves an inducing factor, Std1, which stably associates with Pma1 in the [GAR$^+$] form. Pma1 and Std1 remain at the plasma membrane to cause [GAR$^+$]. Changes in Pma1 oligomer size correlate with altered [GAR$^+$] frequency, and Pma1 alters its association with a signaling protein, from Mth1 in [gar$^-$] to Std1 in [GAR$^+$]. We therefore hypothesize that [GAR$^+$] causes its phenotype, glucosamine resistance, by altering signaling through a glucose signaling pathway. This signal does not affect the Snf1 pathway, otherwise greater transcriptional differences between [gar$^-$] and [GAR$^+$] would be observed in our microarray experiments. It does, however, affect the Snf3/Rgt2 signaling pathway as shown by the change in *HXT3* expression. We thus speculate that [GAR$^+$] increases the ability of Std1 to silence *HXT3*, perhaps by increasing its affinity for that DNA binding protein Rgt1 (figure 2.6). The increased association of Std1 and Pma1 could allow for this change either directly, by, for example, preventing degradation of Std1, or indirectly. We have also uncovered a novel role for Pma1 in glucose sensing, as shown by alterations in Hxt3-GFP signal in Pma1 mutants. Although Pma1 was known to respond to and be controlled by glucose (Morsomme et al, 2000), it has not previously been linked to glucose signaling itself.

[GAR$^+$] is semi-dominant

[GAR$^+$] is also unusual for being semi-dominant in a mating rather than dominant, as [PSI$^+$] are [URE3] are. Instead, a mixed population of large (strong) and

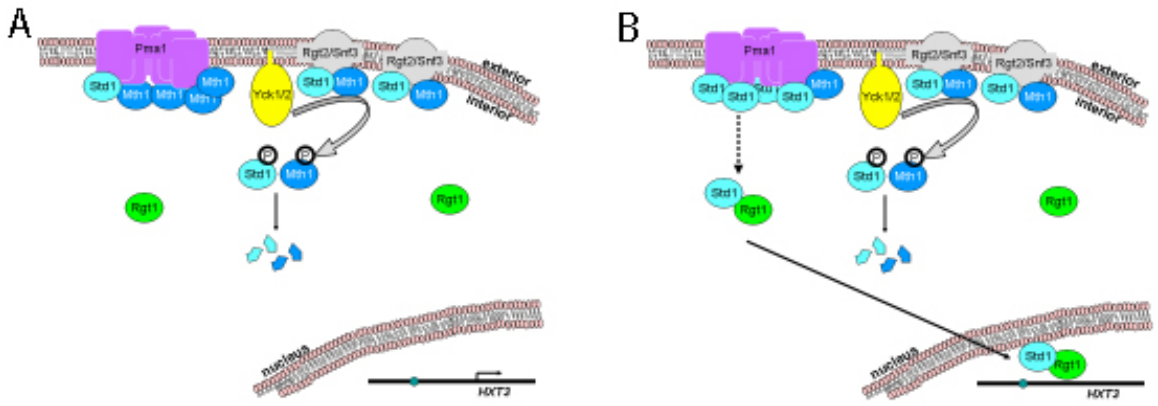

Figure 2.6: Pma1 and the Rgt2/Snf3 glucose signaling pathway

We propose that Pma1 acts as a part of the Rgt2/Snf3 signaling pathway. a) In [gar$^-$] glucose-grown cells, Pma1 associates with Mth1. The glucose signal is propagated through Snf3 and Rgt2 to Yck1 and Yck2, which phosphorylate Mth1 and Std1. This phosphorylation marks Mth1 and Std1 for degredation, leaving their interacting partner, Rgt1, free in the cytosol, where it does not repress transcription at the *HXT3* locus. b) Under [GAR$^+$] conditions, *HXT3* transcription is repressed, which resembles that of cells grown in a carbon source other than glucose. Pma1 associates with Std1, which somehow facilitates the repression of *HXT3*, possibly by altering the affinity of Std1 for Rgt1. Association with Std1 has previously been shown to facilitate the binding of Rgt1 to DNA (Lakshmanan et al., 2003).

small (weak) colonies appear on glucosamine medium following mating. Strong [GAR$^+$] is very stable in meiosis and weak [GAR$^+$] somewhat so, not because weak [GAR$^+$] switches to [gar$^-$] but because it converts to strong [GAR$^+$]. Weak [GAR$^+$] also converts to strong [GAR$^+$] under standard mitotic conditions within approximately 25 generations. This could be because formation of strong [GAR$^+$] is a multi-step process and weak [GAR$^+$] represents an unstable intermediate on the pathway to strong [GAR$^+$]. Std1 promotes [GAR$^+$], as transient overexpression of *STD1* induced [GAR$^+$], and Mth1 has an inhibitory affect, as transient overexpression of *MTH1* blocked [GAR$^+$] (table 2.1). Inhibition of [GAR$^+$] formation by *MTH1* is likely due in part to transcriptional repression of *STD1*, which has been observed when *MTH1* is overexpressed (Kim et al., 2006). The association of Mth1 with Pma1 in the [gar$^-$] state could prevent Std1 from associating with Pma1 and thus forming the [GAR$^+$] state. Another possibility is that [gar$^-$] and [GAR$^+$] are both self-propagating states involviong their associations with Mth1 and Std1, respectively, and weak [GAR$^+$] is a middle state that can convert to either [gar$^-$] or strong [GAR$^+$], which are then self-perpetuating. Regardless, the opposing results from *STD1* and *MTH1* overexpression are intriguing because *MTH1* and *STD1* are paralogs that are ~61% identical on the amino acid level (Hubbard et al., 1994). Neither, however, have any known enzymatic activity or domains identifiable by BLAST analysis.

Pma1 is conserved across the fungal kingdom and is even found in some plants. Our discovery that an extremely well-conserved transmembrane protein can act as a prion

raises questions whether the prion phenomenon is widespread. Pma1 forms high molecular weight oligomers in plants and is regulated by nutrients (Duby and Boutry, 2008) and might therefore have a [GAR$^+$]-like form. Because Pma1 is so well-conserved it would be extremely interest to investigate whether the [GAR$^+$] prion is found in a variety of organisms. Further, if [GAR$^+$] is common, this lends support to the idea that self-propagating protein-based genetic elements might be more common as a mechanism of regulation than previously thought.

Experimental Procedures

Yeast strains and genetic manipulations

Strain construction and manipulation followed standard yeast techniques. A list of strains and plasmids used in this study is available in table S1. Unless otherwise stated, data shown is from genetic background W303. Five-fold dilutions were used for all spotting assays. Growth rate was measured in the Bioscreen C (Growth Curves USA) at 30°C with intensive, intermittent shaking with the OD_{600} measured every 15 minutes.

[GAR$^+$] frequency assays and isolation of [GAR$^+$]

Cultures for [GAR$^+$] frequency assays were grown overnight in 2% glucose, either YPD or SD, subcultured in the same, then grown to early exponential phase (OD_{600} = 0.2-0.4). Cultures plated straight to GGM (1% yeast extract, 2% peptone, 2% glycerol, 0.05% D-(+)-glucosamine [Sigma G4875]) and diluted 10^{-4} for plating to YPD. To isolate [GAR$^+$]

for further study, colonies from GGM were restreaked once to GGM then used in downstream applications.

Western blotting

Protein samples were run on 4-12% SDS gels from Invitrogen and blotted to PVDF using standard techniques. All samples to be tested for Pma1 were incubated in loading buffer (4% SDS, 50mM Tris pH 6.8, 2% β-mercaptoethanol, 10% glycerol) for 10min at 37°C. Monoclonal αPma1 mouse antibody was obtained from EnCor Biotechnology. Polyclonal αPma1 rabbit antibody was a gift from Amy Chang. Polyclonal αSec61 antibody was a gift from Tom Rapaport. Immune complexes were visualized by ECL.

Microarray analysis

PolyA RNA was produced using standard methods (cite). Samples were labeled and hybridized to Affymetrix S98 arrays using standard methods (cite).

Hxt3-GFP analysis

Hxt3-GFP signal was observed starting at OD_{600} = 0.7 in an S288C background.

Immunoprecipitation

IPs were performed using standard in IP buffer (50mM HEPES pH 7.5, 150mM NaCl, 2.5mM EDTA, 1% V/VTriton X-100, 40mM NEM, 3mM PMSF, 1 Protease Inhibitor Cocktail Tablet per 5ml buffer [Roche]). Cells were lysed either by bead beating (9 x 30sec with 15sec on ice between) or spheroplasting (30min at 30°C in 1M D-sorbitol,

0.1M EDTA, 0.5mg/ml zymolase) with comparable results. Lysates were adjusted for protein concentration, incubated with protein G agarose beads (Roche) for 30min at 4°C, centrifuged at 3300 x g for 2min, and the supernatant collected. The supernatant was then incubated with 10μg mouse αHA antibody (Sigma) for 1 hour at 4°C followed by incubation with 50μl protein G beads (Roche) for 1 hour at 4°C. Samples then washed six times in chilled IP buffer and run on a 4-12% SDS gel. Gels were either stained with colloidal Coomassie (Invitrogen) or blotted for Pma1.

Native gels

Midlog cultures (150ml, OD_{600}~0.5) were lysed by bead beating (9 x 30sec with 15sec on ice between) into sorbitol buffer (250mM sorbitol, 50mM Tris pH 7.5, 3mM PMSF, 1 Protease Inhibitor Cocktail Tablet per 5ml buffer [Roche]). Samples were equalized at a concentration of 15μg/μl in 650μl, a "total" sample collected, and centrifuged at 16000 x g for 30min at 4°C. The supernatant was removed, a sample saved for downstream analysis, and the pellet washed once in sorbitol buffer. The pellet was resuspended in sorbitol buffer (200μl), and an aliquot (95μl) incubated 20min on ice with digitonin to 1% (Calbiochem). These samples were then centrifuged at 16000 x g at 4°C for 30min and separated into supernatant ("digitonin soluble") and pellet ("digitonin insoluble") fractions. 15μl of the soluble fraction was incubated with Coomassie G-250 at a detergent to dye ratio of 8:1 for 10min on ice then loaded onto 3-12% Blue Native gel (Invitrogen) and run at 4°C as per the manufacturer's instructions.

Trypsin digestion

Cells were grown to mid exponential phase (OD_{600}~0.5), washed three times in water, then lysed by bead beating (9 x 30sec with 15sec on ice between) into sorbitol buffer (250mM sorbitol, 50mM Tris pH 7.5, 3mM PMSF, 1 Protease Inhibitor Cocktail Tablet per 5ml buffer [Roche]). Samples were centrifuged at 16000 x g for 30min at 4°C, the supernatant removed, then washed three times in sorbitol buffer with protease inhibitors and three times in sorbitol buffer without protease inhibitors. For trypsin reactions, 10μg protein and 4μg trypsin (Worthington) were used in a total volume of 20μl. Reactions were incubated at 30°C and stopped after the designated point in time by addition of 2μl soybean trypsin inhibitor (10mg/ml stock, from Sigma) then immediately frozen in an ethanol/dry ice bath. Samples were run on gels as described above, probed with monoclonal αPma1, stripped, and re-probed with polyclonal αSec61.

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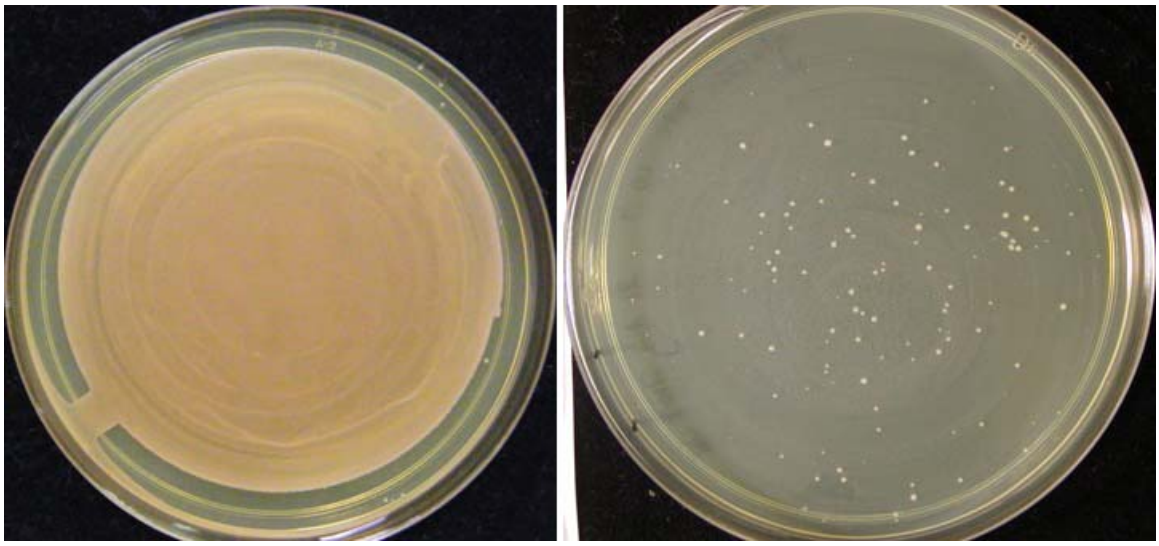
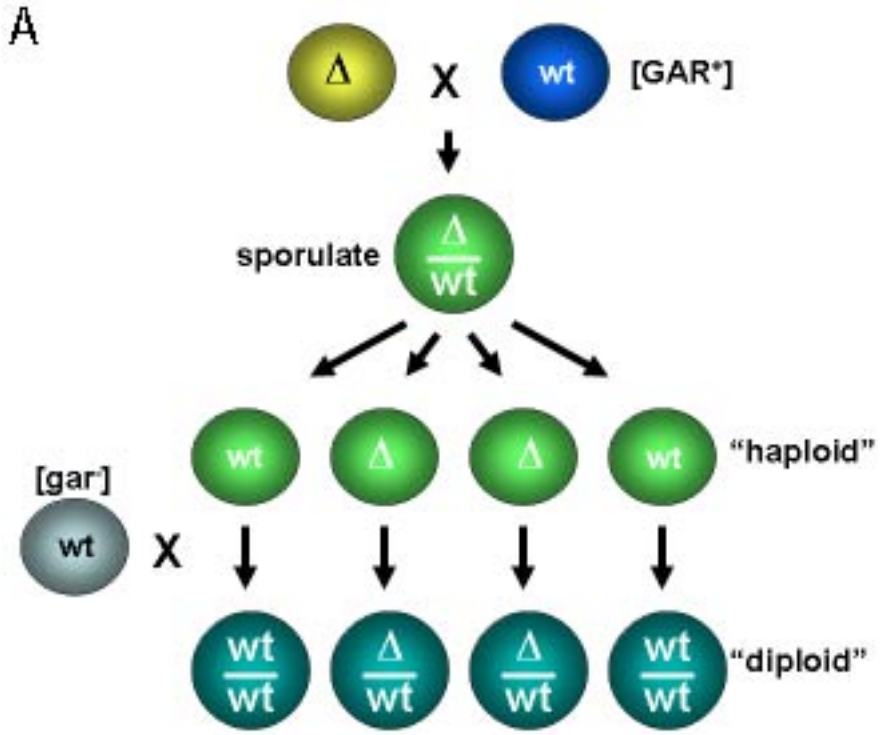


Figure S2.01: Spontaneous glucosamine resistance

Exponential phase yeast grown in YPD (2% glucose) were plate to 2% glucose (left) or 2% glycerol + 0.05% glucosamine (GGM; right). Spontaneous gluocosamine-resistant colonies are visible on the GGM plate; these are used in [GAR^+] studies.


A
Δ
X
wt
[GAR+]
sporulate
Δ/wt
[gar-]
wt
X
wt
Δ
Δ
wt
"haploid"
wt/wt
Δ/wt
Δ/wt
wt/wt
"diploid"

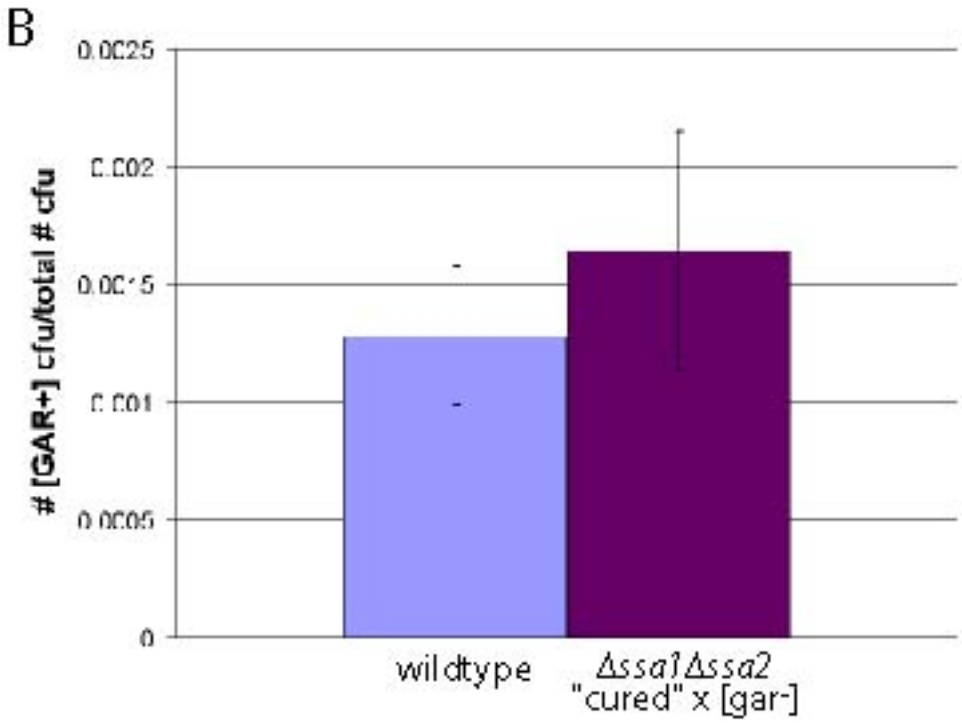

B
[GAR+] cfu/total # cfu
0.0025
0.002
0.0015
0.001
0.0005
0
wildtype
Δssa1Δssa2
"cured" x [gar-]

Figure S2.02: Hsp70-dependent curing of [GAR$^+$] is reversible

a) The crosses involved in a [GAR$^+$] propagation assay are shown. Cells carrying [GAR$^+$] were mated to [gar$^-$] cells carrying a mutation of interest ("Δ"), here Δ*ssa1*Δ*ssa2*. Diploids were selected for, then sporulated. These spores ("haploids") were then crossed to wildtype [gar$^-$] cells and we then selected for the resultant diploids ("diploids"). Both haploids and diploids were tested for glucosamine resistance; if diploids were sensitive to glucosamine, then the [GAR$^+$] heritable element cannot be propagated through the mutant of interest and [GAR$^+$] is therefore "cured" to [gar$^-$]. b) [GAR$^+$] frequency within a population of wildtype [gar$^-$] cells or cells "cured" of [GAR$^+$] by deletion of *ssa1* and *ssa2*, then crossed to [gar$^-$]. The final cross to [gar$^-$] demonstrates whether [GAR$^+$] can propagate through Δ*ssa1*Δ*ssa2* mutants, as outlined in part a. [GAR$^+$] frequency is measured in the cells that result from this cross. Because [GAR$^+$] appears spontaneously at the same frequency as wildtype, Δ*ssa1*Δ*ssa2* mutants reversibly cure [GAR$^+$]. Also, this demonstrates that [GAR$^+$] is not "cryptic" in Δ*ssa1*Δ*ssa2* mutants, otherwise all cells would be [GAR$^+$] and the measured frequency approaching 1.0.

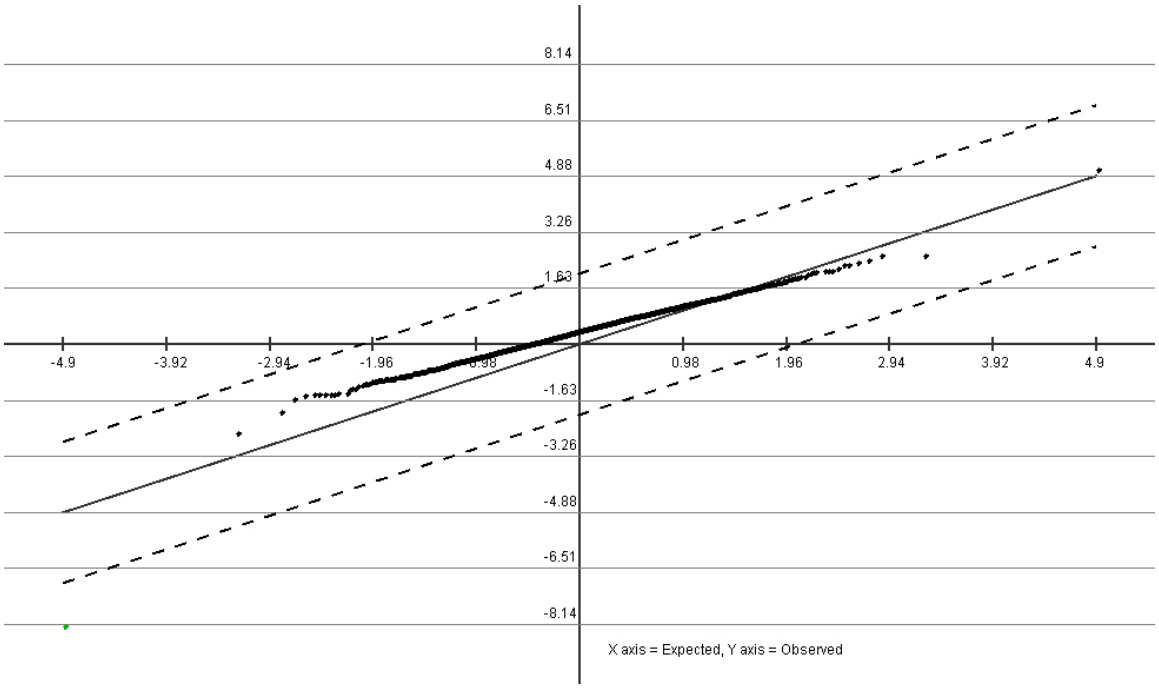


Figure S2.03: Transcriptional profiling of [gar$^-$] and [GAR$^+$] cells

A SAM plot of Affymetrix microarrays comparing [gar$^-$] and [GAR$^+$] cells grown in glucose. A single point (green) in the bottom left corner represents the only transcript that exhibits a significant change in abundance: *YDR345C* (*HXT3*).

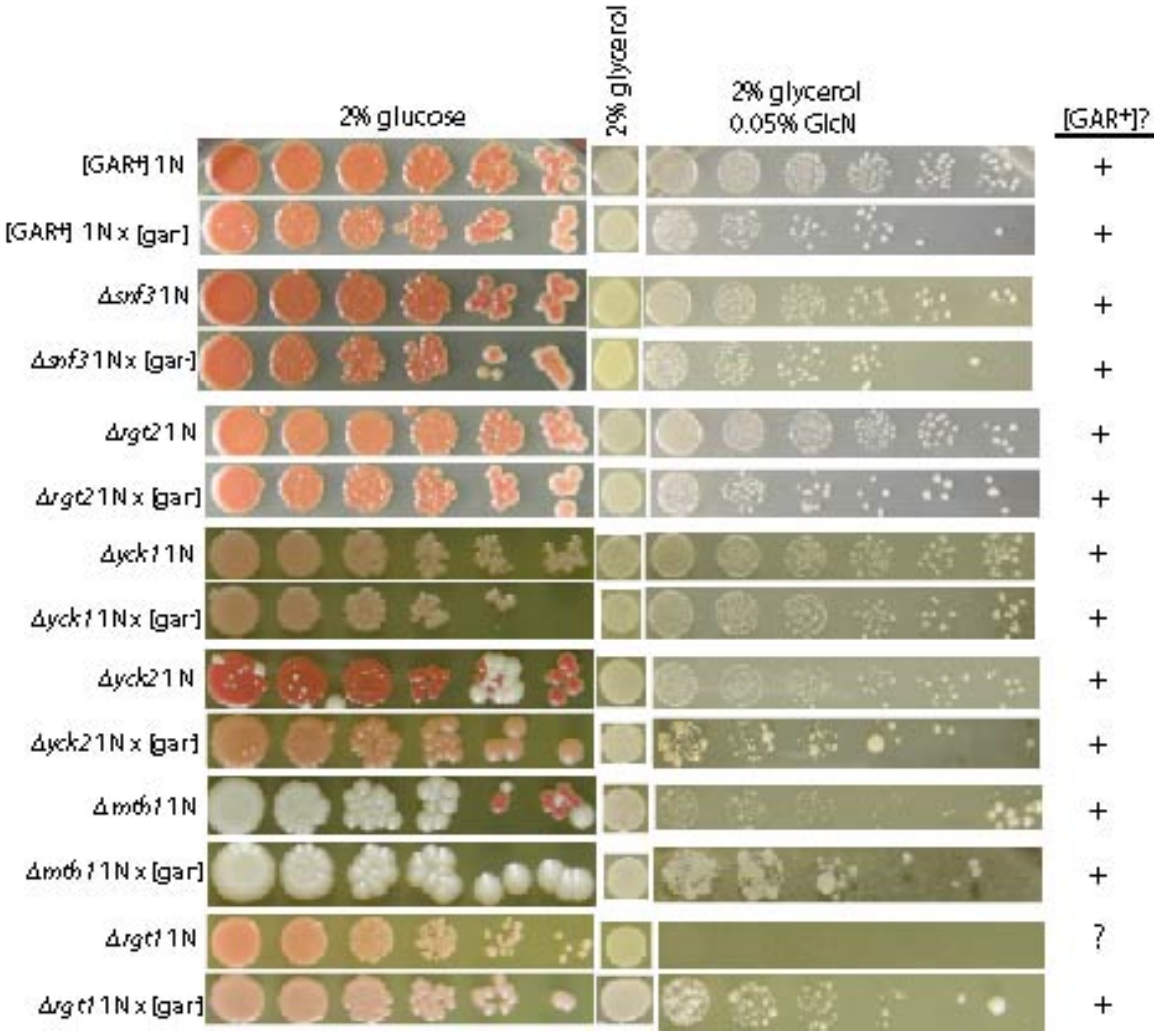


Figure S2.04: Knockout mutants of Rgt2/Snf3 pathway members propagate [GAR$^+$]

[gar$^-$] strains in which various members of the Rgt2/Snf3 pathway were knocked out were crossed to [GAR$^+$] cells, then sporulated and dissected. These spores ("1N") were tested for glucosamine resistance and then crossed to [gar$^-$] haploids to determine whether [GAR$^+$] can be propagated through these mutants ("2N") (see S2.02 for outline of crosses). Δ*rgt1* 1N cells are not glucosamine-resistant but 2N cells are, demonstrating that [GAR$^+$] is cryptic in Δ*rgt1* haploid cells. However, *RGT1* is not the causal agent of [GAR$^+$] because [GAR$^+$] can be propagated from Δ*rgt1* to wildtype cells.

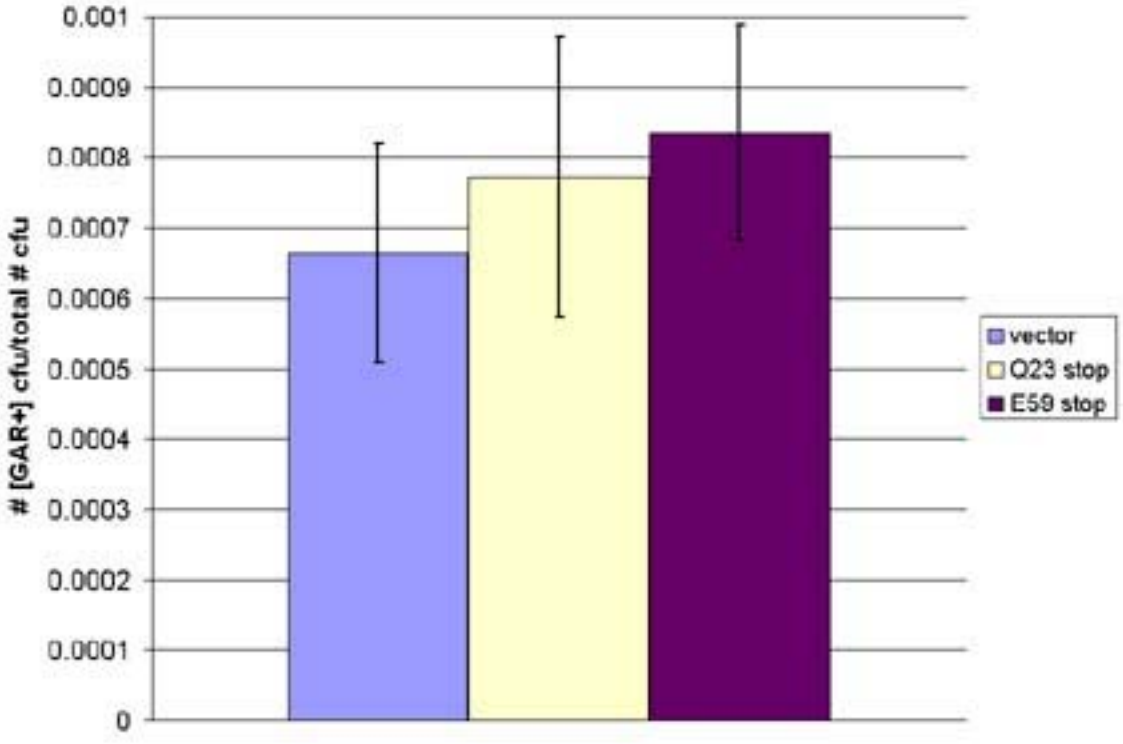


Figure S2.05: *PMA1* nonsense mutations do not induce [GAR$^+$]

The *PMA1* ORF containing nonsense mutations at Q23 or E59 was transiently overexpressed. This did not induce [GAR$^+$] relative to vector, demonstrating that the increase in [GAR$^+$] due to *PMA1* overexpression (figure 2.3b) is specific to the Pma1 protein.

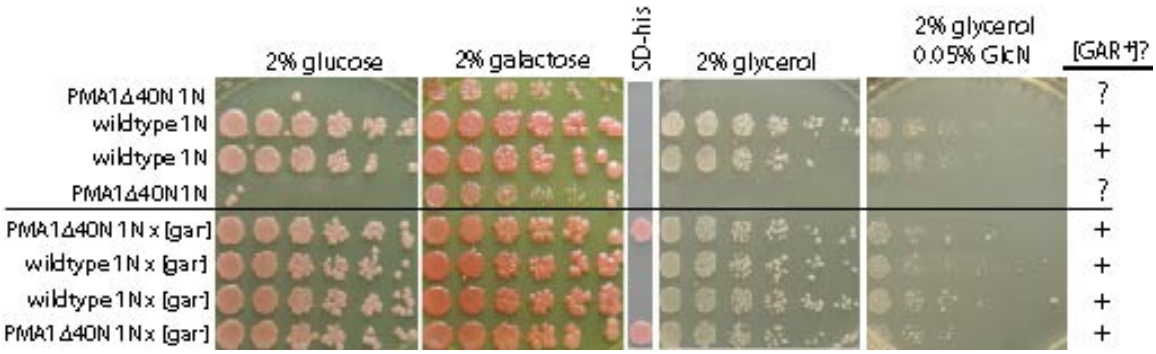


Figure S2.06: PMA1Δ40N propagates [GAR$^+$]

Top: tetrad spores from a [GAR$^+$] diploid with the genotype *GAL-PMA1Δ40N/PMA1*. The *pma1* mutation is marked with His$^+$. Wildtype spores grown on glucosamine-containing medium but *pma1* mutants cannot grown on any medium lacking galactose, so grown on glycerol-glucosamine cannot be measured. Bottom: spores from top crossed to a [gar$^-$] strain containing a wildtype *PMA1* allele. *PMA1Δ40N* spores grow on glycerol-glucosamine medium and therefore can propagate [GAR$^+$] to wildtype [gar$^-$] yeast.

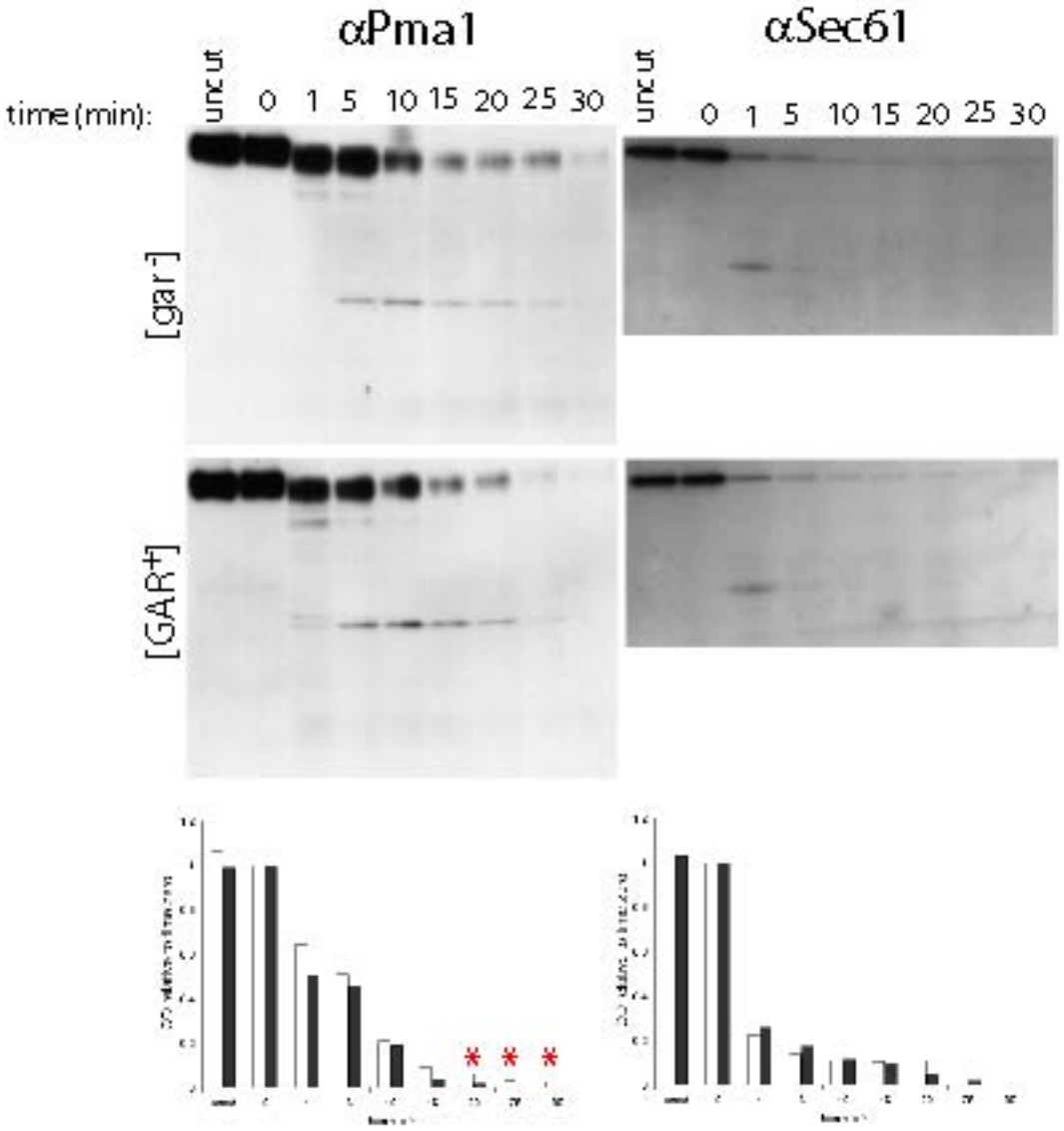
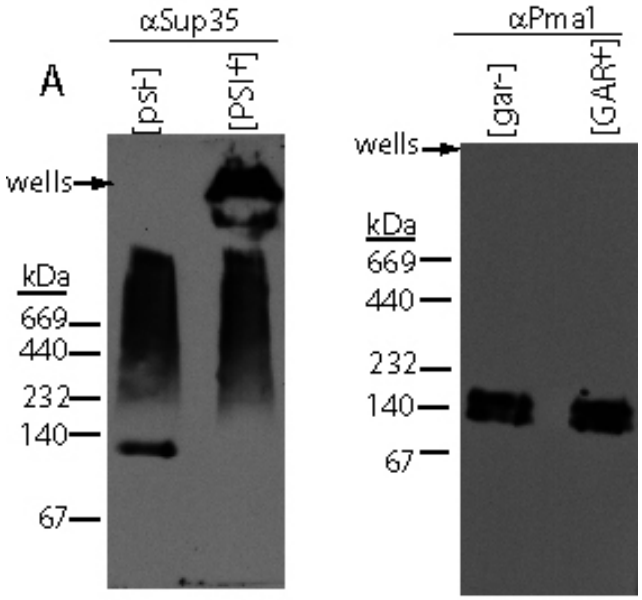


Figure S2.07: Pma1 from [GAR$^+$] is more sensitive to trypsin than [gar$^-$] Pma1

Trypsin digestion of Pma1 (left) or Sec61 (right) from [gar$^-$] (top) or [GAR$^+$] (middle). A total of six blots were averaged (bottom) and the amount of uncut Pma1 or Sec61 measured and graphed relative to t = 0. Graph represents mean (n = 6) of (t=n)/(t=0) and p-value was calculated using a paired Wilcoxon test. A red asterisks marks statistically significant points (p = 0.03).


A
αSup35
[psi-]
[PSI+]
wells
kDa
669
440
232
140
67
αPma1
[gar-]
[GAR+]
wells
kDa
669
440
232
140
67

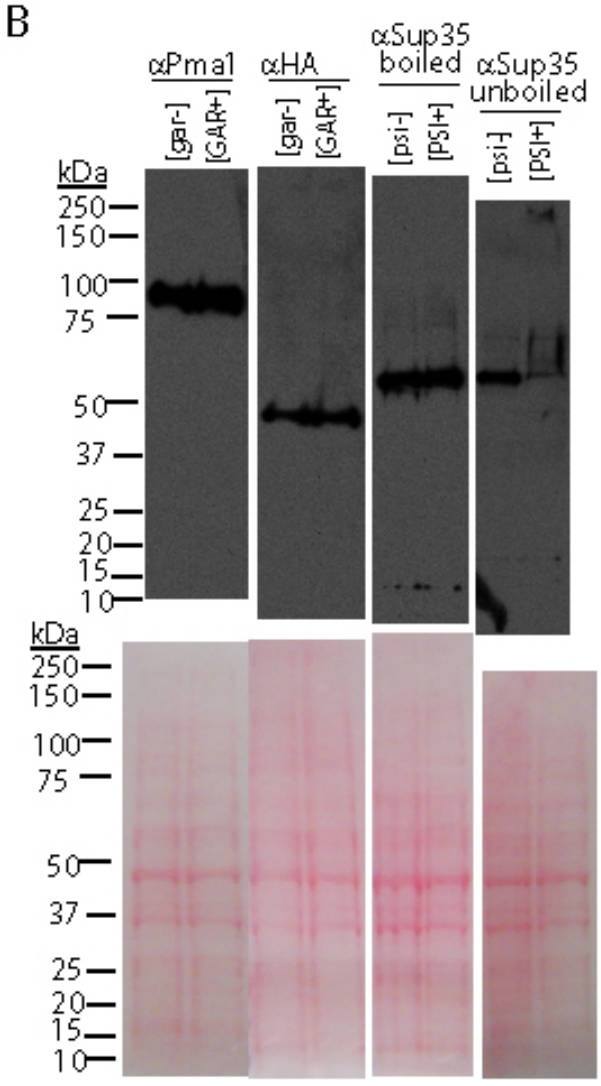

B
αPma1
[gar-]
[GAR+]
αHA
[gar-]
[GAR+]
αSup35 boiled
[psi-]
[PSI+]
αSup35 unboiled
[psi-]
[PSI+]
kDa
250
150
100
75
50
37
25
20
15
10
kDa
250
150
100
75
50
37
25
20
15
10

Figure S2.08: Pma1 and Std1 do not form SDS-resistant species

a) SDS-treated protein samples from [psi$^-$] and [PSI$^+$] (left) and [gar$^-$] and [GAR$^+$] (right) were run on Blue Native gels. Samples were incubated 10min in 4% SDS at 37°C before running, transferred by standard Western techniques, then probed with αSup35 (left) or αPma1 antibodies. Sup35 shows protein in the well in [PSI$^+$] but not in [psi$^-$], indicated a difference in SDS-solubility. This is expected because Sup35 forms amyloid in [PSI$^+$]. Pma1, however, does not show any difference in SDS-solubility between [gar$^-$] and [GAR$^+$], indicating that Pma1 does not enter into an amyloid state. b) Samples run on SDS gels and blotted for the protein of interest (top) or stained with Ponceau as a loading control (bottom). [gar$^-$] and [GAR$^+$] samples were probed with αPma1 (far left) or αHA (second left; to detect Std1-6HA). There were no differences in mobility in Pma1 or Std1 between [gar$^-$] and [GAR$^+$] samples following incubation in 4% SDS for 10min at 37°C. When [psi$^-$] and [PSI$^+$] protein samples were treated this way, however, (far right: 37°C for 10min), Sup35 protein from [PSI$^+$] runs higher than that from [psi$^-$] and does not resolve well. When protein samples are boiled, however (second right), Sup35 shows no difference in mobility between [psi$^-$] and [PSI$^+$]. Sup35 therefore behaves like an amyloid in [PSI$^+$] whereas neither Pma1 nor Std1 exhibit the SDS resistance characteristic of amyloids in [gar$^-$] or [GAR$^+$].

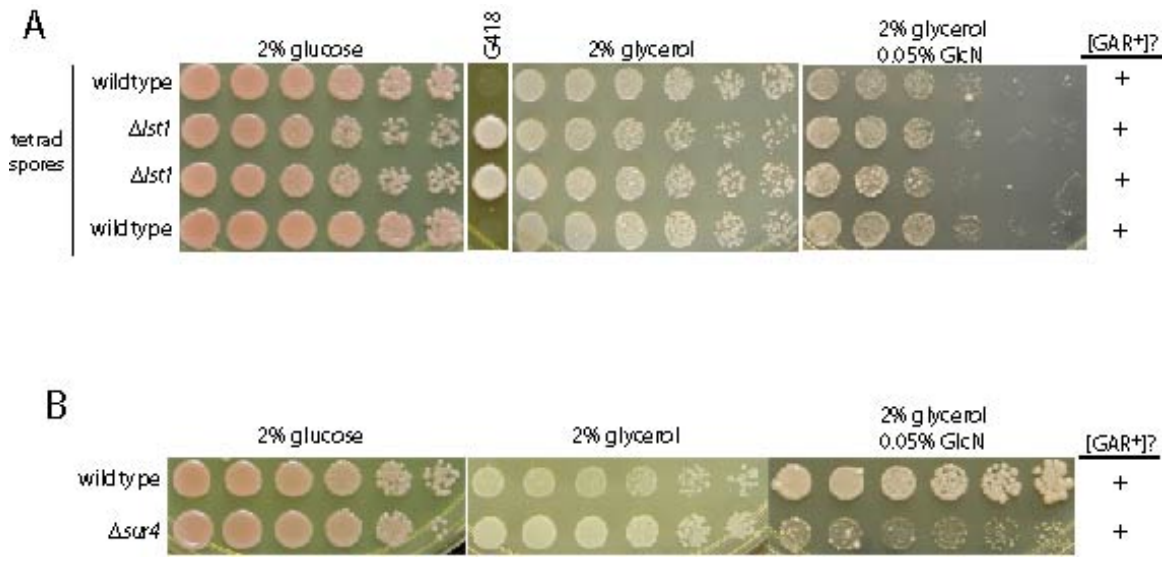


Figure S2.09: Δ*lst1* and Δ*sur4* both carry [GAR$^+$]

[gar$^-$] strains in which either *lst1* (part a) or *sur4* (part b) were knocked out were crossed to [GAR$^+$] cells, then sporulated and dissected. These spores were tested for glucosamine resistance. All spores grown on glycerol-glucosamine plates, demonstrating that Δ*lst1* and Δ*sur4* can hold [GAR$^+$].

```
      1* *****     *   *                *                   50
Scer  MTDTSSSSSS SS.ASSVSAH QPTQEKPAKT YDDAASESSD DDDIDALIEE
Spar  MADTSSSSSS SSSASSVSAH QPTQEKPAKT YDDAASESSD DDDIDALIEE
Sbay  ...MTDNTSS SSSASSASAH QPTQEKPAKT FDDAASESSD DDDIDALIDE

      51  *   ** * **           *    *                   100
Scer  LQSNHGVDDE DSDNDGPVAA GEARPVPEEY LQTDPSYGLT SDEVLKRRKK
Spar  LQSNHGVDDE GSDDDGPVAA GEARPVPEEY LQTDPSYGLT SDEVLKRRKK
Sbay  LQSNPGVDGS ESEDDGPVAA GEARLVPEEL LQTDPSYGLT SDEVLKRRKK

      101    ***    **  *                             150
Scer  YGLNQMADEK ESLVVKFVMF FVGPIQFVME AAAILAAGLS DWVDFGVICG
Spar  YGLNQMADEK ESLVVKFVMF FVGPIQFVME AAAILAAGLS DWVDFGVICG
Sbay  YGLNQMAENN ESLIIKFIMF FVGPIQFVME AAAILAAGLS DWVDFGVICG

      151        *          *                          200
Scer  LLMLNAGVGF VQEFQAGSIV DELKKTLANT AVVIRDGQLV EIPANEVVPG
Spar  LLMLNAGVGF VQEFQAGSIV DELKKTLANT AVVIRDGQLV EIPANEVVPG
Sbay  LLMLNAGVGF IQEFQAGSIV EELKKTLANT AVVIRDGQLV EIPANEVVPG

      201                                           * 250
Scer  DILQLEDGTV IPTDGRIVTE DCFLQIDQSA ITGESLAVDK HYGDQTFSSS
Spar  DILQLEDGTI IPTDGRIVTE DCFLQIDQSA ITGESLAVDK HYGDQTFSSS
Sbay  DILQLEDGTI IPTDGRIVTE ECFLQIDQSA ITGESLAVDK HYGDQAFSSS

      251                                *              300
Scer  TVKRGEGFMV VTATGDNTFV GRAAALVNKA AGGQGHFTEV LNGIGIILLV
Spar  TVKRGEGFMV VTATGDNTFV GRAAALVNKA AGGQGHFTEV LNGIGIILLV
Sbay  TVKRGEGFMV VTATGDNTFV GRAAALVNKA SGGQGHFTEV LNGIGIILLV

      301*   *                                       350
Scer  LVIATLLLVW TACFYRTNGI VRILRYTLGI TIIGVPVGLP AVVTTTMAVG
Spar  LVVATLLLVW TACFYRTNGI VRILRYTLGI TIIGVPVGLP AVVTTTMAVG
Sbay  LVIITLLVVW TACFYRTNGI VRILRYTLGI TIIGVPVGLP AVVTTTMAVG

      351                                           400
Scer  AAYLAKKQAI VQKLSAIESL AGVEILCSDK TGTLTKNKLS LHEPYTVEGV
Spar  AAYLAKKQAI VQKLSAIESL AGVEILCSDK TGTLTKNKLS LHEPYTVEGV
Sbay  AAYLAKKQAI VQKLSAIESL AGVEILCSDK TGTLTKNKLS LHEPYTVEGV

      401                                 *         450
Scer  SPDDLMLTAC LAASRKKKGL DAIDKAFLKS LKQYPKAKDA LTKYKVLEFH
Spar  SPDDLMLTAC LAASRKKKGL DAIDKAFLKS LKQYPKAKDA LTKYKVLEFH
Sbay  SADDLMLTAC LAASRKKKGL DAIDKAFLKS LIQYPKAKDA LTKYKVLEFH

      451                                           500
Scer  PFDPVSKKVT AVVESPEGER IVCVKGAPLF VLKTVEEDHP IPEDVHENYE
Spar  PFDPVSKKVT AVVESPEGER IVCVKGAPLF VLKTVEEDHP IPEDVHENYE
Sbay  PFDPVSKKVT AVVESPEGER IVCVKGAPLF VLKTVEEDHP IPEDVHENYE

      501                                           550
Scer  NKVAELASRG FRALGVARKR GEGHWEILGV MPCMDPPRDD TAQTVSEARH
Spar  NKVAELASRG FRALGVARKR GEGHWEILGV MPCMDPPRDD TAQTVSEARH
Sbay  NKVAELASRG FRALGVARKR GEGHWEILGV MPCMDPPRDD TAQTVSEARH
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      551                                                        600
Scer  LGLRVKMLTG DAVGIAKETC RQLGLGTNIY NAERLGLGGG GDMPGSELAD
Spar  LGLRVKMLTG DAVGIAKETC RQLGLGTNIY NAERLGLGGG GDMPGSELAD
Sbay  LGLRVKMLTG DAVGIAKETC RQLGLGTNIY NAERLGLGGG GDMPGSELAD

      601                                                        650
Scer  FVENADGFAE VFPQHKYRVV EILQNRGYLV AMTGDGVNDA PSLKKADTGI
Spar  FVENADGFAE VFPQHKYRVV EILQNRGYLV AMTGDGVNDA PSLKKADTGI
Sbay  FVENADGFAE VFPQHKYRVV EILQNRGFLV AMTGDGVNDA PSLKKADTGI

      651                                                        700
Scer  AVEGATDAAR SAADIVFLAP GLSAIIDALK TSRQIFHRMY SYVVYRIALS
Spar  AVEGATDAAR SAADIVFLAP GLSAIIDALK TSRQIFHRMY SYVVYRIALS
Sbay  AVEGATDAAR SAADIVFLAP GLSAIIDALK TSRQIFHRMY SYVVYRIALS

      701                *                                       750
Scer  LHLEIFLGLW IAILDNSLDI DLIVFIAIFA DVATLAIAYD NAPYSPKPVK
Spar  LHLEIFLGLW IAILDNSLNI DLIVFIAIFA DVATLAIAYD NAPYSPKPVK
Sbay  LHLEIFLGLW IAILDNSLDI DLIVFIAIFA DVATLAIAYD NAPYSPKPVK

      751              *  *                                    800
Scer  WNLPRLWGMS IILGIVLAIG SWITLTTMFL PKGGIIQNFG AMNGIMFLQI
Spar  WNLPRLWGMS IILGIILAVG SWITLTTMFL PKGGIIQNFG ALNGIMFLQI
Sbay  WNLPRLWGMS IILGIVLAVG SWITLTTMFL PKGGIIQNFG AMNGIMFLQI

      801                    *                                  850
Scer  SLTENWLIFI TRAAGPFWSS IPSWQLAGAV FAVDIIATMF TLFGWWSENW
Spar  SLTENWLIFI TRAAGPFWSS IPSWQLAGAV FAVDIIATMF TLFGWWSENW
Sbay  SLTENWLIFI TRAAGPFWSS VPSWQLAGAV FAVDIIATMF TLFGWWSENW

      851                                      *       *         900
Scer  TDIVTVVRVW IWSIGIFCVL GGFYYEMSTS EAFDRLMNGK PMKEKKSTRS
Spar  TDIVTVVRVW IWSIGIFCVL GGFYYEMSTS EAFDRVMNGK PMKEKKSTRS
Sbay  TDIVTVVRVW IWSIGIFCVL GGFYYEMSTS EAFDRMMNGK PAKEKKSTRS

      901              919
Scer  VEDFMAAMQR VSTQHEKET
Spar  VEDFMAAMQR VSTQHEKET
Sbay  VEDFLAAMQR VSTQHEKEA
```

Figure S2.10: Pma1 alignment

Alignment of Pma1 from *Saccharomyces* cerevisiae, *S. paradoxus*, and *S. bayanus*. Identical amino acids are marked in blue and different amino acids in red. Red dots mark gaps.

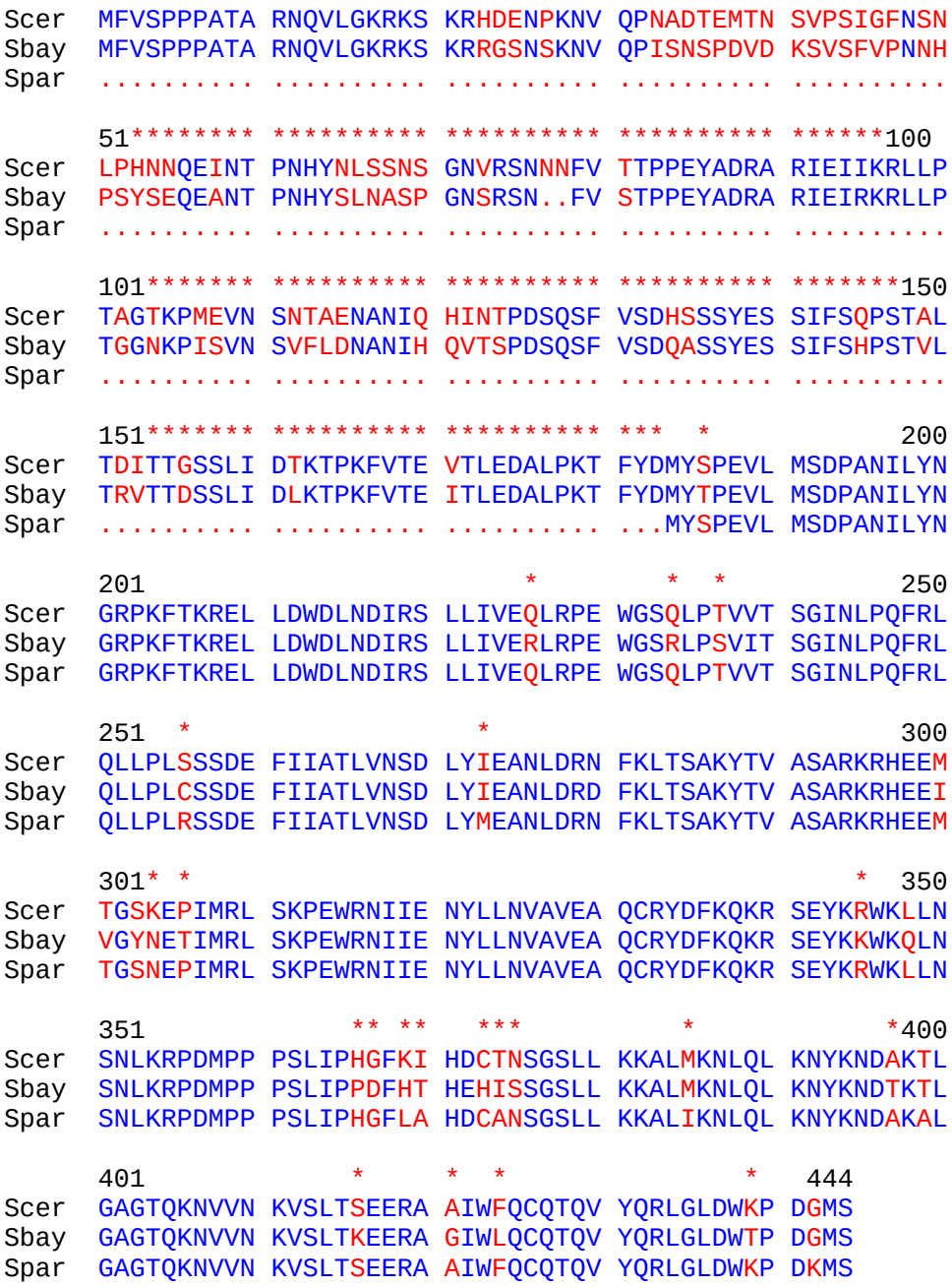

Figure S2.11: Std1 alignment

Alignment of Std1 from *Saccharomyces* cerevisiae, *S. paradoxus*, and *S. bayanus*. Identical amino acids are marked in blue and different amino acids in red. Red dots mark gaps. Note that the N-terminal region of Std1 from *S. paradoxus* is missing.

ORF number	gene name	ORF number	gene name
YAL056W	GPB2	YIL148W	RPL40A
YBL079W	NUP170	YJL003W	COX16
YCL036W	GFD2	YJL179W	PFD1
YCR044C	PER1	YJR039W	
YCR050C		YJR055W	HIT1
YCR085W		YJR058C	APS2
YDL006W	PTC1	YJR118C	ILM1
YDL160C	DHH1	YKL073W	LHS1
YDL194W	SNF3	YKR024C	DBP7
YDL232W	OST4	YKR055W	RHO4
YDR074W	TPS2	YLR402W	
YDR129C	SAC6	YML048W	GSF2
YDR521W		YML063W	RPS1B
YER115C	SPR6	YML094W	GIM5
YER131W	RPS26B	YML115C	VAN1
YGL015C		YML129C	COX14
YGL084C	GUP1	YMR074C	
YGL127C	SOH1	YMR307W	GAS1
YGL197W	MDS3	YNL133C	FYV6
YGR036C	CAX4	YNL238W	KEX2
YGR071C		YNR052C	POP2
YGR159C	NSR1	YOL081W	IRA2
YGR180C	RNR4	YOR175C	
YGR229C	SMI1	YOR253W	NAT5
YHL019C	APM2	YPL090C	RPS6A
YHL033C	RPL8A	YPL178W	CBC2
YHR075C	PPE1	YPL179W	PPQ1
YHR087W		YPR129W	SCD6
YIL040W	APQ12	YPR170C	
significant GO categories: signal transduction (p = 0.013)			

Table S2.1: ORFs that exhibit glucosamine-resistance when knocked out

ORF number	gene name	significant GO terms	p-value	genes
YAL013W	DEP1	organelle organization and biogenesis	0.002	DEP1
YBL061C	SKT5			MIS1
YBR084W	MIS1			RPP1A
YBR120C	CBP6			RTF1
YDL081C	RPP1A			SUR4
YDR017C	KCS1			RPL13B
YGL244W	RTF1			DMA2
YJL165C	HAL5			CSE2
YKL038W	RGT1	protein modification process	0.034	DEP1
YLR372W	SUR4			MIS1
YMR142C	RPL13B			RTF1
YNL040W				DMA2
YNL116W	DMA2	translation	0.001	MIS1
YNR010W	CSE2			CBP6
YOL023W	IFM1			RPP1A
YOR333C				RPL13B
YDR277C	MTH1			IFM1
YOR047C	STD1	ligase activity	0.039	MIS1
				DMA2
		transcription regulator activity	0.005	DEP1
				RTF1
				RGT1
				CSE2

Table S2.2: ORFs that show low [GAR^+] levels when knocked out

ORF number	gene name
YGL028C	SCW11
YGL041C	
YGL138C	
YGR027C	RPS25A
YHR046C	INM1
YJL198W	PHO90
YKL092C	BUD2
YLR032W	RAD5
YNL168C	FMP41
YOL092W	
YOR108W	LEU9
YOR275C	RIM20
YPR159W	KRE6
YHR046C	

significant GO terms	p-value	genes
carbohydrate metabolic processing	0.049	INM1
		KRE6
cytokinesis	0.02	SCW11
		BUD2
hydrolase activity	0.032	INM1
		KRE6
		SCW11
		RAD5

Table S2.3: ORFs that show high [GAR^{+}] levels when knocked out

plasmid name	backbone	contains	source
pPMA1 ura+	pRS316	-1700 to +2950 PMA1	this study
pPMA1 trp+	pRS314	-1700 to +2950 PMA1	this study
pPMA1 S. bay.	pRS314	5' UTR of S. cerevisiae PMA1 (to -1700) fused to S. bayanus PMA1 ORF	this study
pPMA1 S. par.	pRS314	5' UTR of S. cerevisiae PMA1 (to -1700) fused to S. paradoxus PMA1 ORF	this study
pPMA1 S899A	pRS314	pPMA1 mutated at S899	this study
pPMA1 S899D	pRS314	pPMA1 mutated at S899	this study
pPMA1 S911A	pRS314	pPMA1 mutated at S911	this study
pPMA1 S911D	pRS314	pPMA1 mutated at S911	this study
pPMA1 T912A	pRS314	pPMA1 mutated at T912	this study
pPMA1 T912D	pRS314	pPMA1 mutated at T912	this study
pPMA1 911A912A	pRS314	pPMA1 mutated at S911 and T912	this study
pPMA1 911D912D	pRS314	pPMA1 mutated at S911 and T912	this study
pRGT2	p413GPD	RGT2 ORF under control of a GPD promoter (high expression)	this study
pSNF3	p413GPD	SNF3 ORF under control of a GPD promoter	this study
pYCK1	p413GPD	YCK1 ORF under control of a GPD promoter	this study
pYCK2	p413GPD	YCK2 ORF under control of a GPD promoter	this study
pSTD1	p413GPD	STD1 ORF under control of a GPD promoter	this study
pMTH1	p413GPD	MTH1 ORF under control of a GPD promoter	this study
pRGT1	p413GPD	RGT1 ORF under control of a GPD promoter	this study
pHXT3	p413GPD	HXT3 ORF under control of a GPD promoter	this study
pPMA1-OX	p414GPD	PMA1 ORF under control of a GPD promoter	this study
pPMA1Δ40N-OX	p414GPD	PMA1Δ40N ORF under control of a GPD promoter	this study
pPMA1Δ104N-OX	p414GPD	PMA1Δ104N ORF under control of a GPD promoter	this study
pPMA1Δ40C-OX	p414GPD	PMA1Δ40C ORF under control of a GPD promoter	this study
pPMA1Q23stop	p414GPD	pPMA1-OX with nonsense mutation at Q23	this study
pPMA1E59stop	p414GPD	pPMA1-OX with nonsense mutation at E59	this study

Table S24: Plasmids used in Chapter 2.

strain name	purpose	source
W303 [gar-]		R. Rothstein
W303 [GAR+]		this study
W303 Δpma1 pPMA1	genomic copy of pma1 replaced with KanMX, covered by plasmid pPMA1 ura+	this study
W303 Δrgt2	[GAR+] propagation studies	this study
W303 Δsnf3	[GAR+] propagation studies	this study
W303 Δyck1	[GAR+] propagation studies	this study
W303 Δyck2	[GAR+] propagation studies	this study
W303 Δstd1	[GAR+] propagation studies	this study
W303 Δmth1	[GAR+] propagation studies	this study
W303 Δrgt1	[GAR+] propagation studies	this study
W303 Δhxt3	[GAR+] propagation studies	this study
W303 Δsur4	Pma1 oligomerization studies	this study
W303 Δlst1	Pma1 oligomerization studies	this study
W303 Δlcb3	Pma1 oligomerization studies	this study
W303 Δlcb4	Pma1 oligomerization studies	this study
W303 Δdpl1	Pma1 oligomerization studies	this study
W303 Δatg19	Pma1 oligomerization studies	this study
W303 Δerg5	Pma1 oligomerization studies	this study
W303 GAL-Δ40N	[GAR+] propagation studies	Liu et al., 2006
W303 GAL-Δ40N Δstd1	[GAR+] propagation studies	this study
S288c HXT3-GFP	monitoring Hxt3 protein levels in [gar-] and [GAR+]	Huh et al., 2003
S288c/W303 HXT3-GFP Δpma1 pPMA1	monitoring Hxt3 protein levels in [gar-] and [GAR+]	this study

Table S2.5: Yeast strains used in Chapter 2.

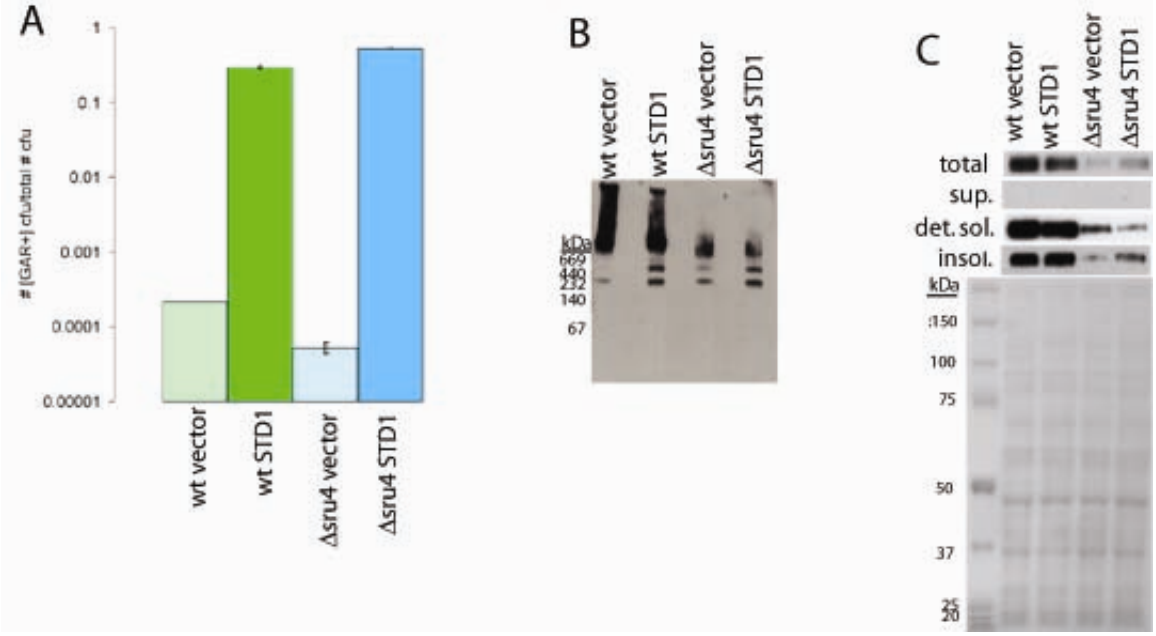

Figure S2.12: Overexpression of *STD1* rescues Δ*sur4*'s inability to form [GAR$^+$]

a) Constitutive overexpression of *STD1* rescues the inability of Δ*sur4* to become [GAR$^+$]. Note that the y-axis is a logarithmic scale. b) Native gel of digitonin-soluble protein extracts from wildtype (wt) or Δ*sur4* backgrounds carrying an empty vector or a vector overexpressing *STD1* and probed with α-Pma1. Note that HMW oligomers of Pma1 are not rescued when *STD1* is overexpressed. c) Total protein stained with Ponceau S (bottom) and Western of fractions using an α-Pma1 antibody. Note that the amount of Pma1 in the fractions in the Δ*sur4 STD1* sample only increased slightly, despite the increase in [GAR$^+$] frequency. Low levels of Pma1 protein therefore do not affect [GAR$^+$] formation.

Chapter Three:

Natural Variation, the Environment and the [GAR^{+}] Prion

Introduction

Microbes live in dynamic environments with widespread competition and limited resources. Among other things, they are subject to changing temperatures, toxins, nutrient starvation, and osmotic changes. The number and variety of competing organisms is increased in "easy" niches, so decreased environmental stress begets increased competition. The ability to survive diverse stress and to outgrow competitors is therefore crucial to fitness.

In order for organisms to survive in fluctuating environments, they must be responsive to changes in them. This is particularly important for plants and fungi, which are immobile or relatively immobile and therefore have no choice but to adapt to circumstances (Winkelmann, 2007). Two major mechanisms for adaptation are phenotypic variation and pleiotropy. Prions are an unusual source of pleiotropy and phenotypic variation that may contribute to environmental adaptation (Eaglestone et al., 1999; True and Lindquist, 2000; Masel and Bergman, 2003).

Fungal prions are protein-based elements of genetic inheritance. They involve proteins with an unusual ability to acquire an altered conformation that is associated with an altered function. Most particularly, these changes in conformation and function are propagated to newly synthesized proteins of the same type in an extremely stable manner.

Mother cells pass the prion proteins to their daughters perpetuating the change in function, and its associated phenotypes, from generation to generation. Proteins can enter the prion state spontaneously at a low rate. And occasionally the prion state is not passed on to daughter cells (Uptain and Lindquist, 2002; Chien et al., 2004; Wickner et al., 2004; Shorter and Lindquist, 2005; Tuite and Cox, 2006). These means that colonies arising from cells without the prion, will generally contain some cells that have it, and colonies arising from cells with the prion will generally contain some cells that have lost it. This creates a diversity of phenotypes within a colony even though the genome of each cell is identical. By increasing the number of phenotypes associated with that genome, we have suggested that prions might increase the chance that that a particular genome will survive in a changing environment (Eaglestone et al., 1999; True and Lindquist, 2000; Masel and Bergman, 2003; True et al., 2004).

The work of several groups has shown that the prion known as [PSI^+] confers a growth advantage compared to [psi^-] under a variety of different environmental conditions (Eaglestone et al., 1999; True and Lindquist, 2000). Of about 100 conditions tested, 50 showed no difference, [psi^-] had a growth advantage in 25%, and [PSI^+] had a growth advantage in 25% (True and Lindquist, 2000). The spontaneous appearance of PSI therefore provides a temporary survival advantage under some conditions. The [PSI^+]-dependent survival advantage can become fixed by genetic change, so that [PSI^+] can subsequently be lost but the [PSI^+]-dependent phenotype maintained (True et al., 2004).

[PSI^+] is both induced and lost at an increased rate under stressful conditions, such as when cells are exposed to changes in osmotic conditions and diverse chemicals

((Eaglestone et al., 1999; Tyedmers et al., unpublished). This is almost certainly due to the increase in protein misfolding and the induction of chaperone protein, under these conditions (Eaglestone et al., 1999). Thus, the prion-forming ability of Sup35, the causal agent of [PSI^+], allows it to act as a "contingency locus". Under stressful conditions, when cells are inherently not well adapted to their environment, the rate at which cells switch between prions states increases from one in 10^6 to one in 10^4 or 10^5 cells. This increases the likelihood that that might sample and advantageous phenotype, but most cells in the culture retain their initial prion state (Tyedmers et al., unpublished). If [PSI^+] is advantageous in that environment, then the proportion of [PSI^+] in the population can increase allowing survival of that genotype. If it is disadvantageous, only a small number of cells will perish.

The idea that a prion could be advantageous is, however, fairly controversial. Neither [PSI^+] nor [URE3] have been found in environmental sampling experiments (n = 70), although the selfish 2μ plasmid was found in 54% of yeast tested. The argument was that [PSI^+] and [URE3] are therefore more detrimental than the mildly detrimental 2μ plasmid (Nakayashiki et al., 2005). However, [PSI^+] is envisioned as being only temporarily advantageous and only in some conditions (True and Lindquist, 2000; True et al., 2004). Thus one could not expect to find [PSI^+] or [URE3] in environmental isolates. [PSI^+]-dependent phenotypes can also be fixed quite easily (True et al., 2004). In this case the loss of [PSI^+] would not be disadvantageous, even if the original [PSI^+]-dependent trait is advantageous. Furthermore, [RNQ^+] was found in 16% of these isolates (Nakayashiki et al., 2005). As the only known phenotype of [RNQ^+] is to allow

the induction of [PSI$^+$], this suggests that a subpopulation of the yeast isolates tested maintain the ability to induce [PSI$^+$] even if they do not currently carry [PSI$^+$].

Arguing in favor of a beneficial role for this prion, the ability of Sup35 to form [PSI$^+$] has been conserved over a billion years of fungal evolution (Nakayashiki et al., 2001; Tanaka et al., 2005; Harrison et al., 2007). The precise sequence of the prion-determining region (PrD) is not conserved but the highly unusual amino acid composition of the prion-determining region, which allows it to switch into the prion state has been conserved across the basidiomyces (Harrison et al., 2007). Sup35 alleles from *K. lactis*, *C. albicans*, and other fungi are demonstrably capable of forming [PSI$^+$] in *S. cerevisiae*. *K. lactis*, *S. bayanus*, and *S. paradoxus* Sup35 also form [PSI$^+$] in their species of origin (Nakayashiki et al., 2001; Tanaka et al., 2005). Of course, it might be argued that Sup35 PrD has been conserved to provide some other function. However, the nature of its conservation, which allows it to assume an amyloid, prion conformation, rather than to assume a more normal, globular fold, argues against it.

Saccharomyces cerevisiae is used in the production of bread, alcoholic beverages, and industrial alcohol. It has been selected over millennia for such purposes, resulting in a variety of strains with vastly different properties. Indeed, these optimizations have resulted in such specialized strains that many breweries and wineries regard them as industrial secrets (Landry et al., 2006). *S. cerevisiae* can also be isolated from the environment; samples have been found in soil and on plant bark, leaves, and fruits. Occasionally *S. cerevisiae* infects immunocompromised patients, although *S. cerevisiae* infection is considerably less common than infection by other fungal species such as *Candida albicans* or *Aspergillus fumigatus*. Overall, *S. cerevisiae* is capable of adapting

to a wide variety of ecological niches and their accompanying stressors. These include nutrient starvation, low pH, fluctuating environments, high ethanol (fermentation) (Querol et al., 2003), heat stress, and hypoxia (animal infection) (Brown et al., 2007). Unsurprisingly, *S. cerevisiae* isolates from these different environments exhibit a wide variety of phenotypes and considerable genetic variation (Fay and Benavides, 2005; Landry et al., 2006).

The phenotype resulting from the presence of the [GAR^+] prion, resistance to glucosamine, involves changes in the fundamental process of carbon metabolism. We wondered if [GAR^+] might play a role in the adaptation of *S. cerevisiae* to distinct biological niches. We asked whether [GAR^+] is found in wild yeast isolates, whether [GAR^+] can be induced in wild yeasts, and what environmental and genetic factors affect the appearance of [GAR^+]. We found great variation in [GAR^+] frequency between isolates with particular enrichment for higher [GAR^+] frequency in fruit isolates. We also identified two potential genetic mechanisms that might explain variation in [GAR^+] frequency: natural variation in *PMA1* sequence and genome-wide genetic differences. We sequenced *PMA1* and upstream regions from 45 different *S. cerevisiae* isolates. We also performed quantitative trait locus (QTL) analysis on the 103 genotyped progeny from a cross between a vineyard isolate and a lab strain, using [GAR^+] frequency as a quantitative trait (Brem et al., 2002). From this we hypothesized that we would both identify *PMA1*-independent and *PMA1*-linked regulators of [GAR^+].

Our findings suggest that [GAR^+] plays an important role in the *S. cerevisiae* lifestyle. The frequency of the [GAR^+] prion varies with ecological niche. We tested diploid samples of *S. cerevisiae* used to brew beer ("brewery") or isolated from fruit, soil,

or human patients ("clinical"). The phenotype of glucosamine-resistance is common in one niche, soil, but we have yet to determine whether this is the result of [GAR^+]. Polymorphisms within the *PMA1* sequence did not correlate with the [GAR^+] frequency but we did note a surprisingly high degree of heterozygosity at the *PMA1* locus (~45% of samples) in diploid isolates of *S. cerevisiae*.

Using [GAR^+] frequency as a quantitative trait, we identified two genomic regions that are correlated with high rate of appearance of [GAR^+] by QTL analysis. A difference in copy number of two genes that confer 2-deoxyglucose resistance when overexpressed (Randez-Gil et al., 1995), *DOG1* and *DOG2*, are the most likely source of increased [GAR^+] frequency in one of these regions. Finally, [GAR^+] cells have a competitive advantage over [gar^-] cells in rich medium. However, [GAR^+] is more sensitive to glucose starvation than [gar^-] in defined medium. Overall, these data suggest that [GAR^+] is beneficial in certain ecological niches and environmental conditions and [GAR^+] serves as a source of phenotypic flexibility.

Results

[GAR^+] frequency varies with ecological niche

Saccharomyces cerevisiae can survive a wide variety of ecological niches and has been isolated from a number of different environments. As utilization of carbon sources is fundamental to survival, we hypothesized that [GAR^+] could be important for survival in particular environments. We therefore tested a number of *S. cerevisiae* isolates from a variety of environments for resistance to glucosamine. If isolates were glucosamine-sensitive, we measured the frequency of appearance of [GAR^+].

Table 3.1: Yeast strains and their source

strain name	category	source	sample origin
Abbey Ale	beer	brewing	White Labs
Belgian Ale	beer	brewing	White Labs
English Ale	beer	brewing	White Labs
Forbidden Fruit Ale	beer	brewing	Wyeast
Irish Ale	beer	brewing	White Labs
Northwest Ale	beer	brewing	Wyeast
Trappist Ale	beer	brewing	White Labs
Urquell Pilsner	beer	brewing	Wyeast
Y-7327	beer	Tibetan beer starter	Agricultural Research Service Collection
YJM521	clinical	patient	John McCusker
YJM522	clinical	patient	John McCusker
YJM273	clinical	patient	John McCusker
YJM310	clinical	patient	John McCusker
YJM311	clinical	patient	John McCusker
YJM436	clinical	patient	John McCusker
YJM440	clinical	patient	John McCusker
YJM454	clinical	patient	John McCusker
YJM210	clinical	patient	John McCusker
YJM455	clinical	patient	John McCusker
Y-27806	clinical	patient	Agricultural Research Service Collection
Y-27788	clinical	patient	Agricultural Research Service Collection
Y-502	clinical	patient	Agricultural Research Service Collection
Y-492	clinical	patient	Agricultural Research Service Collection
Y-10988	clinical	patient	Agricultural Research Service Collection
YJM128	clinical	patient	John McCusker
YJM309	clinical	patient	John McCusker
YB-4081	fruit	guava	Agricultural Research Service Collection
Y-5511	fruit	coconut	Agricultural Research Service Collection
YB-399	fruit	cherries	Agricultural Research Service Collection

strain name	category	source	sample origin
Y-382	fruit	grain	Agricultural Research Service Collection
Y-1537	fruit	grapes	Agricultural Research Service Collection
Y-7568	fruit	papaya	Agricultural Research Service Collection
YB-210	fruit	banana	Agricultural Research Service Collection
YB-3121	fruit	mimosa	Agricultural Research Service Collection
YB-4082	fruit	papaya	Agricultural Research Service Collection
YB-432	fruit	pineapple	Agricultural Research Service Collection
Y-35	fruit	*Ilex aquifolium*	Agricultural Research Service Collection
Y-139	fruit	grape	Agricultural Research Service Collection
Y-12657	fruit	olive	Agricultural Research Service Collection
OP1	soil	Occoneechee Park, VA	Dietzmann, Dietrich
OP2	soil	Occoneechee Park, VA	Dietzmann, Dietrich
OP3	soil	Occoneechee Park, VA	Dietzmann, Dietrich
OP4	soil	Occoneechee Park, VA	Dietzmann, Dietrich
OP6	soil	Occoneechee Park, VA	Dietzmann, Dietrich
OP7	soil	Occoneechee Park, VA	Dietzmann, Dietrich
OP8	soil	Occoneechee Park, VA	Dietzmann, Dietrich
OP9	soil	Occoneechee Park, VA	Dietzmann, Dietrich
SM1	soil	Stone Mountain, GA	Dietzmann, Dietrich
SM2	soil	Stone Mountain, GA	Dietzmann, Dietrich
SM12	soil	Stone Mountain, GA	Dietzmann, Dietrich
SM17	soil	Stone Mountain, GA	Dietzmann, Dietrich
SM66	soil	Stone Mountain, GA	Dietzmann, Dietrich
SM69	soil	Stone Mountain, GA	Dietzmann, Dietrich

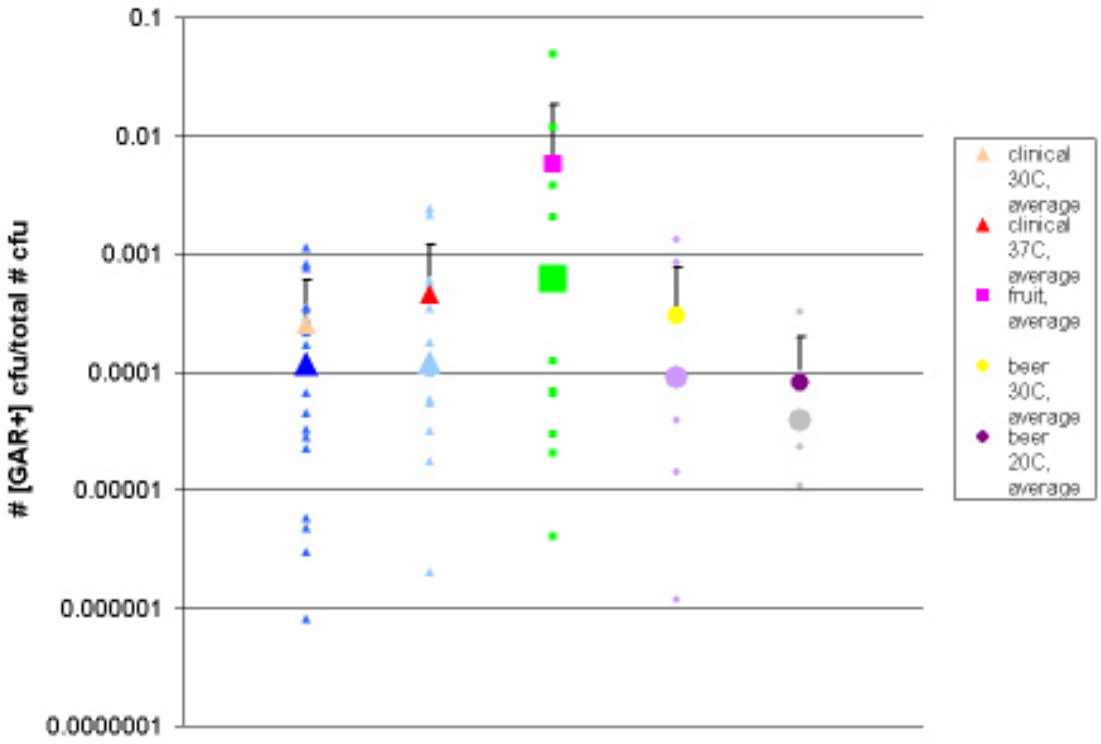

Figure 3.1: [GAR$^+$] frequency varies with ecological niche.

[GAR$^+$] frequency was measured for each yeast strain listed in table 3.1. Three independent cultures were grown at the temperature specified and each culture was plated in duplicate. Small points represent an individual strain within a category (dark blue: clinical, 30°C; light blue: clinical, 37°C; green: fruit, 30°C; purple: beer, 30°C; gray: beer, 20°C). Larger shapes represent the average for the category (peach: clinical, 30°C; orange: clinical, 37°C; pink: fruit, 30°C; yellow: beer, 30°C; purple: beer, 20°C). Error bars represent the standard deviation of the [GAR$^+$] frequency for the category as a whole. The average [GAR$^+$] frequency for the set of strains isolate from fruits is approximately 20 fold higher than the average for the clinical strains at either 30°C or 37°C. The large data point in each series that is the same color as the rest of the series is the median point.

site	variation	S288c sequence	amino acid	heterozygosity
-1557	G insertion		N/A	yes
-1038	A, G	G	N/A	yes
-704	C, T	T	N/A	yes
-611	C, T	T	N/A	yes
-314	A, G	A	N/A	yes
-287	C, G, T	G	N/A	yes
-272	C, T	T	N/A	no
-257	C, T	C	N/A	yes
-116	T insertion	T	N/A	yes
-111	C, T	C	N/A	no
-13	A, G	A	N/A	yes
156	C, T	T	synonymous	no
221	C, T	C	P-->L	yes
246	C, T	T	synonymous	yes
348	C, T	C	synonymous	no
349	C, G	G	V-->L	yes
426	C, T	C	synonymous	no
454	A, C	A	M-->L	yes
625	A, G	G	V-->I	yes
891	A, G	A	synonymous	yes
904	A, G	A	I-->V	yes
1380	C, G, T	C	synonymous	yes
1563	A, G	A	synonymous	yes
2055	C, T	T	synonymous	no
2152	A, G	G	D-->N	yes
2241	A, C	C	synonymous	yes
2283	C, T	T	synonymous	yes
2286	A, G	G	synonymous	yes
2289	C, T	C	synonymous	yes
2292	A, T	A	synonymous	yes
2293	A, G	G	V-->I	yes
2298	A, G	A	synonymous	yes
2301	C, T	T	synonymous	yes
2302	A, G	A	I-->V	yes
2304	A, C	A		yes
2310	A, T	T	synonymous	yes
2460	C, T	C	synonymous	yes
2466	A, C	C	synonymous	yes
2475	A, G	G	synonymous	yes
2506	G, T	G	A-->S	yes

Table II: Polymorphisms in *PMA1* from -1700 to +2950
Common polymorphisms found within the *PMA1* sequence. Polymorphism had to be found in at least five of 45 total samples to be included in this table. "Heterozygosity" indicates that any single strain was heterozygous at the site in question.

We measured [GAR$^+$] frequency from 45 different yeast isolates (table 3.1). We found that the rate of appearance of [GAR$^+$] varied with ecological niche of the host (figure 3.1). *S. cerevisiae* lab strains, brewery strains, and clinical isolates all showed approximately the same average [GAR$^+$] frequencies, around 3 in 10^3 cells. Samples from each niche exhibited high variance in [GAR$^+$] frequency and therefore different medians. The median [GAR$^+$] frequencies of clinical isolates was around 1 in 10^4 cells, whereas the median [GAR$^+$] frequency of beer isolates was around 4 in 10^5. Temperature did not affect [GAR$^+$] frequency in clinical isolates but it decreased with increasing temperatures in brewery isolates. Yeast samples from fruits showed a much higher appearance of [GAR$^+$], on average 20 fold higher than the average [GAR$^+$] frequency and five fold higher than the median [GAR$^+$] frequency in clinical isolates. Fruit yeasts also show a greater variance in [GAR$^+$] frequency than clinical and brewery isolates.

One possible origin of this variation in [GAR$^+$] frequency is changes in the sequence of *PMA1* or its surrounding region. We therefore sequenced the *PMA1* locus, 1700bp 5' of the gene, and 250bp 3' of the gene to determine whether any polymorphisms correlate with [GAR$^+$] frequency. Initial data from direct sequencing suggested that some samples were heterozygous, so we switched to subcloning our region of interest and sequencing four separate samples from each yeast strain. We did not find any single change that is necessary for high [GAR$^+$] frequency (polymorphisms are listed in table 3.2) or any clear haplotypes.

Sequencing *PMA1* from a variety of *S. cerevisiae* isolates showed that *PMA1* exhibits a surprising degree of heterozygosity. Because *S. cerevisiae* is homothallic, it is

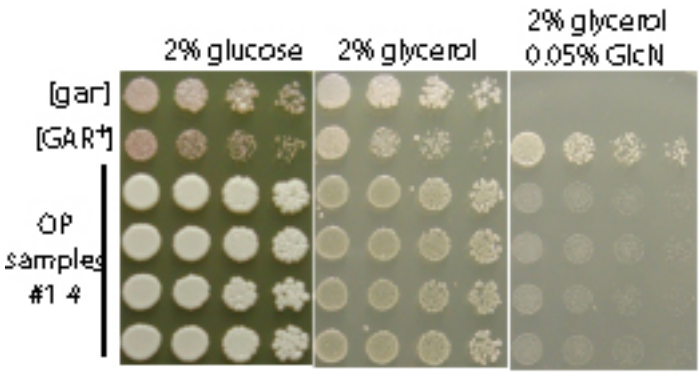

Figure 3.2: Does [GAR$^+$] exist in wild yeast?

a) Spot tests of [gar$^-$], [GAR$^+$], and four soil isolates; the remaining ten showed the same growth patterns. Each serial dilution factor is five-fold. Cultures were diluted and spotted to 2% glucose, 2% glycerol, and glycerol-glucosamine medium (GGM).

known that diploid *S. cerevisiae* samples are mostly homozygous, since haploids could switch mating types then mate with the neighboring, genetically identical cell. Population analysis of the closely related *S. paradoxus*, which is also homothallic, predicts a 99% inbreeding rate in the wild (Johnson et al., 2004). However, *PMA1* was heterozygous in 20 of our 45 samples, usually at multiple sites in each heterozygous strain. Some of these changes are nonsynonymous, including an alanine to serine change at amino acid 835 that is found in nine samples and is heterozygous in three more.

Soil isolates are resistant to glucosamine and an environmental isolate induces [GAR$^+$]

We tested 14 *S. cerevisiae* samples isolated from the soil at two different locations in the Southeastern United States. All of these showed glucosamine-resistance (figure 3.2). Whether this is the result of [GAR$^+$] or caused by a genetic mutation we cannot yet say because isolates are homothallic and therefore not genetically tractable. Experiments are underway to analyze the segregation patterns of this phenotype.

Various Staphylococcus *species induce [GAR$^+$]*

We serendipitously discovered that a bacterial species is capable of inducing glucosamine-resistance in *S. cerevisiae* when it appeared as a contaminant on an agar plate. [gar$^-$] cells grown on a plate next to the bacterial cells acquired resistance to glucosamine over time (figure 3.3a). When the initially [gar$^-$] samples are removed from this plate and grown on glycerol/glucosamine without the bacteria present, they remain glucosamine-resistant. This induced resistance is semi-dominant in a mating test, suggesting that its resistance to glucosamine is caused by [GAR$^+$] and not a genetic

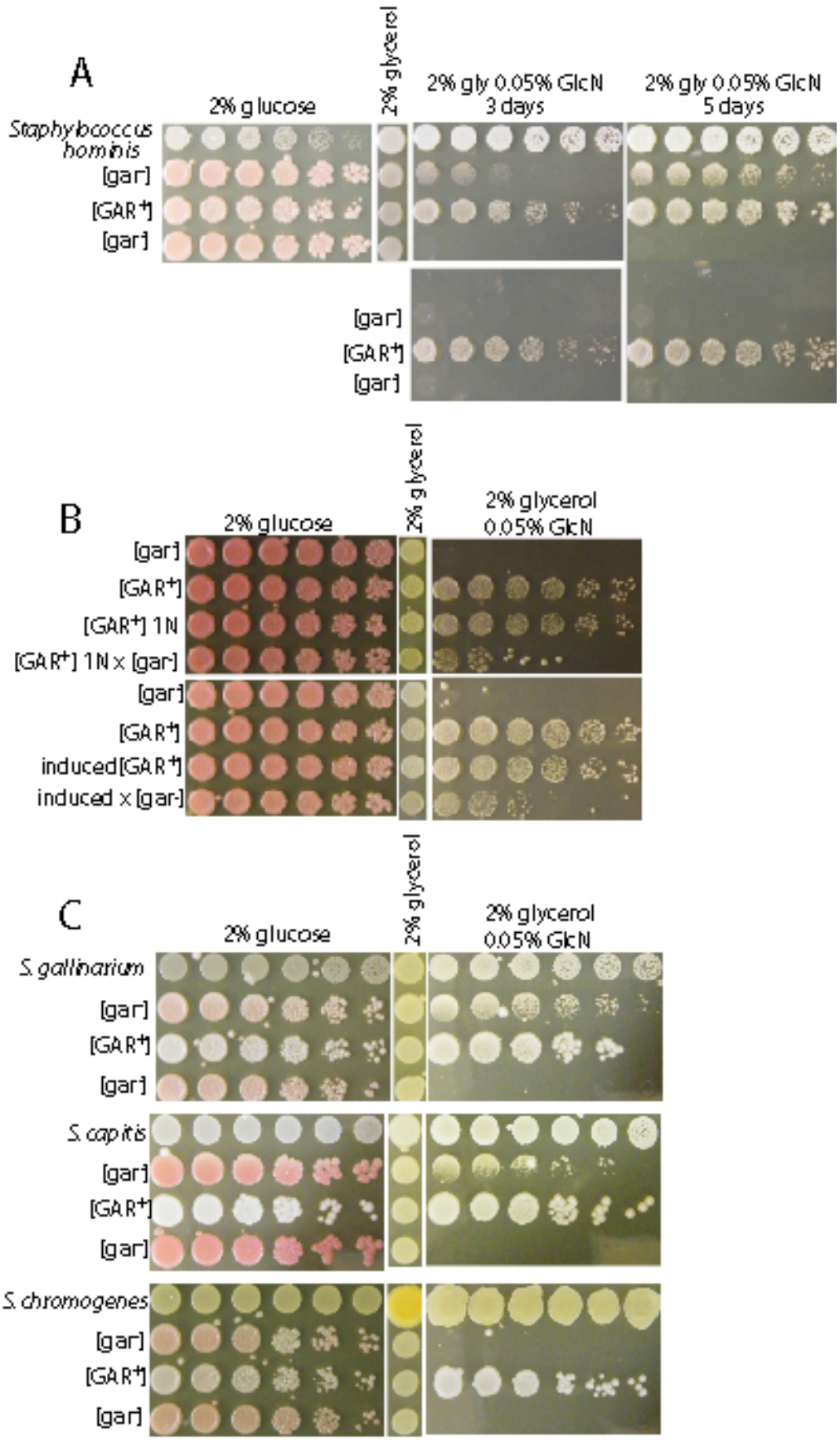
A
2% glucose
2% glycerol
2% gly 0.05% GlcN 3 days
2% gly 0.05% GlcN 5 days
Staphylococcus hominis
[gar-]
[GAR+]
[gar-]
[gar-]
[GAR+]
[gar-]
B
2% glucose
2% glycerol
2% glycerol 0.05% GlcN
[gar-]
[GAR+]
[GAR+] 1N
[GAR+] 1N x [gar-]
[gar-]
[GAR+]
induced [GAR+]
induced x [gar-]
C
2% glucose
2% glycerol
2% glycerol 0.05% GlcN
S. gallinarium
[gar-]
[GAR+]
[gar-]
S. capitis
[gar-]
[GAR+]
[gar-]
S. chromogenes
[gar-]
[GAR+]
[gar-]

Figure 3.3: *Staphylococcus* species induce the conversion of [gar$^-$] to [GAR$^+$]

a) Induction of glucosamine resistance by *Staphylococcus hominis*. Cultures were grown in YPD, diluted in five-fold serial dilutions, then spotted to 2% glucose (YPD), 2% glycerol, and GGM. GGM plates were incubated five days before being photographed. Note that the [gar$^-$] sample adjacent to the unknown bacteria grew on GGM proportional to the plating cell density. b) Mating of the induced glucosamine-resistant strains in part a to [gar$^-$] to test for dominance. Induced glucosamine-resistance yeast show semi-dominant glucosamine-resistance, suggesting that the induced strain is [GAR$^+$] rather than a genetic change. c) [gar$^-$] samples grown adjacent to additional *Staphylococcus* species isolates. Five of nine *Staphylococcus* species induced the conversion of [gar$^-$] to [GAR$^+$]. *S. gallinarium* induced [GAR$^+$] at high efficiency (top), *S. capitis* induced [GAR$^+$] with medium efficiency (middle), and *S. chromogenes* did not induce [GAR$^+$] (bottom).

alteration (figure 3.3b). The [GAR$^+$]-inducing bacterium was identified as *Staphylococcus hominis* by 16S rDNA sequencing (figure S3.1).

Staphylococcus is a ubiquitous genus of gram positive bacteria capable of growing in a wide variety of ecological niches, particularly on animals. *Staphylococcus* is even occasionally in the soil (Madigan and Martinko, 2006). We also tested eight other *Staphylococcus* species (out of a total of 31 species) and found that six of these induced [GAR$^+$] when cultured with *S. cerevisiae*, albeit with varying efficiency (figure 3.3c).

Genetic factors influence [GAR$^+$] frequency

The rate of appearance of [GAR$^+$] varied widely between yeast strains and their ecological niche. As the frequency of appearance of [GAR$^+$] is a quantitative trait, we sought to identify factors that influence [GAR$^+$] frequency by quantitative trait locus (QTL) mapping. We used a set of genotyped segregants from a cross between a vineyard isolate, RM11, and the lab strain BY4617, which is virtually identical to S288c (Brem et al., 2002). The BY4617 parent showed a higher frequency of [GAR$^+$] appearance than the RM11 strain. Parents and segregants were grown in 2% glucose then plated to medium containing glycerol and glucosamine. Growth on glucosamine medium was tracked over the course of twelve days and density of growth was taken as a measure of [GAR$^+$] frequency (figure 3.4a).

As a control to ensure we were working with [GAR$^+$] and not Mendelian genetic mutations, glucosamine-resistant samples were mated to a [gar$^-$] strain and diploids selected. [GAR$^+$] is semi-dominant in a mating (see chapter two) but Mendelian

mutations would most likely be recessive. All glucosamine-resistant segregants for which we could select diploids showed semi-dominant glucosamine-resistance following mating (figure 3.4b). This demonstrates that the glucosamine-resistance we measured is likely the result of [GAR$^+$] and not a Mendelian mutation.

We identified two regions correlated with high appearance of [GAR$^+$] (figure 3.4c). One of these regions associated with a high rate of appearance of [GAR$^+$] is on chromosome XIV, bp 412,000 to 450,000 (figure 3.4c, top). The causal polymorphisms are less blatant; there are a number of polymorphisms in a leucine tRNA upstream of *PHO23* and a number within the 350 bp region 5' of *PHO23*. For these reasons and because this region is only slightly above the significance cutoff for LOD scores (figure 3.4c), we focused on the second significant region.

The second region is located to bp 180,000 to 220,000 on chromosome VIII (top). The region that confers higher [GAR$^+$] frequency corresponded with that from the BY4617 parent (bottom). This region contains genes *DOG1* and *DOG2*, which were originally identified from a screen for mutations that confer resistance to the non-hydrolyzable glucose analog 2-deoxyglucose (Sanz et al., 1994). The RM11 parents lacks *DOG2* whereas the BY4617 carries both *DOG1* and *DOG2* (figure S3.2). Since *DOG1* and *DOG2* confer 2-deoxyglucose resistance when overexpressed (Randez-Gil et al., 1995), the copy number difference could well account for the difference in frequency of [GAR$^+$].

Additionally, we screened ~5000 *S. cerevisiae* ORFs to identify candidates that induce [GAR$^+$] following transient overexpression. Three genes were found to induce

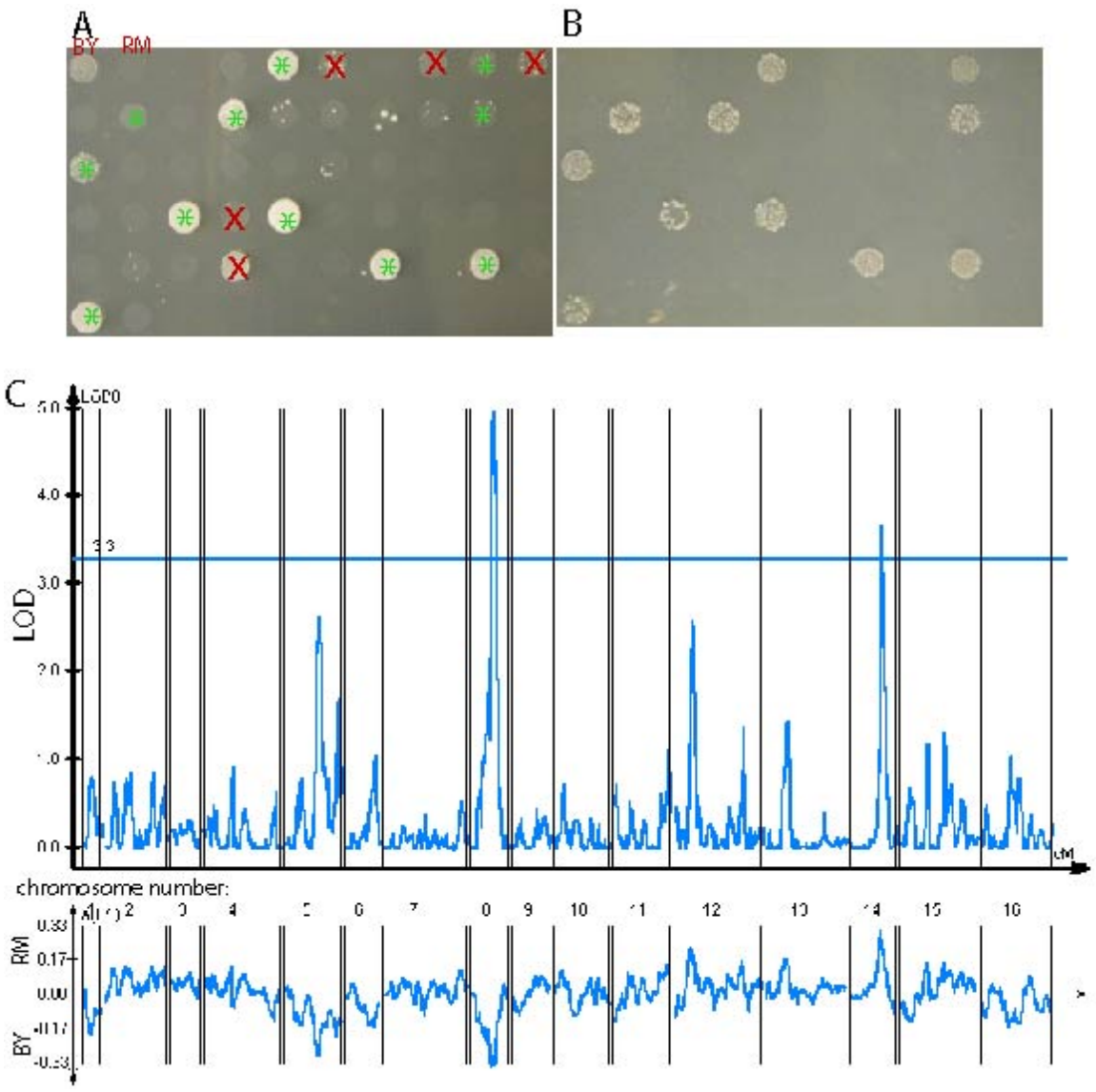
A
BY RM
B
C
LOD0
4.0
3.3
3.0
LOD
chromosome number:
RM
BY

Figure 3.4: QTL analysis of [GAR$^+$] frequency

a) Growth of segregants on GGM after five days. Samples that grew on GGM were mated to a [gar$^-$] strain with appropriate auxotrophies to select for diploids. Segregants marked with a red 'X' are completely prototrophic and so were not mated to [gar$^-$]; those with a green asterisks contained auxotrophies that allowed for selection of diploids. Parental strains were also not mated. b) Segregants from part a were pinned to GGM following mating and selection of diploids. However, prototrophic segregants were not included. Diploids grew on GGM, demonstrating that the glucosamine-resistant phenotype displayed is semi-dominant and therefore is likely caused by [GAR$^+$] rather than a genetic mutation. c) Chromosome map (x-axis, top) with associated LOD scores (y-axis, top) from QTL analysis. A significant peak is visible on chromosome VIII and chromosome XIV (top). The bottom area of the graph shows the extent of association with either parent (BY is negative, RM11 is positive) and the trait of interest. Note that the QTL on chromosome VIII is enriched for BY and the QTL on chromosome XIV is enriched for RM11.

glucosamine-resistance: *STD1*, *DOG2*, and *SPO23* (figure S3.3). *STD1* was the first factor identified to induce [GAR$^+$] (see chapter two). *DOG2* was also identified by QTL analysis, suggesting that its affects on [GAR$^+$] are real and important in the natural biology of the organism.

[GAR$^+$] cells have a competitive advantage in rich medium but not in minimal medium

To determine whether [GAR$^+$] might have a growth advantage over [gar$^-$], we measured growth rate (change in OD_{600}) in varying carbon sources (figure 3.5a). The growth rates of [gar$^-$] and [GAR$^+$] were almost identical in 2% glucose and 2% galactose. In the mixture of glucose and galactose, [GAR$^+$] reached a high maximum growth rate and did not show as much of a decrease in growth rate as [gar$^-$] did when transitioning between carbon sources during the diauxic shift. This suggests that [GAR$^+$] might exhibit a competitive advantage under particular environmental conditions. To test this, we co-cultured [gar$^-$] and [GAR$^+$] in both rich and defined media containing 2% glucose; a mixture of 0.1% glucose and 1.9% galactose, 2% galactose, and 0.2% glucose. Co-culture conditions allow for direct competition, as both [gar$^-$] cells and [GAR$^+$] cells have access to the same nutrients. Throughout the experiments, cells are plated to rich medium to determine density (colony-forming units, or cfu) then replica plated to glycerol-glucosamine medium to score the number of [GAR$^+$] cfu.

[GAR$^+$] cells outcompeted [gar$^-$] cells in all conditions involving a rich media base (1% yeast extract, 2% peptone). [GAR$^+$] cells compromise between 60% culture (in glucose medium) and 90% (in a mixture of glucose and galactose) of all cfu in the culture

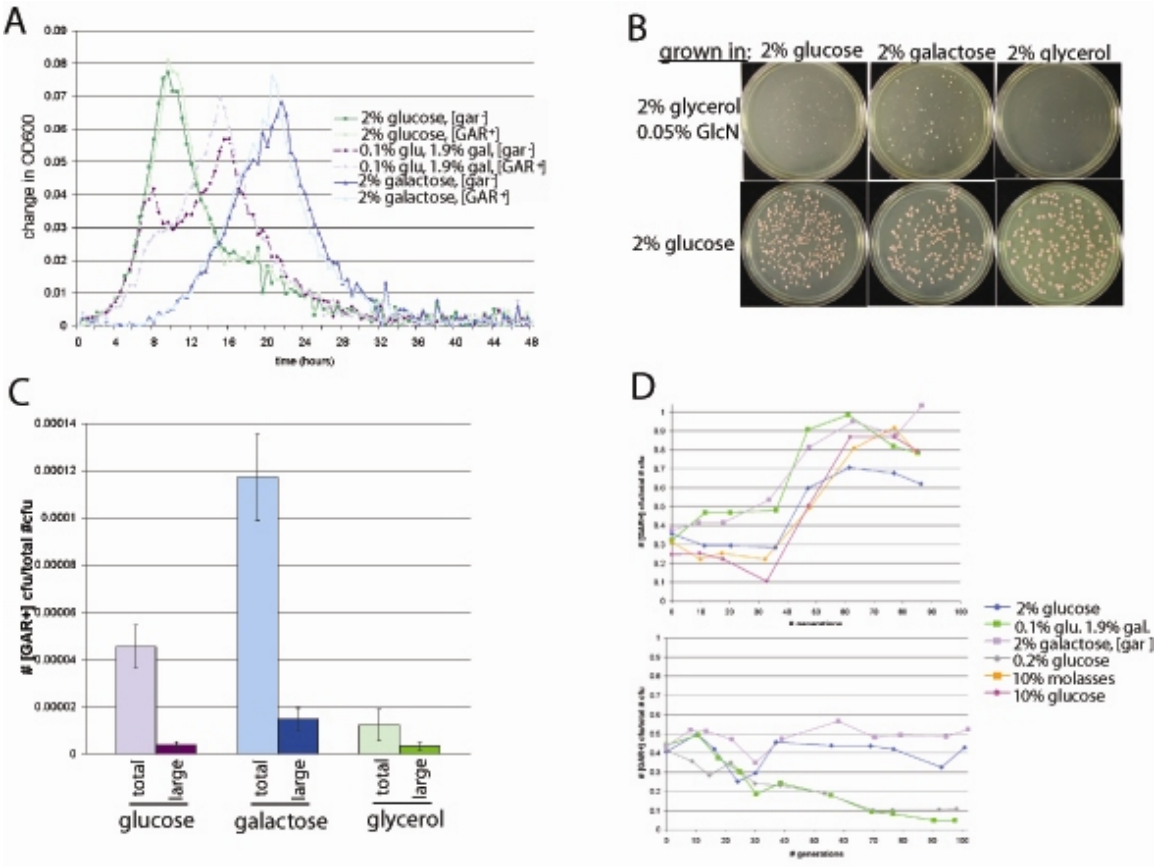

Figure 3.5: Do [GAR$^+$] cells have a competitive advantage over [gar$^-$] cells in some conditions?

a) [GAR$^+$] grows faster than [gar$^-$] in a glucose/galactose mixture. Growth rate, as represented by change in OD_{600}, is shown on the y-axis. [GAR$^+$] and [gar$^-$] cells show virtually identical growth rates in 2% glucose and 2% galactose but [GAR$^+$] does not decrease growth rate at six hours as [gar$^-$] does. Data shown are the mean of four difference samples b,c) Pictures (b) and quantification (c) of [GAR$^+$] frequency and strain ("large") following growth in different carbon sources. "Large" colonies are indicative of strong [GAR$^+$] strains. Large colonies are defined as those having a diameter of 1mm or more. Graph represents the mean +/- the standard deviation. d) Competition between [gar$^-$] and [GAR$^+$] cells in rich (top) or minimal (bottom) media. The Y-axis represents the ratio of [GAR$^+$] cfu to [gar$^-$] cfu and the x-axis represents the number of generations. Three separate samples were competed, each was plated in duplicate, and all six plates were average for each data point.

at the end of the experiment but started at ~35% of cfu (figure 3.5b, top). [GAR$^+$] did less well in 2% glucose than the other conditions; 2% glucose was the only condition in which [GAR$^+$] never increased to more than 71% of the total population of yeast in the culture from a starting point of approximately 35%.

[GAR$^+$] was at best neutral compared to [gar$^-$] in defined medium (yeast nitrogen base + amino acids) (figure 3.5b, bottom). Cultures grown in 2% glucose maintained the starting point of approximately 40% [GAR$^+$] cfu for 100 generations. [GAR$^+$] showed a slight advantage in 2% galactose. It attained about 50% of the *S. cerevisiae* population by 50 generations and maintained that until 100 generations. However, [GAR$^+$] fared poorly in the glucose/galactose mixture; it was less than 5% of total yeast by 100 generations. [GAR$^+$] also did not survive limiting glucose conditions well, and constituted only 10% of that yeast population after 100 generations. Data points from competition experiments represent the average of three independent cultures. Experiments were repeated three times.

The rate of [GAR$^+$] appearance varies with environmental conditions

Pma1 conformation and ATPase activity are regulated by environmental conditions, particularly carbon source (Morsomme et al., 2000). Therefore, because Pma1 is a causal agent of [GAR$^+$], we hypothesize that the GAR prion would also be sensitive to environmental conditions. To test this, we grew [gar$^-$] yeast in rich media with a variety of different carbon sources. The [GAR$^+$] frequency (figure 3.45) and strain ("strong" vs. "weak") (figure 3.5d) varied with carbon source. This suggests that GAR, like Pma1, responds to the host environment.

[GAR$^+$] alters the cell wall architecture and affects susceptibility to anti-fungal drugs

The *S. cerevisiae* cell wall is largely composed of glucose polymers and changes in environmental conditions alter the total amount of various components of the wall (Klis et al., 2006). Because [GAR$^+$] alters glucose utilization and is environmentally responsive, we hypothezied that [GAR$^+$] might affect the cell wall. First we stained by β-glucans using aniline blue, which binds to glucan but not chitin or mannoprotein. (Paul and Johnson, 1977). As β-glucan is completely masked in a normal cell wall (Klis et al., 2006) and, as expected, [gar$^-$] cells did not shown aniline glue staining. Surprisingly, we found that [GAR$^+$] cells showed some aniline blue staining, implying exposure of β-glucan. [gar$^-$] cells did not show aniline blue staining (figure 3.6a).

We further investigated differences in the cell walls of [GAR$^+$] cells compared to [gar$^-$] cells by measuring responses to cell wall-inhibiting drugs. The antifungal drug caspofungin targets yeasts by inhibit β-glucan synthase (Denning, 2003), and [GAR$^+$] was more sensitive to caspofungin than [gar$^-$]. [GAR$^+$] is also more sensitive to fluconozole (figure 3.6b), which permealizes the fungal plasma membrane by inhibiting ergosterol synthesis (Odds et al., 2003).

Discussion

[GAR$^+$] might play a role in yeast ecology

We observed a 20-fold higher average rate of appearance of [GAR$^+$] and a five-fold higher median rate of appearance in fruit isolates than in clinical or brewery isolates, regardless of temperature. This suggests that [GAR$^+$] might be advantageous in conditions resembling those of fruit isolates, such as low pH. As Pma1, a component of

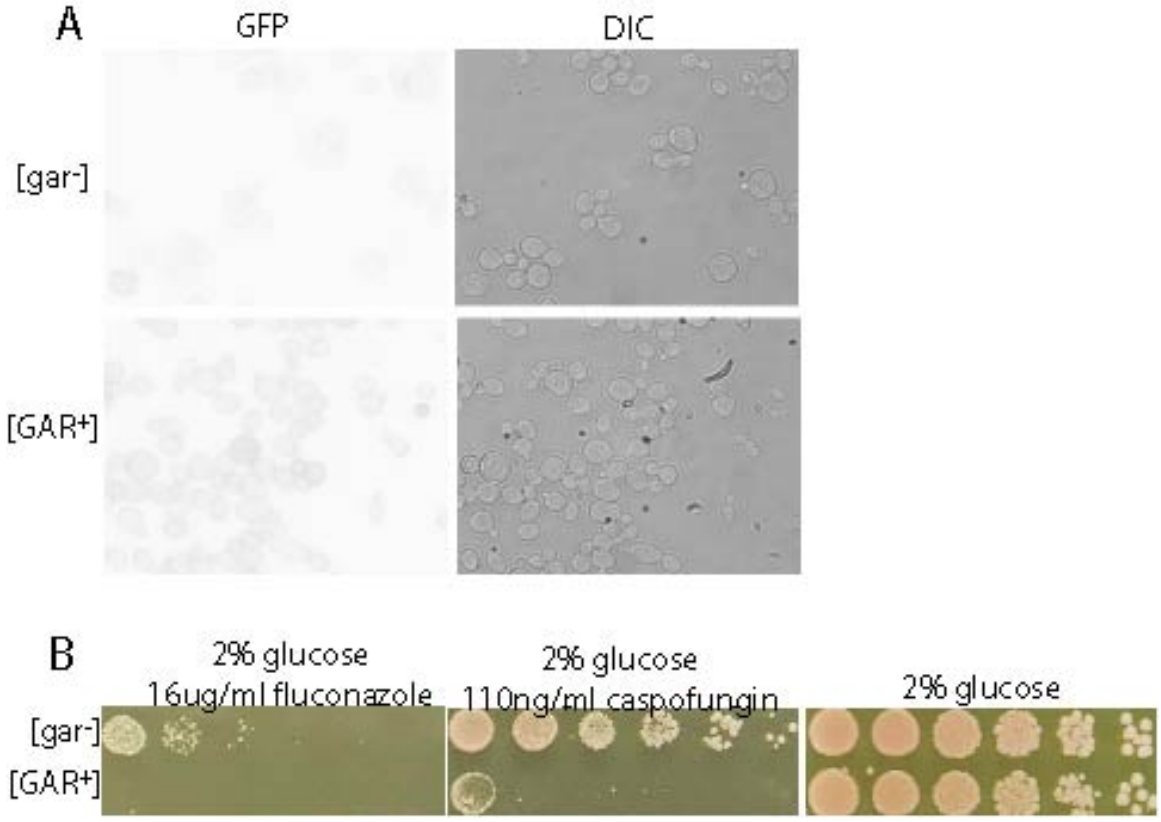

Figure 3.6: The cell wall architecture differs between [gar$^-$] and [GAR$^+$] cells

a) Aniline blue staining of β-glucans (left). [GAR$^+$] cells (bottom) exhibit more staining than [gar$^-$] cells (top), suggesting that the β-glucan is more exposed in [GAR$^+$] cells. b) [GAR$^+$] cells are more sensitive to antifugal drugs than [gar$^-$] cells. Cells were grown to midlog in YPD, serially diluted 5-fold, then spotted to the drug plates shown. [GAR$^+$] cells are more sensitive to caspofungin than [gar$^-$] cells. As caspofungin targets β-glucan synthase, this supports the data from part a. [GAR$^+$] cells are also more sensitive to fluconazole, implying that [GAR$^+$] cells are extra-sensitive to membrane stress.

[GAR$^+$], is a proton pump and is the major controller of cytoplasmic pH (Morsomme), it would not be surprising if [GAR$^+$] were sensitive to pH. We also demonstrated that the [GAR$^+$] prion is sensitive to the host environment, as [GAR$^+$] frequency and strain varies with carbon source. The [GAR$^+$] prion phenotype that is environmentally responsive.

PMA1 exhibits high levels of heterozygosity

Sequencing of *PMA1* from these wild yeast isolates did not yield any single polymorphism associated with [GAR$^+$] frequency. However, a large number of our yeast samples were heterozygous for *PMA1*, which was surprising. S. *cerevisiae* is predominantly homothallic (capable of switching mating type) and is thought to frequently inbreed due to intratetrad matings. Heterozygosity should thus be rare, as inbreeding leads to homozygosity (Kirby, 1984). The probability of obtaining homozygosity at a particular locus increases if that locus is centromeric (Zakharov, 2005). This is the case with *PMA1*, which is only 4cM away from the centromere of chromosome VII (Capieaux et al., 1991).

However, we found that 20 of our 45 samples (44%) were heterozygous at the *PMA1* locus. In contrast, one group who sequenced 27 samples at four loci found that 2 (7%) contained heterozygosity (Aa et al., 2006). Heterozygous samples were observed in isolates from all ecological niches, although only one fruit isolate showed heterozygosity. Heterozygosity complicated analysis of the *PMA1* sequence, however, as heterozygous samples were not included in Wilcoxon calculations of the association between polymorphisms and rate of appearance of [GAR$^+$].

[GAR$^+$] is influenced by other organisms

We demonstrated that *Staphylococcus* species are capable of switching [gar$^-$] *S. cerevisiae* cells to [GAR$^+$]. This suggests that [GAR$^+$] could well be found in wild yeast, as *Staphylococcus* species are commonly found in a wide variety of ecological niches, including in close association with humans (Madigan and Martinko, 2006). As *S. cerevisiae* is used in a variety of industrial processes, it is certainly possible that yeast would encounter *Staphylococcus* frequently.

[GAR$^+$] has a competitive advantage over [gar$^-$] under some conditions

Initial data suggest that [GAR$^+$] has an advantage in rich medium independent of carbon source but that [GAR$^+$] is neutral or disadvantageous in defined medium. Similarly, the [PSI$^+$] prion is advantageous in some conditions and disadvantageous in others (Eaglestone et al., 1999; True and Lindquist, 2000). Because [GAR$^+$] appears quite frequently in yeast strains isolated from fruit and because we have yet to identify an *S. cerevisiae* strain that cannot become [GAR$^+$], it seems likely that [GAR$^+$] occasionally serves some advantage.

Efficient carbon source utilization and energy production are among the most important processes for a cell. [GAR$^+$] alters carbon source utilization by conferring on its host the ability to use alternative carbon sources when glucose is present. Because processing of carbon sources is so important for a cell, we suggest that [GAR$^+$] is an excellent example prion for addressing the question of whether fungal prions play any role in microbe biology. The observation that an unknown bacterium seems to induce

[GAR$^+$] further supports the idea that [GAR$^+$] is environmentally responsive and might even be a plasticity factor.

Overall, our data suggest that [GAR$^+$] could have a competitive advantage under certain environmental conditions and that the GAR phenotype is sensitive to its environment. [GAR$^+$] exhibits a faster growth rate compared to [gar$^-$] under certain environmental conditions. Also, since [GAR$^+$] appears at a frequency of up to 1 in 20 cells in some yeast strains isolated from fruit, [GAR$^+$] could well confer some sort of advantage in this niche. Further experiments will attempt to address these questions.

Experimental procedures

[GAR+] frequency assays

[gar$^-$] cells were grown to midlog in YPD (2% glucose unless otherwise stated). Samples were diluted appropriately and plated to YPD and medium containing 2% glycerol and 0.05% glucosamine (GGM). Colony forming units (CFU) were counted after 2 days on YPD and after 6 days on GGM. Colony size was measured by using Scion Image.

Yeast strains and genetic manipulations

Strain construction and manipulation followed standard yeast techniques. Growth rate was measured in the Bioscreen C (Growth Curves USA) at 30°C with intensive, intermittent shaking with the OD_{600} measured every 15 minutes.

Sequencing of PMA1

PCR products of -1700 to +2950 were amplified using a high fidelity LA taq polymerase (Takara). Samples were subcloned using TA cloning methods into pCR2.1 (Invitrogen), transformed into DH5α *E. coli*, and selected for the presence of the insert using X-gal as per the manufacturer's instructions. A minimum of four plamids from two independent transformations were sequenced by the Northwoods DNA facility. Sequencing reactions were analyzed in Sequencher 4.7. Strains were considered heterozygous if one of the two conditions were met: two samples showed one nucleotide and two another or a single sample consistently differed from the other three at a rate higher than the observed error rate. In the latter case, more samples were usually sequenced unless the outlier sample showed heterozygosity only at sites of previously observed heterozygous polymorphisms.

QTL analysis

Segregants were grown in YPD in 96 well plates to midlog, then spotted to GGM. Growth density of spots was determined using Scion Image. Data were analyzed using WinQTLCartographer and JMP 5.0.

Cell wall staining

Cells were stained for chitin by growing in YPD or CSM until midlog, washing once in water, then incubating 5 minutes in a solution of 1% aniline blue (Sigma). Samples were washed twice in water then imaged on a Zeiss axioplan microscope.

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Staph. hominis gatgaacgctggcggcgtgcctaatacatgcaagtcgagcgaacagacgaggagcttgct
Unknown gatg::acgctggcggcgtgcctaatacatgcaagtcgagcgaacagacgaggagcttgct

Staph. hominis cctttgacgttagcggcggacgggtgagtaacacgtgggtaacctacctataagactggg
Unknown cctttgacgttagcggcggacgggtgagtaacacgtgggtaacctacctataagactggg

Staph. hominis ataacttcgggaaaccggagctaataccggataatatttcgaaccgcatggttcgatagt
Unknown ataacttcgggaaaccggagctaataccggataatatttcgaaccgcatggttcgatagt

Staph. hominis gaaagatggctctgctatcacttatagatggacctgcgccgtattagctagttggtaagg
Unknown gaaagatggctctgctatcacttatagatggacctgcgccgtattagctagttggtaagg

Staph. hominis taacggcttaccaaggcaacgatacgtagccgacctgagagggtgatcggccacactgga
Unknown taacggcttaccaaggcaacgatacgtagccgacctgagagggtgatcggccacactgga

Staph. hominis actgagacacggtccagactcctacgggaggcagcagtagggaatcttccgcaatgggcg
Unknown actgagacacggtccagactcctacgggaggcagcagtagggaatcttccgcaatgggcg

Staph. hominis aaagcctgacggagcaacgccgcgtgagtgatgaaggtcttcggatcgtaaaactctgtt
Unknown aaagcctgacggagcaacgccgcgtgagtgatgaaggtcttcggatcgtaaaactctgtt

Staph. hominis attagggaagaacaaacgtgtaagtaactgtgcacgtcttgacggtacctaatcagaaag
Unknown attagggaagaacaaacgtgtaagtaactgtgcacgtcttgacggtacctaatcagaaag

Staph. hominis ccacggctaactacgtgccagcagccgcggtaatacgtaggtggcaagcgttatccggaa
Unknown ccacggctaactacgtgccagcagccgcggtaatacgtaggtggcaagcgttatccggaa

Staph. hominis ttattgggcgtaaagcgcgcgtaggcggtttttaagtctgatgtgaaagcccacggctc
Unknown ttattgggcgtaaagcgcgcgtaggcggtttttaagtctgatgtgaaagcccacggctc

Staph. hominis aaccgtggagggtcattggaaactggaaaacttgagtgcagaagaggaaagtggaattcc
Unknown aaccgtggagggtcattggaaactggaaaacttgagtgcagaagaggaaagtggaattcc

Staph. hominis atgtgtagcggtgaaatgcgcagagatatggaggaacaccagtggcgaaggcgactttct
Unknown atgtgtagcggtgaaatgcgcagagatatggaggaacaccagtggcgaaggcgactttct

Staph. hominis ggtctgtaactgacgctgatgtgcgaaagcgtggggatcaaacaggattagataccctgg
Unknown ggtctgtaactgacgctgatgtgcgaaagcgtggggatcaaacaggattagataccctgg

Staph. hominis tagtccacgccgtaaacgatgagtgctaagtgttagggggtttccgcccccttagtgctgc
Unknown tagtccacgccgtaaacgatgagtgctaagtgttagggggtttccgcccccttagtgctgc

Staph. hominis agctaacgcattaagcactccgcctggggagtacgaccgcaaggttgaaactcaaaggaa
Unknown agctaacgcattaagcactccgcctggggagtacgaccgcaaggttgaaactcaaaggaa

Staph. hominis ttgacggggacccgcacaagcggtggagcatgtggtttaattcgaagcaacgcgaagaac
Unknown ttgacggggacccgcacaagcggtggagcatgtggtttaattcgaagcaacgcgaagaac

Staph. hominis cttaccaaatcttgacatcctttgacccttctagagatagaagtttccccttcgggggac
Unknown cttaccaaatcttgacatcctttgacccttctagagatagaagtttccccttcgggggac

Staph. hominis aaagtgacaggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttgggttaagtcc
Unknown aaagtgacaggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttgggttaagtcc

Staph. hominis cgcaacgagcgcaacccttaagcttagttgccatcattaagttgggcactctaagttgac
Unknown cgcaacgagcgcaacccttaagcttagttgccatcattaagttgggcactctaagttgac

Staph. hominis tgccggtgacaaaccggaggaaggtggggatgacgtcaaatcatcatgccccttatgatt
Unknown tgccggtgacaaaccggaggaaggtggggatgacgtcaaatcatcatgccccttatgatt

Staph. hominis tgggctacacacgtgctacaatggacaatacaaagggcagcgaaaccgcgaggtcaagca
Unknown tgggctacacacgtgctacaatggacaatacaaagggcagcgaaaccgcgaggtcaagca

Staph. hominis aatcccataaagttgttctcagttcggattgtagtctgcaactcgactacatgaagctgg
Unknown aatcccataaagttgttctcagttcggattgtagtctgcaactcgactacatgaagctgg

Staph. hominis aatcgctagtaatcgtagatcagcatgctacggtgaatacgttcccgggtcttgtacaca
Unknown aatcgctagtaatcgtagatcagcatgctacggtgaatacgttcccgggtcttgtacaca

Staph. hominis ccgcccgtcacaccacgagagtttgtaacacccgaagccggtggagtaaccatttggagc
Unknown ccgcccgtcacaccacgagagtttgtaacacccgaagccggtggagtaaccatttggagc

Staph. hominis tagccgtcgaaggtgggacaaatgattg
Unknown tagccgtcgaaggtgggacaaatgattg

Figure S3.1: 16S rDNA alignment of unknown and *Staphylococcus hominis*

16S rDNA was PCR amplified from the contaminating microorganisms that induced [gar$^-$] to switch to [GAR$^+$] cells. The PCR product was sequenced as described and BLAST analysis performed against the GenBank database. The unknown sample was 99% identical to *Staphylococcus hominis* (1467 out of 1468 bases, the one difference being a gap).

S288c ttttattttcatttttaatgatcgcgattattctgttggaaataacg
RM11 ttttattttcatttttaatgatcgcgattattctgttggaaataacg

S288c ttctgatggagattgttggttacgttgccactcacgtaagaagttc
RM11 ttctgatggagattgttggttacgttgccactcacgtaagaagttc

S288c aaaggataatggcagaattttcagctgatctatgtctttttgacct
RM11 aaaggataatggcagaattttcagctgatctatgtctttttgacct
DOG1 atggcagaattttcagctgatctatgtctttttgacct

S288c agatggtaccatagtgagtacaacagtggccgcagagaaagcatg
RM11 agatggtaccatagtgagtacaacagtggccgcagagaaagcatg
DOG1 agatggtaccatagtgagtacaacagtggccgcagagaaagcatg

S288c gaccaagttgtgttacgaatacggtgttgatccttccgagttattt
RM11 gaccaagttgtgttacgaatacggtgttgatccttccgagttattt
DOG1 gaccaagttgtgttacgaatacggtgttgatccttccgagttattt

S288c aagcattctcatggtgcaagaacacaagaggttttgagaaggtttt
RM11 aagcattctcatggtgcaagatcacaagaaatgatgaagaaatttt
DOG1 aagcattctcatggtgcaagaacacaagaggttttgagaaggtttt

S288c tccctaaattggatgatacagacaataaaggtgttcttgctctaga
RM11 ttccaaaattggacaataccgataataaaggtgttcttgcgttaga
DOG1 tccctaaattggatgatacagacaataaaggtgttcttgctctaga

S288c aaaagatat tgcccatagttacttggacacagtaagccttattcct
RM11 aaaggatatggcagataattatttggacacagtaagccttatccct
DOG1 aaaagatattgcccatagttacttggacacagtaagccttattcct

S288c ggtgcagagaacttactgttatcgttagatgtagatactgagactc
RM11 ggtgcagagaatttattgttatcgttagatgtaaatactgagactc
DOG1 ggtgcagagaacttactgttatcgttagatgtagatactgagactc

S288c aaaaaaagttacctgaaaggaaatgggctatcgttacctctggttc
RM11 aaaaaaagttacctgaaaggaaatgggctatcgttacctctggttc
DOG1 aaaaaaagttacctgaaaggaaatgggctatcgttacctctggttc

S288c tccatatttggcattttcatggttcgagacaatattgaaaaatgttg
RM11 tccctatttggcattttcatggttcgagacaatattgaaaaatgttg
DOG1 tccatatttggcattttcatggttcgagacaatattgaaaaatgttg

S288c gaaagcccaaagttttcattactgggtttgacgtgaagaacggtaa
RM11 gaaagcccaaagttttcattactgggtttgacgtgaagaacggtaa
DOG1 gaaagcccaaagttttcattactgggtttgacgtgaagaacggtaa

S288c gcctgatcccgagggttattcaagagctcgtgatttattgcgtcaa
RM11 gcctgatcccgagggttattcaagagctcgtgatttattgcgtcaa
DOG1 gcctgatcccgagggttattcaagagctcgtgatttattgcgtcaa

S288c gatttgcaattaactggtaaacaggatctgaagtatgttgtcttcg
RM11 gatttgcaattaactggtaaacaggatctgaagtatgttgtctttg
DOG1 gatttgcaattaactggtaaacaggatctgaagtatgttgtcttcg

S288c aagatgcacccgtgggcataaaggccggcaaagcaatgggcgcca
RM11 aagatgcacccgtgggcataaaggccggtaaagcaatgggcgcaa
DOG1 aagatgcacccgtgggcataaaggccggcaaagcaatgggcgcca

S288c ttactgtgggtataacatcctcgtatgacaagagcgttttatttgac
RM11 ttactgtgggtataacatcctcgtatgataagagcgttttatttgac
DOG1 ttactgtgggtataacatcctcgtatgacaagagcgttttatttgac

S288c gcaggagcagattatgtagtctgtgatttgacacaggtttccgtgg
RM11 gcaggtgcagattatgtagtctgtgatttgacacaggtttccgtgg
DOG1 gcaggagcagattatgtagtctgtgatttgacacaggtttccgtgg

S288c ttaagaacaatgaaaacggtattgtcatccaggtaaacaacccttt
RM11 ttaagaacaatgagaacggtatcgttatccaggtaaacaacccttt
DOG1 ttaagaacaatgaaaacggtattgtcatccaggtaaacaacccttt

S288c gacaagggcctgagtaaacaaaaatgtgacaaaagaacgaatata
RM11 :::
DOG1 gacaagggcctga

S288c tatagatgtaaaacatatggacaagcaaaaagtcgaattatgtatg
RM11 :::

S288c tcattttaggtactgaagaggtaagatttttttgagtttttcttcg
RM11 :::

S288c aagatggttgtgtggttatatgttaatcttccttagcgcaaaacact
RM11 :::

S288c tccatcaactgtatttcgttggaatgctttgtattcagttttgtatca
RM11 :::::::::::::::::::::::::: ::::::::::::::::::::::::::::::::::::

S288c ttatctttaatcacaattgcgtcaggatgtaagaactacgtaatgat
RM11 ::

S288c cttattatttctcgtagagaatagttccgtagattgaatacgctccg
RM11 ::

S288c tcattatttttaaatgtggggaaggggtaattctcgaggatttttca
RM11 ::

S288c aaaacttaaaatgcgctggcaacatcttctttggtgaaaacaaatg
RM11 ::

S288c ctaaaaggagactaagagtacttttgttattcactatagtattagc
RM11 ::

S288c caacacgttatcgatacatttactgctatatacataaaaaatttacg
RM11 ::

S288c tcaaaaaaataaaaaaaaaaaatgccacaattttcagtagatcttt
RM11 ::
DOG2 atgccacaattttcagtagatcttt

S288c gtctttttgacctagatgggactattgtcagcacaacaactgcagcg
RM11 ::
DOG2 gtctttttgacctagatgggactattgtcagcacaacaactgcagcg

S288c gaaagtgcctggaaaaaattatgccgtcagcatggggttgatcctg
RM11 ::
DOG2 gaaagtgcctggaaaaaattatgccgtcagcatggggttgatcctg

S288c ttgagttattcaagcattcccatggtgcaagatcacaagaaatgatg
RM11 ::
DOG2 ttgagttattcaagcattcccatggtgcaagatcacaagaaatgatg

S288c aagaaatttttttccaaaattggacaataccgataataaaggtgttct
RM11 ::
DOG2 aagaaatttttttccaaaattggacaataccgataataaaggtgttct

S288c tgcgttagaaaaggatatggcagataattatttggacacagtaagcc
RM11 ::
DOG2 tgcgttagaaaaggatatggcagataattatttggacacagtaagcc

S288c ttatccctggtgcagagaatttattgttatcgttagatgtagatactg
RM11 ::
DOG2 ttatccctggtgcagagaatttattgttatcgttagatgtagatactg

S288c agactcaaaaaaagttacctgaaaggaaatgggctatcgttacctct
RM11 ::
DOG2 agactcaaaaaaagttacctgaaaggaaatgggctatcgttacctct

S288c ggttctccatatttggcattttcatggttcgagacaatattgaaaaat
RM11 ::
DOG2 ggttctccatatttggcattttcatggttcgagacaatattgaaaaat

S288c gttggaaagcccaaagttttcattactggatttgacgtgaagaacgg
RM11 ::
DOG2 gttggaaagcccaaagttttcattactggatttgacgtgaagaacgg

S288c taagcctgatcccgagggttactcaagagctcgtgatttattgcgtc
RM11 ::
DOG2 taagcctgatcccgagggttactcaagagctcgtgatttattgcgtc

S288c aagatttgcaattaactggtaaacaggatctgaagtatgttgtcttt
RM11 ::
DOG2 aagatttgcaattaactggtaaacaggatctgaagtatgttgtcttt

S288c gaagatgcacccgtgggcataaaggccggcaaagcaatgggcgcaa
RM11 ::
DOG2 gaagatgcacccgtgggcataaaggccggcaaagcaatgggcgcaa

S288c ttactgtgggtataacatcctcgtatgataagagcgttttatttgacg
RM11 ::
DOG2 ttactgtgggtataacatcctcgtatgataagagcgttttatttgacg

S288c caggtgcagattatgtggtctgtgatttgacacaggtttccgtggtt
RM11 ::
DOG2 caggtgcagattatgtggtctgtgatttgacacaggtttccgtggtt

S288c aagaacaatgagaacggtatcgttatccaggtaaacaacccctttgac
RM11 ::
DOG2 aagaacaatgagaacggtatcgttatccaggtaaacaacccctttgac

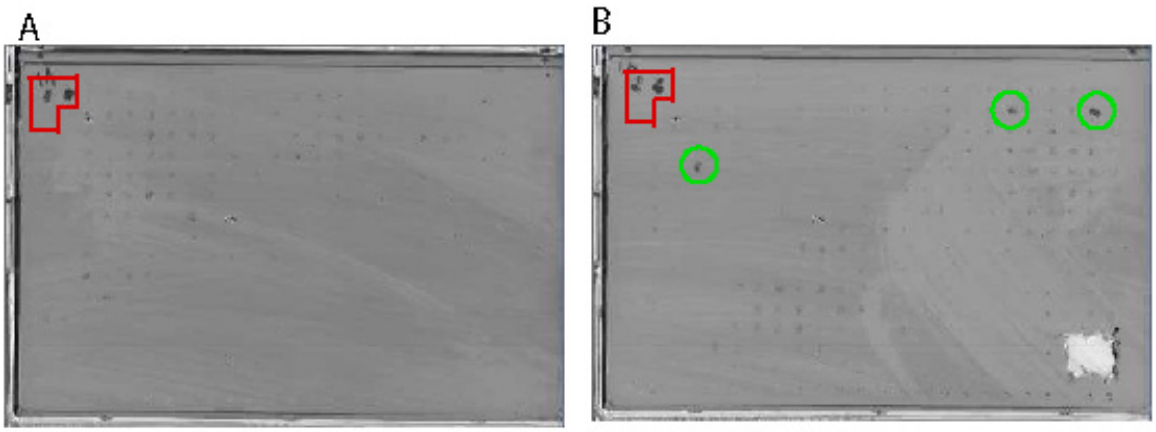
S288c gagagattaaataaataaggacatcgcagaagcacgaatatata
RM11 ::::::::: gacgagagattaaataaataaggacatcgcagaagcacga
DOG2 gagagattaa

S288c agataaaattgtatgtaaaagcaaaagttga::::::::::actgcgtatga
RM11 agataaaattgtatgtaaaagcaaaagttgattgaactgcgtatga

S288c tttcttatagtgagtatgaaatttttttttttttt:::::ggttatctaact
RM11 tttcttatagtgagtatgaaatttttttttttttttttggttatctaact

S288c tatttttctt
RM11 tatttttctt

Figure S3.2: Alignment of S288c and RM11 in the region surrounding *DOG1* and *DOG2*
Region on chromosome VIII that corresponds with altered [GAR^+] frequency by QTL mapping (figure 3.4). The RM11 parent has lost *DOG2* and its upstream region, whereas BY4716 contains both *DOG1* and *DOG2*. As the *DOG* genes confer resistance to 2-deoxyglucose, this region could be responsible for the difference in [GAR^+] frequency observed between BY and RM11.


A
B

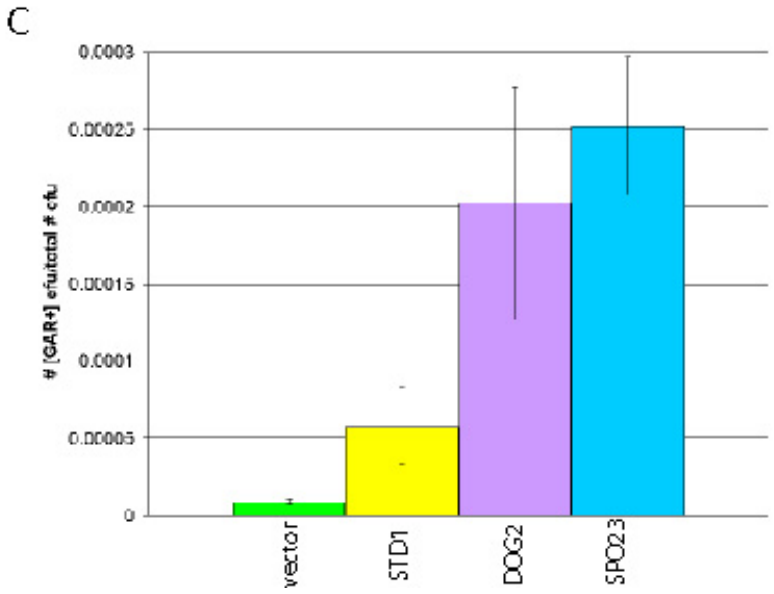

C
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
[GAR+] efuitotal # cfu
vector
STD1
DOG2
SPO23

Figure S3.3: Screen for inducers of [GAR$^+$]

A library of ~5000 ORFs was overexpressed as previously described using the inducible *GAL1* promoter combined with the estradiol system (Louvion et al., 1993; Quintero et al., 2007). Following 48 hours growth in inducing medium, cells were plated to glycerol-glucosamine medium to select for [GAR$^+$] cells. a) A typical glycerol-glucosamine plate from this screen. Control cells are in the upper left corner in the red box. *STD1* under control of the GPD promoter (top left) and the *GAL*-estradiol system (top right) grew on glycerol-glucosamine medium, as expected. Cells containing empty vectors (bottom left) did not grow. No ORF tested on this plate induced [GAR$^+$]. b) Controls (red box) are the same as in part a. On this plate three ORFs induced [GAR$^+$] following overexpression (green circles): *STD1* (left), *DOG2* (middle), and *SPO23* (right). c) Quantification of the increase in [GAR$^+$] frequency following overexpression of *STD1*, *DOG2*, or *SPO23*. The increase in [GAR$^+$] cells is seven fold, 23 fold, or 28 fold relative to vector, respectively. Error bars are +/- standard deviation.

Chapter Four:

Conclusions and Further Experiments

When we began this work, fungal prions were limited in number, involved an amyloid-based structure only, and tended to be rich in glutamines and asparagines. [PSI^+], [URE3], and [Het-s] were long-standing, previously unexplained phenotypes that had been identified as prions based on genetic characteristics. All three proteins aggregated in their [$PRION^+$] form and entered into insoluble, SDS-resistant heritable aggregates. Chaperone activity, particularly that of Hsp104, fragmented aggregates to create heritable prion seeds (Shorter and Lindquist, 2005). To the best of anyone's knowledge, fulfillment of the genetic attributes of prions required an aggregation- and amyloid-based mechanism of inheritance.

Most of the early attempts to identify additional fungal prions were based on sequence, particularly looking for N- and Q-rich proteins. These identified [RNQ^+] (Sondheimer and Lindquist, 2000) and [NU^+] (Michelitsch and Weissman, 2000; Osherovich et al., 2004) but pre-disposed the results to amyloid-based prions.

Prions in general were originally defined only as a protein-based infectious element and fungal prions were defined by their genetic characteristics, albeit with the suggestion that they could be caused by a conformational change (Wickner, 1994; Prusiner, 1998; Shorter and Lindquist, 2005). However, because the initial set of fungal prions aggregated in the [$PRION^+$] form, the first decade or so of work on fungal prions

focused on how aggregation was linked to heritability. Recent work in the fungal prion field has considered whether genetic elements that do not involve amyloids might act as prions (Wickner et al., 2007). The first of these was a self-activating protease, [β], which under artificial conditions is self-propagating. However, because [β] is only self-propagating in a Δ*pep4* background, it serves more as a proof-of-principle than a physiologically important non-amyloid based fungal prion (Roberts and Wickner, 2003). Another putative non-amyloid prion, *Crippled growth* (*C*), seems to be caused by a self-sustaining MAP kinase cascade. However, data on the mechanism of *C* propagation or even which proteins are involved are limited (Kicka and Silar, 2004; Kicka et al., 2006).

Our work on [GAR$^+$] advances the prion field by demonstrating the existence of a non-amyloid, composite prion consisting of a transmembrane protein and a signaling protein that likely alter a signaling cascade in the [PRION$^+$] form. [GAR$^+$] fulfills all the genetic characteristics of a prion: it exhibits semi-dominant, non-Mendelian, infectious inheritance, appears spontaneously at a very high frequency, and is susceptible to chaperones. However, [GAR$^+$] is otherwise quite different from the other fungal prions.

The signaling protein *STD1* strongly induces a stable [GAR$^+$] state following transient overexpression but is not essential for [GAR$^+$] propagation. [PSI$^+$] and [URE3] are both induced by transient overexpression of their prion-determining proteins but these proteins are then necessary for prion propagation (Wickner et al., 1995). The closest situation to that of [GAR$^+$] and *STD1* is that [RNQ$^+$] is necessary for [PSI$^+$] induction but not propagation (Derkatch et al., 2000; Derkatch et al., 2001).

[GAR$^+$] is also novel among fungal prions because Pma1, one protein involved in [GAR$^+$], is a transmembrane protein. PrP, the mammalian prion protein, is GPI-

anchored, but it changes location in the disease-associated form and enters into extracellular aggregates (Prusiner, 1998). [GAR$^+$] therefore expands the category of potential prions to include transmembrane proteins and proteins that do not change localization or solubility between [prion$^-$] and [PRION$^+$].

Finally, [GAR$^+$] appears to be involved in a signaling cascade. Std1 is part of the Rgt2/Snf3 signaling pathway in [gar$^-$] cells (Schmidt et al., 1999) and seems to still be associated with that pathway in [GAR$^+$] cells. When *S. cerevisiae* is grown in glucose, a signal is propagated through the Snf3/Rgt2 pathway that prevents transcriptional repression of *HXT3*. However, in *HXT3* is prematurely silenced in [GAR$^+$]. Mutations in Pma1 that alter the rate of appearance of [GAR$^+$] also show increased silencing at *HXT3*. This silencing is greater than can be accounted for by the increased [GAR$^+$] frequency alone. Therefore, we hypothesize that Pma1 is involved in the Rgt2/Snf3 signaling pathway and that [GAR$^+$] represents an altered signaling cascade through the Rgt2/Snf3 pathway. This is quite different from [PSI$^+$], [URE3], [Het-s], and [RNQ$^+$].

There are four outstanding questions about [GAR$^+$] that we find particularly interesting: do Pma1 and Std1 act together to create [GAR$^+$]; how is [GAR$^+$] passed from mother to daughter cells; is the transition between [gar$^-$] and [GAR$^+$] regulated and if so, how; and is [GAR$^+$] found in other species or outside fungi. These are related to the larger question of what sort of proteins can be heritable and how and whether prions are common or found in a variety of species.

How are Pma1 and Std1 involved in [GAR$^+$] propagation and induction?

PMA1 is sufficient for a species barrier in [GAR$^+$] and replacing *PMA1* from *S. cerevisiae* with *PMA1* from other *Saccharomyces* species is sufficient to block [GAR$^+$] propagation. These data demonstrate the importance of Pma1 in [GAR$^+$] propagation. Swapping *PMA1* between species, however, could also disrupt protein interactions involving Pma1. The species barrier experiment there implicates Pma1 but does not eliminate the involvement of other proteins. Further, mutations in both Pma1 and Std1 are required for preventing [GAR$^+$] propagation and neither mutation alone is sufficient to cure [GAR$^+$]. Is Std1 also involved in [GAR$^+$] propagation?

Random mutagenesis of Std1 and Pma1 could help determine how the two interact and what makes [GAR$^+$] heritable. If one could isolate a form of Std1 that increases the frequency of [GAR$^+$] appearance, whether the mutant Std1 acted by increasing association with Pma1 or by some other mechanism would be informative. Mutational analysis should be extended to the N-terminal region of Pma1 to determine whether association with Std1 or oligomerization of Pma1 is more important for the formation of [GAR$^+$]. The N-terminus of Pma1 is thought to be involved in but is not sufficient for Pma1 homooligomerization (Kuhlbrandt et al., 2002; Liu et al., 2006). Transient overexpression of a *PMA1* mutant lacking the N-terminal 40 amino acids does not increase [GAR$^+$] frequency, whereas overexpression of wildtype *PMA1* increases [GAR$^+$] appearance. Does the N-terminal truncation change Pma1 oligomers or its association with Std1? Blue Native gel analysis could be performed on the N-terminus mutant to determine whether it is still capable of associating with Std1 and whether Pma1 still forms high molecular weight oligomers. If both oligomers and Std1 association are

disrupted, one could create smaller mutations within the N-terminal region of the endogenous copy of Pma1 in an attempt to identify mutants that no longer stably associate with Std1. These could then be used to determine whether Std1 is necessary for [GAR$^+$] propagation, propagating the signal through the Rgt2/Snf3 pathway, or whether the two are irretrievably linked.

[GAR$^+$] exhibits a strong species barrier, since Pma1 from *S. paradoxus* cannot propagate [gar$^-$] of *S. cerevisiae* Pma1 origin (see chapter two). The sequence of Pma1 differs little between the species but Std1 differs considerably. Is this "species barrier" between *S. paradoxus* and *S. cerevisiae* [GAR$^+$] the result of differences in Std1? This could be shown by substituting *STD1* from *S. paradoxus* for the *S. cerevisiae* version. Also, one could test whether *S. bayanus* Pma1, which weakly propagates *S. cerevisiae* [GAR$^+$] can associate with *S. cerevisiae* Std1 and vice versa.

Another way of probing the Std1/Pma1 relationship would be to determine whether the association in [GAR$^+$] between individual Std1 and Pma1 molecules is long- or short-lived. Our data suggest that the association overall is stable, but if the turnover is high that it suggests a model such Std1 being protected from degradation but still interacting with Rgt1, rather than a long-term association between Std1 and Pma1 creating a new signal or altering the membrane organization through shifting protein complexes. Fluorescent recovery after photobleaching (FRAP) could be used to monitor the association between Std1 and Pma1 and such methods have been used to monitor membrane protein turnover in the past. First tagged Std1 could be bleached and monitored to determine dynamics and whether its association with Pma1 is long- or short-lived. Tagged Pma1 could then be monitored to determine whether Pma1 in

[GAR$^+$] shows different dynamics than Pma1 in [gar$^-$] cells. This could also show whether membrane fluidity is similar in [gar$^-$] cells and [GAR$^+$] cells.

Std1 is involved in propagating the glucose signal (Schmidt et al., 1999; Kaniak et al., 2004) and our data suggest that Pma1 is also involved in signaling through the Rgt2/Snf3 pathway. What role does Mth1, the homolog of Std1 (Hubbard et al., 1994), play in [GAR$^+$] propagation? Mth1 blocks [GAR$^+$] appearance when transiently overexpressed, possibly due to transcriptional repression of *STD1* (Kaniak et al., 2004), and we see Mth1 associating with Pma1 oligomers in the [gar$^-$] form. Does the association of Mth1 physically block the association of Std1, thus preventing the formation of [GAR$^+$]? Or does Mth1 set up a [gar$^-$] state that, like [GAR$^+$], is also self-supporting? One could perform mutation analysis of Mth1 and Std1 by domain swapping to determine which regions are important for the association with Pma1. Mth1 and Std1 have been shown to be degraded when glucose is present but Std1 is then newly transcribed, whereas *MTH1* transcription is repressed (Flick et al., 2003). Is this turnover of the cellular Std1 somehow important for [GAR$^+$]? Which regions in Std1 and Mth1 are involved in their different associations with Pma1? The N-terminal half is most probably, as that is the divergent region; the C-terminal portions are fairly similar to each other (supplemental). Chimeric proteins could be overexpressed to determine which promote and which block [GAR$^+$] appearance.

Finally, the role of the N- and C-termini of Pma1 in formation of the [GAR$^+$] heritable element is not completely understood. Engineering chimeric proteins containing either the N- or the C-terminus fused to a stable protein containing a single membrane-spanning domain would help address this question. The constructs could be

overexpressed, presumably without the toxicity that results from *PMA1* overexpression, to definitively establish whether the N- or C-terminus can induce [GAR^+]. Random mutagenesis of the Pma1 N- and C-terminal regions of these constructs could be used to identify contacts important for [GAR^+] formation and propagation. That information could then be used to investigate the importance of the Pma2 oligomer for [GAR^+] and further strengthen the argument that Pma1 is responsible for [GAR^+].

How is [GAR^+] heritable?

Previously described yeast prions form amyloid aggregates in the [$PRION^+$] form; transmission of these aggregates from mother cells to daughter cells renders the [$PRION^+$] protein conformation heritable (Cox et al., 2003). However, [GAR^+] does not form aggregates, so how is it propagated from mother cell to daughter cell? This question would be relatively easy to answer for amyloid-based prions, but no one has observed a heritable phenotype dependent on a membrane protein before. Is [GAR^+] heritable due to Pma1/Std1 prion "seeds" or is it heritable because it creates a self-propagating signaling cascade (figure 4.1)?

The ideal way to answer the mechanism of heritability would be by protein transformation. The transformation of *S. cerevisiae* cells from [psi^-] to [PSI^+] by transformation of Sup35 protein fibers provided definitive proof of protein-only inheritance and thus the prion hypothesis (Tanaka et al., 2004). Since these experiments, researchers have been able to induce [URE3] (Brachmann et al., 2005), [RNQ^+] (Patel and Liebman, 2007), [Het-s] (Ritter et al., 2005), and [Cin] (Collin et al., 2004) by protein transformation. Protein transformation of [GAR^+] by Pma1 would

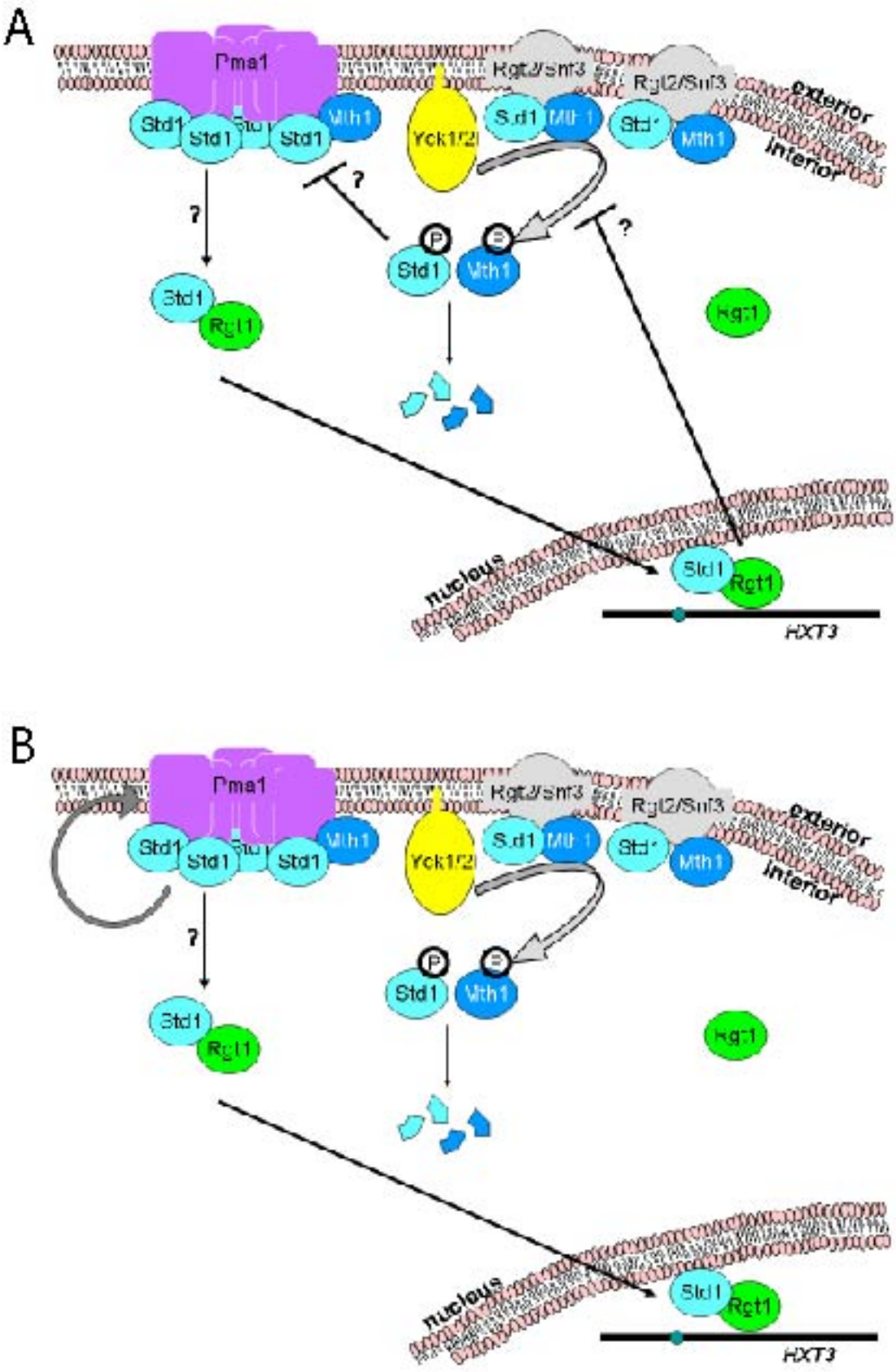

A
Pma1
Rgt2/Snf3
exterior
interior
Std1
Mth1
Yck1/2
?
P
Ub
Rgt1
nucleus
HXT3
B
Pma1
Rgt2/Snf3
exterior
interior
Std1
Mth1
Yck1/2
?
P
Ub
Rgt1
nucleus
HXT3

Figure 4.1: How is [GAR$^+$] heritable?

a) The [GAR$^+$] element could be heritable because it establishes a self-perpetuating signaling pathway. For instance, transcriptional repression caused by Std1 and Rgt1 could repress genes in addition to *HXT3* that, for example, might degrade Std1 protein or repress the *STD1* gene or a gene involved in the glucosamine-resistant phenotype. This could setup a feedback loop, in which Pma1 and Std1 alter glucose signaling in such a way that the new signal strengthens the association between Pma1 and Std1, thus sustaining the original signal. b) A second model for [GAR$^+$] proposes that Pma1 enters into a self-propagating protein conformation, possibly due to its association with Std1. This conformation could alter signaling through the Rgt2/Snf3 signaling pathway, which accounts for the transcriptional repression of *HXT3* in glucose-grown [GAR$^+$] cells.

incontroversially establish Pma1 as a causal agent of [GAR$^+$] but show whether the heritable form is modified, enzymatically active, etc. These would help determine whether [GAR$^+$] is heritable solely because of conformation or because of activity such as a self-propagating cascade. Unfortunately, protein transformation with Pma1 presents a number of difficulties. A large protein with 10 transmembrane domains would be hard to purify from *E. coli*. One could purify just the N-terminal region, which when deleted prevents induction of [GAR$^+$] by transient overexpression of *PMA1*. However, one lacks a method of inducing this peptide to enter into the [GAR$^+$] conformation, assuming the [GAR$^+$] form results from protein conformation and not posttranslational modification.

If a protein transformation protocol could be established for [GAR$^+$], it could be used to determine what is necessary and sufficient for [GAR$^+$]. Is Std1 necessary for transformation of Pma1 or does Std1 just make the conversion more efficient? Do posttranslational modifications facilitate the switch between [gar$^-$] cells and [GAR$^+$] cells? The creation of [GAR$^+$] *in vitro* might also provide enough material for structural studies.

If a protein transformation protocol cannot be established, one could attempt to address the question of conformation or signaling cascade using some of the mutational analysis described in the previous section. The question of how posttranslational modification affects Pma1 and [GAR$^+$] could be addressed with alanine- and/or glutamic acid-scanning mutagenesis of the N-terminal region of Pma1.

What causes the switch between [gar$^-$] and [GAR$^+$] and is it regulated?

It is not known whether or how the switch from [prion$^-$] cells to [PRION$^+$] cells is regulated. Chaperones are necessary for the maintenance and inheritance of prion phenotypes and conditions that alter chaperone levels affect the appearance and propagation of prions (Jones and Tuite, 2005). For example, a number of chemical stress conditions increase the frequency of [PSI$^+$] appearance (Tyedmers et al., unpublished). As all prions are sensitive to chaperones, are all prions similarly sensitive to environmental conditions? If so, this would strengthen the argument that prions can occasionally be beneficial, as it would suggest that prions represent an environmentally responsive mechanism of phenotypic sampling.

[GAR$^+$] suggests an additional mechanism of prion regulation: phosphorylation. Pma1 enzyme activity is regulated by phosphorylation and serine to alanine mutations in phosphorylation sites increase [GAR$^+$] frequency. Can phosphorylation or dephosphorylation induce Pma1 to switch between different prion states? A number of kinases have been shown to phosphorylate Pma1; the expression level of some of these could be altered to determine whether they alter [GAR$^+$] frequency. Several of kinases that regulate Pma1 are members of Npr/Hal5 family, which regulate nutrient transporters (Goossens et al., 2000). [GAR$^+$] might therefore have a level of regulation beyond what other prions have, and be sensitive both to nutrient-sensitive phosphorylation and changes in chaperone levels.

As Pma1 is already known to be phosphorylated, why would it be subject to the additional level of regulation that is this stable, heritable propagation of the [GAR$^+$] prion phenotype? Phosphorylation acts rapidly and responds quickly to environmental

conditions, which renders [GAR$^+$] seemingly redundant. However, a possible advantage of [GAR$^+$] is that the entire population of cells, or even all of the protein in a single cell, does not enter into the [GAR$^+$] form. Instead, a subset switches to [GAR$^+$] but the population as a whole does not suffer from the negative consequences of [GAR$^+$]. This would be more difficult to obtain through kinase activity, which tends to have high penetrance.

Does the [GAR$^+$] heritable element confer a benefit to its host?

Whether prions are found in wild yeast and whether they confer any benefit to their hosts are points of ongoing debate (True and Lindquist, 2000; True et al., 2004; Wickner et al., 2007). The [GAR$^+$] element provides a particularly interesting subject for these questions because glucose metabolism is central to cell survival. [GAR$^+$] appears spontaneously at extremely high frequency, up to one in 100 cells in some strains. Yeast isolated from a particular ecological niche, fruit, showed enrichment for high [GAR$^+$] frequency, suggesting that [GAR$^+$] might occasionally be advantageous. Experiments directly competing [gar$^-$] cells and [GAR$^+$] cells suggest the same conclusion. [GAR$^+$] can be induced by a number of *Staphylococcus* species, making it probable that yeast cells carrying the [GAR$^+$] element could be found in the wild.

However, we do not currently know whether [GAR$^+$] is found in other fungi or plants. If [GAR$^+$] is conserved, it is more likely to be beneficial. The plant equivalent of Pma1 exists in many isoforms, including 11 in *Arabidopsis* and seven in tomato. The enzyme activity and sometimes expression levels of many of these are sensitive to

environmental conditions, including glucose, the hormone auxin, and light (Portillo, 2000).

How widespread is [GAR$^+$] and, by extension, prions in general?

Pma1 is a highly conserved protein that is found in both fungi and plants. Whether Pma1's prion-forming ability is also conserved is potentially very interesting, particularly since prions have not yet been identified in plants. Pma1 in other fungi also show interesting properties. For instance, Pma1 from *C. albicans* species is responsive to the clinically important dimorphic switch (Monk et al., 1993).

One difficulty in assaying for [GAR$^+$] outside of *S. cerevisiae* is that they phenotype of [GAR$^+$] requires glucose repression. While many fungi exhibit some sort of carbon catabolite repression, it is very efficientt in *S. cerevisiae* and *S. pombe*. These other fungi do, however, carry *PMA1* (Portillo, 2000). In many cases, the predicted Pma1 protein has a long, unstructured, cytoplasmic N-terminal region that resembles the *S. cerevisiae* one necessary for overexpression-induced increase in [GAR$^+$] frequency. One therefore should not limit searching for [GAR$^+$] in organisms that have glucose repression or *STD1* but instead look at a variety of organisms, particularly those in which Pma1 has unstructured cytoplasmic regions. Regulation of enzyme activity of Pma1 relatives by environmental conditions is found in other fungi and in plants; this could be an important property in whether something can for [GAR$^+$].

To determine whether *PMA1* from other organisms can form a prion, tests can first be perform in *S. cerevisiae*, then developed in the native organism. This has been used before to identify prions; *SUP35* from a variety of fungi has been shown to act as a

prion in *S. cerevisiae*. A gene from the sea slug *Aplysia californica* was successfully tested for prion-like properties in *S. cerevisiae* (Si et al., 2003). Our work suggests that *PMA1* from *Saccharomyces paradoxus* and *Saccharomyces bayanus* can maintain [GAR$^+$] in *S. cerevisiae* even though they cannot propagate [GAR$^+$] to *S. cerevisiae PMA1*. It therefore seems reasonable to test *PMA1* alleles from other organisms, even those that lack *STD1* or *MTH1*, in *S. cerevisiae* for prion-like properties.

If *PMA1* from other organisms can act as a prion in *S. cerevisiae*, it would be interesting to test whether *PMA1* can form a prion in its organism of origin. This is more difficult because the [PRION$^+$] phenotype is unknown. Fortunately Pma1 is a well-studied protein, so one could use known phenotypes of mutants as a starting point to identify a possible [GAR$^+$] phenotype. For example, *S. cerevisiae* [GAR$^+$] is more sensitive to caspofungin in [GAR$^+$] than [gar$^-$]; perhaps *C. albicans* or *C. neoformans* [GAR$^+$] equivalent would exhibit a similar phenotype.

[GAR$^+$] is an interesting new prion partly because its causal agent, Pma1, is well conserved and could be the basis for identifying prions in plants. However, because Pma1 is a transmembrane protein it also could serve as a starting point for identifying transmembrane proteins that can form prions. Recent work showed that the transmembrane protein syntaxin forms large homooligomers via its extracellular regions (Sieber et al., 2007). Pma1 similarly forms large homooligomers through a domain not embedded in the membrane. This leads us to speculate that either the prion phenotype of Pma1 is a byproduct of the oligomerization or the oligomerization leads to its prion-forming ability. Can proteins that form large oligomers in the membrane form prions? One could start by testing transporters in *S. cerevisiae* that show unstructured

cytoplasmic regions for prion-like properties and then perhaps extend the study to other organisms.

We described here a new prion, [GAR$^+$], which involves the transmembrane protein Pma1 and the glucose signaling molecule Std1. These two elements associate in the [GAR$^+$] but do not aggregate, change localization, or form an amyloid. [GAR$^+$] therefore represents a novel type of prion that functions by an unknown mechanism that might involve a signaling cascade. This expands the pool of potential prions to include transmembrane proteins and demonstrates that prions need not aggregate or form amyloid. As Pma1 is a well-conserved protein found in a variety of species it is an excellent candidate for testing the commonality of the prion phenomenon. We also suggest that [GAR$^+$] might be beneficial to its host under some conditions and is induced by co-culturing *S. cerevisiae* with a variety of *Staphylococcus* species. These data suggest that the [GAR$^+$] heritable element could well be found in the wild.

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