Biochemical, Cell Biological, and Genetic Assays to Analyze Amyloid and Prion Aggregation in Yeast

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Simon Alberti[1], Randal Halfmann[1,2] and Susan Lindquist[1,2,3,4]

1. Whitehead Institute for Biomedical Research, Cambridge, MA, USA
2. Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA
3. Howard Hughes Medical Institute, Cambridge, MA, USA
4. Correspondence should be addressed to S.L.

S. Alberti: 617 258 6517 (phone), 617 258 7226 (fax), alberti@wi.mit.edu

R. Halfmann: 617 258 6517 (phone), 617 258 7226 (fax), halfmann@mit.edu

S. Lindquist: 617 258 5184 (phone), 617 258 7226 (fax),

Lindquist_admin@wi.mit.edu

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ABSTRACT

Protein aggregates are associated with a variety of debilitating human diseases, but they can have functional roles as well. Both pathological and non-pathological protein aggregates display tremendous diversity, with substantial differences in aggregate size, morphology and structure. Among the different aggregation types, amyloids are particularly remarkable, because of their high degree of order and their ability to form self-perpetuating conformational states. Amyloids form the structural basis for a group of proteins called prions, which have the ability to generate new phenotypes by a simple switch in protein conformation that does not involve changes in the sequence of the DNA. Although protein aggregates are notoriously difficult to study, recent technological developments and, in particular, the use of yeast prions as model systems, have been very instrumental in understanding fundamental aspects of aggregation. Here, we provide a range of biochemical and yeast genetic methods that are currently used in our laboratory to study protein aggregation and the formation of amyloids and prions.

1. Introduction

More than 40 years ago an unusual nonsense suppressor phenotype was reported that was inherited in a non-Mendelian manner (Cox 1965). This phenotype, [*PSI*+], was later found to be caused by a change in the conformation of the translation termination factor Sup35p (Patino *et al.* 1996; Paushkin *et al.* 1996). Shortly after the discovery of [*PSI*+], a different genetic element, [*URE3*], was isolated (Lacroute 1971) and the causal agent was afterwards indentified as a conformationally altered form of the nitrogen catabolite repressor Ure2p (Wickner 1994). Ure2p and Sup35p are the founding members of an intriguing class of yeast proteins that can act as protein-based epigenetic elements. Also known as prions, these proteins can interconvert between at least two structurally and functionally distinct states, at least one of which adopts a self-propagating aggregated state. A switch to the aggregated prion state generates new phenotypic traits, which increase the phenotypic heterogeneity of yeast populations (Shorter and Lindquist 2005; Alberti *et al.* 2009)..

Sup35 is a translation termination factor. When Sup35p switches into a prion state, a large fraction of the cellular Sup35p is sequestered into insoluble aggregates. The resulting reduction in translation termination activity causes an increase in ribosomal frame-shifting (Namy *et al.* 2008; Park *et al.* 2009) and stop codon read-through (Liebman and Sherman 1979; Patino *et al.* 1996; Paushkin *et al.* 1996). The sequences downstream of stop codons are highly variable, and this, in turn, facilitates the sudden generation of new phenotypes by the uncovering of previously hidden genetic variation (Eaglestone *et al.* 1999; True

and Lindquist 2000; True *et al.* 2004). The other well-understood prion protein, Ure2p, regulates nitrogen catabolism through its interaction with the transcriptional activator Gln3p. Its prion state, [*URE3*], causes the constitutive activation of Gln3p and consequently gives cells the ability to utilize poor nitrogen sources in the presence of a rich nitrogen source (Aigle and Lacroute 1975; Wickner 1994).

The epigenetic properties of Sup35p and Ure2p reside in structurally independent prion-forming domains (PrDs) with a strong compositional bias for residues such as glutamine and asparagine (Edskes *et al.* 1999; Li and Lindquist 2000; Santoso *et al.* 2000; Sondheimer and Lindquist 2000). This observation stimulated the first sequence-based query for additional yeast prions (Sondheimer and Lindquist 2000) that ultimately lead to the identification of an additional prion protein called Rnq1. The prion form of Rnq1, [*RNQ+*], was found to underlie the previously characterized non-Mendelian trait [*PIN+*], which facilitates the *de novo* appearance of [*URE3*] and [*PSI+*] (Derkatch *et al.* 2000; Derkatch *et al.* 2001). Rnq1, however, not only induces benign conformational transitions of other prion proteins, it can also induce toxic conformational changes of glutamine-expansion (polyQ) proteins that cause Huntington's disease and several other late-onset neurodegenerative disorders. When polyQ fragments are expressed in yeast, they create a polyQ length-dependent toxicity that is accompanied by the formation of visible amyloid-containing aggregates in a [*RNQ+*]-dependent manner (Krobitsch and Lindquist 2000; Duennwald *et al.* 2006a). Yeast prions have been widely used as model systems for the study of

protein aggregation *in vivo*. They were tremendously useful for unraveling several aspects of protein aggregation, including the formation of structural variants, known as prion strains, or the presence of transmission barriers between related prion proteins (Tessier and Lindquist 2009).

Protein aggregation is a highly complex process that is determined by intrinsic factors, such as the sequence and structure of the aggregation-prone protein, as well as extrinsic factors, such as temperature, salt concentration and chaperones (Chiti and Dobson 2006; Rousseau *et al.* 2006). A particular type of aggregation underlies the self-perpetuating properties of prions. The biochemically well-characterized prions have an amyloid conformation. Amyloid is a highly ordered fibrillar aggregate. The fibril core is a continuous sheet of β-strands that are arranged perpendicularly to the fibril axis. The exposed β-strands at the ends of the fibril allow amyloids to polymerize by the continuous incorporation of polypeptides of the same primary sequence. This extraordinary self-templating ability allows prions to generate and multiply a transmissible conformational state (Ross *et al.* 2005; Shorter and Lindquist 2005; Caughey *et al.* 2009). Prions can spontaneously switch to this transmissible state from a default conformational state that is usually soluble.

In a recent systematic attempt to discover new prions in yeast we used the unusual amino acid biases of known PrDs to predict novel prionogenic proteins in the *S. cerevisiae* proteome. We subjected one hundred such prion candidates to a range of genetic, cell biological and biochemical assays to analyze their prion- and amyloid-forming propensities, and determined that at least twenty-four yeast

proteins contain a prion-forming domain (Alberti *et al.* 2009). Our findings indicate that prions play a much broader role in yeast biology and support previous assumptions that prions buffer yeast populations against environmental changes. The fact that prions are abundant in yeast suggests that prions also exist in other organisms. Moreover, many more examples of functional aggregation, which do not involve an epigenetic mechanism for the inheritance of new traits, are likely to be discovered. The methods and techniques described here have vastly increased our understanding of protein aggregation in yeast. They will allow us to identify additional aggregation-prone proteins in yeast and other organisms and will advance our understanding of the pathological and non-pathological functions of aggregates.

2. Methods

2.1. Detecting protein aggregation in yeast cells

Protein aggregates are formed when large numbers of polypeptides cooperate to form non-native molecular assemblies. These structures are highly diverse, with differences in the amount of β-sheet content, their overall supramolecular organization and their ability to induce the co-aggregation of other proteins (Chiti and Dobson 2006; Rousseau *et al.* 2006). Notwithstanding the multifactorial nature and complexity of protein aggregation, protein aggregates can simplistically be classified as disordered (amorphous) or ordered (amyloid-like). Amorphous aggregates are generally not well characterized due to their tremendous structural plasticity. Recent studies, however, indicate that the

constituent proteins of some amorphous aggregates have a conformation that is similar to their native structure in solution (Qin *et al.* 2007; Vetri *et al.* 2007). Ordered aggregates, on the other hand, contain greater amounts of β-sheet content and form densely packed amyloid fibers. Amyloid-like aggregates can be distinguished from disordered aggregates based on their resistance to physical and chemical perturbations that affect protein structure, such as increased temperature, ionic detergents or chaotropes. In the following section we provide a variety of methods for the analysis of diverse protein aggregates in yeast cells.

2.1.1. Fluorescence microscopy and staining of amyloid-like aggregates

Aggregating proteins coalesce into macroscopic assemblies that can be visualized by fluorescence microscopy. This very convenient method for following aggregation in cells has greatly expanded our understanding of protein misfolding diseases and other non-pathological aggregation-based phenomena such as prions (Johnston *et al.* 1998; Garcia-Mata *et al.* 1999; Kaganovich *et al.* 2008). Two different experimental approaches are available for the direct visualization of protein aggregates in cells: (1) The aggregate-containing cells can be fixed and treated with an antibody specific to the aggregation-prone protein, or (2) the aggregation-prone protein can be expressed as a chimera with a fluorescent protein (FP). Either approach has both advantages and disadvantages. Immunofluorescence microscopy requires an extensive characterization of the antibody to determine its specificity and to ensure that it is able to recognize the aggregated state of the protein. The benefit, however, is

that the aggregation-prone protein can be expressed unaltered. In fact, tagging of an aggregation-prone protein with an FP can severely interfere with its aggregation propensity by changing its overall solubility. The FP tag could also sterically interfere with the formation of the amyloid structure. Moreover, some FPs are known to self-interact. Particularly problematic in this regard are older versions of DsRed, although we have observed that in rare cases GFP fusions can also cause spurious aggregation. Generally, GFP-driven aggregation does not react with thioflavin T (discussed below) and forms a characteristic well-defined high molecular weight species when analyzed by SDD-AGE (described in section 2.1.3). Experiments with FP chimeras therefore require some caution in the interpretation of the results and we recommend performing additional assays to independently establish whether a protein is aggregation-prone or not.

Despite these drawbacks, using FP-chimeras to study protein aggregation has several advantages, particularly cost effectiveness, ease of use and rapid generation of results. Diverse yeast expression vectors are now available for the tagging of aggregation-prone proteins with FPs, most of which are suitable for determining the aggregation propensities of a protein (Krobitsch and Lindquist 2000; Duennwald *et al.* 2006a; Alberti *et al.* 2007; Alberti *et al.* 2009). The choice of the promoter and the copy number of the yeast plasmid are also important parameters that need to be considered carefully. Expression from high copy two-micron plasmids is highly variable, thus allowing the sampling of a range of different protein concentrations. This property is desirable if the goal is to determine whether a protein is generally able to nucleate and enter an amyloid-

like state in a cellular environment. Low-copy CEN-based plasmids and expression cassettes for integration into the genome, however, have more steady expression levels, a property which can be useful if more consistent aggregation behavior is desired. We usually try to avoid constitutive promoters, as aggregation can be associated with toxicity and frequently triggers growth arrest or cell death. Inducible promoters like *GAL1* are more suitable and transient expression for 6-24 hours is usually sufficient to induce aggregation in a significant fraction of the cell population.

Aggregation-prone proteins form foci that can be visualized by fluorescence microscopy. Yeast cells expressing aggregation-prone proteins form two characteristic types of fluorescent foci (Figure 1A): (1) ring-like structures that localize to the vacuole and/or just below the plasma membrane, or (2) punctate structures that can be distributed all over the cytoplasm but preferentially reside close to the vacuole (Derkatch *et al.* 2001; Zhou *et al.* 2001; Ganusova *et al.* 2006; Taneja *et al.* 2007; Alberti *et al.* 2009). The fibrillar appearance of ring-like structures and their reactivity with amyloid-specific dyes suggests that they consist of laterally associated amyloid fibers. Punctate foci, on the other hand, do not always stain with amyloid-binding dyes, suggesting that these structures can also be of the non-amyloid or amorphous type (Douglas *et al.* 2008). The two types of aggregation can be distinguished based on their fluorescence intensity and their pattern of aggregation. Rings and large punctate foci with very bright fluorescence are indicative of highly ordered amyloid fibers,

whereas multiple small puncta with low brightness usually do not react with amyloid-specific dyes and are therefore of the amorphous type.

To conclusively determine whether these foci result from amorphous or amyloid-like aggregation, we use a staining protocol with the amyloid-specific dye Thioflavin T (ThT). ThT has an emission spectrum that is red-shifted upon amyloid-binding, therefore allowing co-localization with aggregation-prone proteins that are tagged with yellow fluorescent protein. However, staining yeast cells with ThT can lead to high background levels and thus we recommend performing ThT co-staining experiments only for proteins with relatively high expression levels. To grow the yeast cells for staining with ThT, a culture is inoculated in the appropriate selective medium for overnight growth, followed by dilution and regrowth until it reaches an OD_{600} of 0.25 - 1.0. Then 8 ml of the culture are transferred onto a 150 ml Nalgene bottle-top filter (45 mm diameter, 0.2 µM pores, SFCA membrane) and the solution is filtered by applying a vacuum. When the solution has passed through the filter, the vacuum is halted and 5 ml of freshly prepared fixing solution (50 mM H_2KPO_4, pH 6.5; 1 mM $MgCl_2$; 4% formaldehyde) is added to the cells on the filter. The cells are resuspended by swirling and the suspension is then transferred to a 15 ml tube. The cells are incubated at room temperature for 2 hrs and vortexed briefly every 30 min.

The fixed cells are collected by a brief centrifugation step (2 min at 2,000 rcf) and the supernatant is removed carefully and completely. The cells are then resuspended in 5 ml buffer PM prepared freshly (0.1 M H_2KPO_4, pH 7.5; 1 mM

$MgCl_2$) and collected again by centrifugation. After the supernatant is removed completely the cells are resuspended in buffer PMST (0.1 M H_2KPO_4, pH 7.5; 1 mM $MgCl_2$; 1 M Sorbitol; 0.1% Tween 20; containing 1 x EDTA protease inhibitor mix from Roche). The volume of the PMST buffer should be adjusted to generate a final cell density of 10 OD_{600}. 100 µl of the cell suspension is then transferred to a 0.5-ml Eppendorf tube. 0.6 µl of ß-mercaptoethanol and 20 µl of 20,000 U/ml yeast lytic enzyme (ICN, or use zymolyase at 1mg/ml) are added and the spheroplasted cells are incubated on a rotating wheel at room temperature for 15 min for spheroplasting. The spheroplasted cells are then resuspended gently in 100 µl PMST, collected by centrifugation and the resuspension step is repeated once. Subsequently, the cells are incubated in PBS (pH 7.4) containing 0.001% ThT for 20 minutes, washed three times with PBS and then used immediately for fluorescence microscopy.

The pattern of aggregation is protein-specific, dependent on the level and duration of expression and regulated by the physiological state of the cell. Many yeast prion proteins, for instance, proceed through a maturation pathway that includes an early stage with ring-like aggregation patterns and a later stage with punctate cytoplasmic distribution (Alberti *et al.* 2009). Other amyloidogenic proteins such as glutamine-expanded versions of huntingtin exon 1 almost exclusively form punctate foci, when expressed at comparable levels. Interestingly, toxic and non-toxic aggregates of glutamine-expanded huntingtin have distinct subcellular aggregation patterns. Toxic huntingtin forms multiple

punctuate foci, whereas the non-toxic structural variant is present in a single cytosolic focus (Duennwald *et al.* 2006a; Duennwald *et al.* 2006b).

Yeast cells have two different aggresome-like compartments to deal with aggregation-prone proteins such as huntingtin (Kaganovich *et al.* 2008). These compartments, called JUNQ and IPOD, contain predominantly soluble or insoluble misfolded proteins, respectively. The JUNQ is believed to provide a subcellular location for the proteasome-dependent degradation of misfolded proteins, whereas the IPOD is enriched for chaperones like Hsp104 and seems to be a place for the sequestration of insoluble protein aggregates. Targeting to either of the compartments most likely influences the localization of aggregation-prone proteins, but the mechanisms of targeting and the factors involved in the maintenance of the compartments remain to be determined.

2.1.2. Sedimentation assay

In addition to their reactivity with amyloid-specific dyes like ThT, other criteria can be used experimentally to determine whether intracellular aggregates are amyloid-like, such as their unusual resistance to chemical solubilization..The detergent insolubility of amyloids has been used to isolate amyloid-containing fractions from yeast cell lysate by centrifugation (Sondheimer and Lindquist 2000; Bradley *et al.* 2002). In a typical amyloid sedimentation experiment 10 ml of yeast cells are grown to mid-logarithmic phase. The cells are collected by centrifugation and washed in water. The cell pellet is then resuspended in 300 µl of lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl, 2 mM EDTA, 5% glycerol). To

inhibit proteolysis we supplement the lysis buffer with 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM N-ethylmaleimide (NEM) and 1 x EDTA-free protease inhibitor mix (Roche). The suspension is transferred to a 1.5 ml Eppendorf tube containing 300 µl of 0.5 mm glass beads. The cells are then lysed using a bead beater at 4°C and are immediately placed on ice. 300 µl of cold RIPA buffer (50 mM Tris, pH 7.0; 150 mM NaCl; 1% Triton X-100; 0.5% Deoxycholate; 0.1% SDS) is added and the lysate is vortexed for 10 seconds. Subsequently, the crude lysate is centrifuged for 2 min at 800 rcf (4°C) to pellet the cell debris. The sedimentation assay is performed by centrifuging 200 µl of the supernatant in a TLA 100-2 rotor for 30 min at 80,000 rpm and 4°C using an Optima TL Beckman ultracentrifuge. Equal volumes of unfractionated (total) and supernatant samples are incubated in sample buffer containing 2% SDS and 2% β-mercaptoethanol at 95°C for 5 min. The pellet is resuspended in 200 µl of a 1:1 mixture of lysis buffer and RIPA buffer containing protease inhibitors and boiled in sample buffer under the same conditions described above. The samples are then analyzed by SDS-PAGE and immunoblotting with an antibody specific to the aggregation-prone protein. If a putative prion is analyzed, it should predominantly be detectable in the supernatant of prion-free cells and in the pellet fraction of prion-containing cells (see Figure 1B for an example).

2.1.3. Semidenaturing detergent-agarose gel electrophoresis (SDD-AGE)

The recent invention of semi-denaturing detergent-agarose gel electrophoresis (SDD-AGE) very conveniently allows the resolution of amyloid polymers based

on size and insolubility in detergent (see Figure 1C for an example) (Bagriantsev *et al.* 2006). We adapted SDD-AGE for large-scale applications, allowing simultaneous detection of SDS-insoluble conformers of tagged proteins in a large number of samples (Halfmann and Lindquist 2008). This advanced version of SDD-AGE enables one to perform high-throughput screens for novel prions and other amyloidogenic proteins.

As a first step it is necessary to cast the detergent-containing agarose gel. Standard equipment for horizontal DNA electrophoresis can be used and the size of the gel casting tray and the comb should be adjusted according to the number and volume of the samples. We usually prepare a 1.5% agarose solution (medium gel-strength, low EEO) in 1 x TAE. The agarose solution is heated in a microwave until the agarose is completely dissolved. Subsequently, SDS is added to 0.1% from a 10% stock. The agarose solution is then poured into the casting tray. After the gel has set, the comb is removed and the gel is placed into the gel tank. The gel is then completely submerged in 1 x TAE containing 0.1% SDS.

The following lysis procedure is optimized for large numbers of small cultures processed in parallel, although it can be easily modified for individual cultures of larger volume. For high-throughput analysis of yeast lysates we use 2 ml cultures grown overnight with rapid agitation in 96-well blocks. The cells are harvested by centrifugation at 2,000 rcf for 5 min and then resuspended in water. After an additional centrifugation step and removal of the supernatant, the cells are resuspended in 250 µl spheroplasting solution (1.2 M D-sorbitol; 0.5 mM

$MgCl_2$; 20 mM Tris, pH 7.5; 50 mM β-mercaptoethanol; 0.5 mg/ml Zymolyase 100T) and incubated for 1 hr at 30°C. The spheroplasted cells are collected by centrifugation at 800 rcf for 5 min and the supernatant is removed completely. The pelleted spheroplasts are then resuspended in 60 µl lysis buffer (20 mM Tris 7.5; 10 mM β-mercaptoethanol; 0.025 U/µl Benzonase; 0.5% Triton X-100; 2 x HALT protease inhibitor from Sigma-Adlrich). The 96-well block is covered with tape and vortexed at high speed for 1 min and then incubated for an additional 10 min at room temperature. The cellular debris is sedimented by centrifugation at 4,000 rcf for 2 min and the supernatant is carefully transferred to a 96-well plate. As a next step, 4 x sample buffer (2 x TAE; 20% glycerol; 8% SDS; bromophenol blue to preference) is added to a final concentration of 1 x, followed by brief vortexing to mix.

In SDS-containing buffers amyloid-like aggregates are stable at room temperature, but can be disrupted by boiling. Therefore, samples are incubated for an additional 10 min at room temperature, or, as a negative control, incubated at 95°C. Most amyloids will be restored to monomers by the 95°C treatment. The samples are then loaded onto the agarose gel. We usually also load one lane with pre-stained SDS-PAGE marker, enabling us to verify proper transfer and to estimate the size of unpolymerized SDS-soluble conformers. In addition, it is important to use protein aggregation standards. [*psi-*] and [*PSI+*] cell lysates or lysates of yeast cells overexpressing the huntingtin length variants Q25 (non-amyloid) and Q103 (amyloid) can be used for this purpose. The electrophoresis is performed at low voltage (≤ 3 V/cm gel length) until the dye front reaches ~1

cm from the end of the gel. It is important that the gel remains cool during the run since elevated temperatures can reduce the resolution.

For the blotting procedure, we prefer a simple capillary transfer using a dry stack of paper towels for absorption. One piece of nitrocellulose and 8 pieces of GB002 blotting paper (or an equivalent substitute) are cut to the same dimensions as the gel. An additional piece of GB002, which serves as the wick, is cut to be about 20 centimeters wider than the gel. The nitrocellulose, wick, and 4 pieces of GB002 are immersed in 1 x TBS (0.1 M Tris-HCl, pH 7.5; 0.15 M sodium chloride). A stack of dry folded paper towels is assembled that is about 2 cm thick and the same length and width of the gel. On top of the stack of paper towels 4 pieces of dry GB002 are placed, then one piece of wet GB002, and finally the wet nitrocellulose. The gel in the casting tray is briefly rinsed in water to remove excess running buffer. It is then carefully moved from the tray onto the stack. We recommend adding extra buffer on the nitrocellulose to prevent bubbles from becoming trapped under the gel. The remaining three pre-wetted GB002 pieces are placed on top of the gel. To ensure thorough contact between all layers, a pipette should be rolled firmly across the top of the stack. The transfer stack is subsequently flanked with two elevated trays containing 1 x TBS and the pre-wetted wick is draped across the stack such that either end of the wick is submerged in 1 x TBS. Finally, the assembled transfer stack is covered with an additional plastic tray bearing extra weight (e.g. a small bottle of water) to ensure proper contact between all layers of the stack. The transfer should proceed for a minimum of three hours, although we generally transfer over night.

After the transfer, the membrane can be processed by standard immunodetection procedures.

2.1.4. Filter retardation assay using yeast protein lysates

Another convenient method for analyzing aggregation is the size-dependent retention of aggregates on non-binding membranes (Figure 1D). This assay was initially developed to investigate amyloid formation of huntingtin in *in vitro* aggregation assays (Scherzinger *et al.* 1997), but it can also be used to detect aggregates in yeast cell lysates. Cells should be processed as for SDD-AGE (section 2.1.3), except that the sample buffer is omitted. Instead, the lysates are treated with the desired detergent- or chaotrope-containing buffer. Generally, we use SDS at 0.1-2%. Samples are incubated at room temperature for 10 min, or, for a negative control, boiled in 2% SDS.

During this incubation period the vacuum manifold is prepared. First, a thin filter paper (GB002) that is soaked in water is placed on the manifold. Then, a cellulose acetate membrane (pore size 0.2 µm) is soaked in PBS containing 0.1% SDS and placed on top of the filter paper on the manifold. The manifold is closed and the samples are loaded into the wells of the manifold. The samples are filtered through the membrane by applying a vacuum and the membrane is washed 5 times with PBS containing 0.1% SDS. The cellulose acetate membrane can then be used for immunodetection with a protein-specific antibody. To demonstrate that equal amounts of protein were present in the samples, the same procedure can be repeated with a protein-binding

nitrocellulose membrane. As with SDD-AGE, it is important for filter retardation experiments to include protein aggregation standards, such as [*psi*-] and [*PSI*+] cell lysates or lysates of yeast cells overexpressing the huntingtin length variants Q25 and Q103.

2.2. Assays for prion behavior

Prions are amyloids that are transmissible. Fragments of amyloid fibrils can be passed between cells or organisms, and the self-templating ability of amyloid results in the amplification of the structure, giving prions an infectious property. The prion properties of yeast prions reside in structurally independent prion-forming domains (PrDs). The PrDs of Sup35p and other prions are modular and can be fused to non-prion proteins, thereby creating new protein-based elements of inheritance (Li and Lindquist 2000). We employ two assays which exploit this property of prions to experimentally determine whether a predicted PrD can confer a heritable switch in the function of a protein. The assays are based on the well-characterized prion phenotypes of the translation termination factor Sup35p and the nitrogen catabolite regulator Ure2p.

2.2.2. Sup35p-based prion assay

Sup35p consists of an N-terminal PrD (N), a highly charged middle domain (M) and a C-terminal domain (C), which functions in translation termination. Both the N and M domains are dispensable for the essential function of Sup35p in translation termination. The charged M domain serves to increase the solubility of

the amyloid-forming N domain, thereby promoting the conformational bistability of the Sup35p protein. In the prion state, a large fraction of the cellular Sup35p is sequestered into insoluble aggregates, resulting in reduced translation termination activity and an increase in the read-through of stop codons. Premature stop codons in genes of the adenine synthesis pathway, which are present in lab strains such as 74D-694, provide a convenient way to monitor switching of Sup35p to the prion state (Figure 2A). In prion-free [*psi*-] cells, translation termination fidelity is high, leading to the production of truncated and non-functional Ade1p. As a consequence the [*psi*-] cells are unable to grow on adenine-free medium and accumulate a byproduct of the adenine synthesis pathway that confers a red colony color when grown on other media such as YPD. Prion-containing [*PSI*+] cells, on the other hand, have a reduced translation termination activity that allows read-through of the *ade1* nonsense allele and the production of functional full-length Ade1p protein, resulting in growth on adenine-deficient medium and the expression of a white colony color.

The modular nature of prion domains enables the generation of chimeras between the C or MC domain of Sup35p and candidate PrDs that can then be tested for their ability to generate [*PSI*+]-like states (Figure 2A). For several years plasmids have been available that allow the cloning of a candidate PrD N terminal to the C or MC domain of Sup35p (Sondheimer and Lindquist 2000; Osherovich and Weissman 2001). These plasmids can be integrated into the yeast genome to replace the endogenous *SUP35* gene. This procedure, however, is very laborious and has a low success rate, as the resulting strains

frequently express the chimera at levels much lower than wild type Sup35p. As a consequence, these strains aberrantly display a white colony color and show constitutive growth on medium lacking adenine, preventing their use in prion selection assays.

To overcome these difficulties, we recently developed a yeast strain (YRS100) in which a deletion of the chromosomal *SUP35* is covered by a Sup35p-expressing plasmid (Alberti *et al.* 2009). When these cells are transformed with expression plasmids for *PrD-SUP35C* chimeras, a *URA3* marker on the covering *SUP35* plasmid allows it to be selected against in 5-FOA-containing medium (plasmid shuffle). The resulting strains contain PrD-Sup35C fusions as their only source of functional Sup35p. This plasmid-based expression system is more versatile than the previous versions, as it easily allows the use of different promoters and therefore a better control over the expression level of PrD-Sup35 proteins. We have generated vectors with four different promoters, which have the following relative strength of expression: *SUP35* < *TEF2* < *ADH1* < *GPD*. To minimize the time needed for strain generation, these vectors enable recombination-based cloning using the Gateway® system. In recent years, the Gateway® system has emerged as a very powerful cloning method that allows for the rapid *in vitro* recombination of candidate genes into diverse sets of expression vectors. We have generated hundreds of Gateway®-compatible yeast expression vectors, each with a different promoter, selectable marker or tag (Alberti *et al.* 2007; Alberti *et al.* 2009). This technological improvement now allows us to perform high-throughput testing of candidate PrD libraries for prion

properties. All Gateway-compatible plasmids described here are available through the non-profit plasmid repository Addgene (www.addgene.org).

The *SUP35C* and related fusion assays were instrumental in identifying new prions and prion candidates (Sondheimer and Lindquist 2000; Osherovich and Weissman 2001; Alberti *et al.* 2009; Nemecek *et al.* 2009). However, it can be difficult to work with PrD-Sup35 chimeras and it is therefore important to know about the shortcomings of this assay. Whether a Sup35p-based prion selection experiment will be feasible or not critically depends on the expression level of the PrD-Sup35C fusion protein and its functional activity in translation termination. Too low or too high levels of active Sup35p result in permanently elevated levels of stop codon read-through, with corresponding [*prion*-] strains that are able to grow on adenine-deficient medium and a colony color that is shifted to white (Figure 2B). It is therefore important to find the right window of expression to generate strains with adequate translation termination fidelity. In our lab we obtained the best expression results with the *ADH1* promoter.

In general, a sufficient level of translation termination activity is indicated by a light to dark red colony color. Strains with pink or white colony colors usually have too high levels of translation termination activity and can thus not be used in prion selection assays that test for the ability to grow on adenine-deficient synthetic media (Figure 2B). In rarer cases it is possible that a high level of read-through is not caused by insufficient expression levels, but by constitutive aggregation of the PrD. To rule out that the PrD-Sup35C chimera is already present in an aggregated state, an SDD-AGE followed by immunoblotting with an

anti-Sup35p antibody should be performed. It is also important to point out that the size of the PrD that is fused to Sup35p is a key factor that determines functionality of the chimera. Based on our experience, PrDs between 60 and 250 amino acids are well tolerated. PrDs above this threshold, however, tend to inhibit the translation termination activity of Sup35p. In some cases it can be important to include a solubilizing domain such as the M domain between the PrD and the C domain, as it could slow down the aggregation kinetics, a property that is particularly desirable if a protein is very aggregation-prone.

To generate a PrD-Sup35C-expressing strain, we introduce the corresponding expression plasmid into the YRS100 strain and select the transformants on appropriate selective plates. The transformants are isolated, grown in liquid medium for a few hours and then plated on 5-FOA plates to counterselect the covering *SUP35* plasmid. Colonies growing on 5-FOA plates are streaked on YPglycerol to select for cells with functional mitochondria and eliminate petite mutants that change the colony color to white. Subsequently, the cells are transferred onto YPD plates to assess the colony color phenotype. In rare cases strain isolates expressing the same construct can vary in colony color. We therefore recommend isolating a number of different colonies and using the isolates with the predominant colony colors for subsequent experiments.

At this stage, some strains might already show switching between a red and a white colony color on YPD plates, indicating that the PrD under investigation has prion properties. The strains can then be plated on SD medium lacking adenine to more thoroughly select for the prion state. However, switching

rates of prions can be as low as 10^{-6} to 10^{-7}. Therefore, in cases where spontaneous switching is not observed, conformational conversion to the prion state should be induced. Amyloid nucleates in a concentration-dependent manner. Thus transient overexpression of the PrD can be used to increase the switching frequency of a prion. To do this, we usually introduce an additional plasmid for expression of a PrD-EYFP fusion under the control of a galactose-inducible promoter. Induction of the prion state is achieved by growing the resulting transformants in galactose-containing medium for 24 hours. The cells are then plated on YPD and SD-ade plates at a density of 200 and 50,000 per plate, respectively. The same strains grown in raffinose serve as a control. A greater number of Ade^+ colonies under inducing conditions suggests that expression of cPrD-EYFP induced a prion switch. In these cases the Ade^+ colonies should be streaked on YPD plates to determine if a colony color change from red to white or pink has occurred. Often several colony color variants can be observed in one particular *PrD-SUP35C* strain. This could be due to the presence of weak and strong prion variants, or “strains”, that have been reported previously for other prions (Tessier and Lindquist 2009). A single prion protein can generate multiple variants that differ in the strength of their prion phenotypes. The underlying basis for this phenomenon is the presence of initial structural differences in the amyloid-forming nucleus that are amplified and maintained through the faithful self-templating mechanism of amyloids.

After having isolated several putative prion strains that exhibit a change in colony color, it is important to determine whether these changes are based on a

conformational conversion of the PrD-Sup35C protein. Known yeast prions critically depend on the chaperone disaggregase Hsp104p for propagation (Ross *et al.* 2005; Shorter and Lindquist 2005). Thus, deletion of the *HSP104* gene or repeated streaking of the putative prion strains on YPD plates containing 5 mM of the Hsp104p inhibitor guanidinium hydrochloride (GdnHCl) are convenient ways of testing whether a color change is due to a prion switch. However, as some prion variants can propagate in the absence of Hsp104p, we suggest testing those that are resistant to Hsp104p inactivation for the presence of aggregated PrD-Sup35C by SDD-AGE and immunoblotting with a Sup35p-specific antibody. We found that many strains are able to switch to a prion-like state that is not based on a conformational change in the PrD-Sup35 protein, but involve other genetic or epigenetic changes of unknown origin. To rule out these false positive candidates, it is very important to rigorously test putative prion strains for the presence of conformationally altered PrDs.

2.2.1. Ure2p-based prion assay

Ure2p is a 354-amino acid protein consisting of an N-terminal PrD and a globular C-terminal region. The C-terminal region of Ure2p shows structural similarity to glutathione transferases and is necessary and sufficient for its regulatory function. Ure2p regulates nitrogen catabolism through its interaction with the transcriptional activator Gln3p. [*URE3*], the prion state of Ure2p, results in the constitutive activation of Gln3p, and the prion-containing cells acquire the ability to utilize poor nitrogen sources in the presence of a rich nitrogen source. One of

the genes activated by Gln3p is the *DAL5* gene. It codes for a permease that is able to transport ureidosuccinate (USA), an essential intermediate of uracil biosynthesis. This ability to take up USA has historically been used to monitor the presence of [*URE3*] (Wickner 1994).

The N-terminal region of Ure2p is required for its prion properties *in vivo* and deletion of the N-terminal region has no detectable effect on the stability or folding of the C-terminal functional part of the protein. Analogous to the Sup35p assay described in the previous section, the Ure2p PrD can be replaced with a candidate PrD and the resulting chimera can then be tested for its ability to create heritable phenotypes that mimic [*URE3*] (Nemecek *et al.* 2009). Assaying [*URE3*] by selection on USA-containing plates has several disadvantages and for this reason we use a strain that contains an *ADE2* reporter gene that is placed under the control of the *DAL5* promoter (Brachmann *et al.* 2006). In prion-free [*ure-o*] cells, the *ADE2* gene expression is repressed and no functional Ade2p protein is produced, resulting in a red colony color and an inability to grow on medium lacking adenine. Derepression of the *DAL5* promoter in [*URE3*] cells, however, results in a switch to a white colony color and the ability to grow on adenine-free medium (Figure 3A).

We have also developed a yeast strain in which the chromosomal copy of the *URE2* gene was deleted in the BY334 background (Brachmann *et al.* 2006). This strain was fully complemented by transformation of an expression plasmid for Ure2p (data not shown). In order to use this strain for the detection of novel prions, we generated Gateway® vectors for the formation of chimeras between a

candidate PrD and Ure2C (amino acids 66-354). The C terminus of Ure2p contains an HA tag that allows the detection of PrD-Ure2C chimeras with HA-specific antibodies. In addition, these vectors are available with three different promoters that can be used to express PrD-Ure2C chimeras at different levels (*TEF2* < *ADH1* < *GPD*). Using these vectors, we tested the PrDs of two well-characterized yeast prions, Sup35p and Rnq1p, for their ability to undergo prion switching when fused to the C-terminal domain of Ure2p. A chimera between the PrD domain of Sup35p and the C-terminal domain of Ure2p showed prion switching and formed weak and strong prion strains (Figure 3B and 3C). A fusion between the PrD of Rnq1p and Ure2C also behaved as a prion, with at least two different color variants (Figure 3D and 3E). Additionally, we found that even the full-length Rnq1p protein, when fused to Ure2C, resulted in a fully functional chimera that showed prion-dependent inactivation (data not shown). This finding suggests that the C domain of Ure2p is much more tolerant of larger PrDs than the C domain of Sup35p. We tested additional previously described PrDs for their ability to induce switching when fused to Ure2C and we found that many of these showed prion switching behavior (data not shown).

Despite its usefulness as a tool for the detection of prion switching behavior, there are disadvantages associated with the Ure2C-based selection system. The selection for the prion state of a PrD-Ure2C chimera on adenine-deficient media is usually very difficult due to high levels of background growth. Therefore, in those cases in which a prion state cannot be isolated by selection, we suggest identifying prion-containing strains based on colony color changes on

media containing adenine. We noticed that the PrD-Ure2C fusions readily enter an aggregated state, which is probably due to the fact that the C domain of Ure2p does not have a solubilizing effect as strong as the C domain of Sup35p. In many cases, the high switching rates of PrD-Ure2C fusions allowed us to readily isolate several prion-containing strains from a single plate. Again, it is important to establish that the putative prion phenotypes are based on a conformationally altered state of the PrD-Ure2C chimera. The methods that are most convenient for testing are repeated streaking on plates containing GdnHCl or SDD-AGE followed by immunoblotting with an HA-specific antibody.

2.2.3. Prion selection assays

All yeast prions described to date cause a loss-of-function when in the prion state, but it is also conceivable that prion switches induce a gain-of-function phenotype, as has been described for the CPEB protein that is involved in long-term memory formation (Si *et al.* 2003). To rigorously establish that a candidate protein operates as a prion in a physiologically relevant manner, robust prion selection assays have to be applied to isolate a prion-containing strain. The functional annotation of the yeast genome is a tremendously powerful resource for unraveling the biology of putative prions. A wealth of data from genome-wide deletion and overexpression screens as well as chemical and phenotypic profiling studies is now available to design functional prion assays (Zhu *et al.* 2003; Cooper *et al.* 2006; Sopko *et al.* 2006; Hillenmeyer *et al.* 2008).

The transcriptional activity of a putative prionogenic transcription factor, for example, can be monitored in a transcriptional reporter assay. We recently used such an approach to verify the prion properties of Mot3p (Alberti *et al.* 2009). Mot3p is a globally acting transcription factor that modulates a variety of processes, including mating, carbon metabolism, and stress response. It tightly represses anaerobic genes, including *ANB1* and *DAN1*, during aerobic growth. To analyze Mot3p transcriptional activity, we created Mot3p-controlled auxotrophies by replacing the *ANB1* or *DAN1* ORFs with *URA3*. The resulting strains could not grow without supplemental uracil due to the Mot3p-mediated repression of *URA3* expression. However, *URA3* expression and uracil-free growth could be restored upon reduction of Mot3p activity, as with *MOT3* deletion or inactivation of Mot3p by prion formation.

To select for the Mot3p prion state, we transiently overexpressed the Mot3p PrD, via a galactose-inducible expression plasmid, and plated the cells onto media lacking uracil. We isolated Ura+ strains whose phenotype persisted even after the inducing plasmid had been lost. These putative prion strains were then analyzed using a variety of prion tests, including curing by inactivation of Hsp104, testing for non-Mendelian inheritance in mating and sporulation experiments and probing for an aggregated form of Mot3p in prion-containing cells. Candidate-tailored prion selection assays analogous to the one developed for Mot3p allow the study of prion in their natural context, thus providing valuable insights into the biological functions of prion conformational switches.

2.2.4. Methods to analyze prion inheritance

Yeast prions are protein-based epigenetic elements that are inherited in a non-Mendelian manner. The unusual genetic properties of yeast prions can be used to determine if a phenotype is based on a prion (Ross *et al.* 2005; Shorter and Lindquist 2005). Diploid cells that result from a mating between [*prion-*] and [*PRION+*] cells are usually uniformly [*PRION+*]. The genetic dominance emerges from the self-perpetuating nature of prions: when a prion-containing cell fuses with a prion-free cell, the efficient prion replication mechanism rapidly consumes non-prion conformers until a new equilibrium is reached that is shifted in favor of the prion conformer. Diploid [*PRION+*] cells that undergo meiosis and sporulation normally generate [*PRION+*] progeny with a 4:0 inheritance pattern. However, stochastic deviations from the 4:0 pattern are possible, if the number of prion-replicating units or propagons is low, causing some progeny to receive no propagons and to correspondingly lose the prion state.

To establish whether a putative prion trait is inherited in a "protein only" cytoplasm-based manner through the cytoplasm, cytoduction can be performed. Cytoduction is an abortive mating in which the cytoplasms of a prion-free recipient cells and a prion-containing donor cell mix without fusion of the nuclei. Daughter cells with mixed cytoplasm but only one nucleus bud off from the zygote and can be selected (Conde and Fink 1976). As a result, donor cells only contribute cytoplasm, whereas recipient cells contribute cytoplasm and nucleus to the progeny. The recipient cells we use are karyogamy-deficient and carry a mitochondrial petite mutation termed rho^0. As a consequence, recipient cells can

not fuse their nuclei with those of the donor cell and are unable to grow on a non-fermentable carbon source, such as glycerol, unless they receive wild-type mitochondria from the donor cytoplasm. Following cytoduction, haploid progeny are selected that retained the nuclear markers of the recipient strain but can also grow in medium containing glycerol. If the aggregated state of the candidate PrD was successfully transmitted through the cytoplasm, the selected cytoductants should display the prion phenotype under investigation. For a more detailed description of cytoduction we direct the reader to two recent articles on this topic (Liebman *et al.* 2006; Wickner *et al.* 2006).

2.2.5. Transformation of prion particles

The principal tenet of the prion hypothesis is that prions replicate in a protein-only manner, without the direction of an underlying nucleic acid template. The most rigorous proof for a prion, then, is to show that nucleic acid-free preparations of aggregated protein are “infectious” in and of themselves. That is, they have the capacity to convert cells to a stable prion state when they are introduced into those cells. Protein transformations have irrefutably established the protein-only nature of prion propagation for a number of yeast prions, and have also been used to show that the prion strain phenomenon results from conformational variations in the underlying amyloid structure.

Protein transformations are generally done by fusing prion particles with recipient cell spheroplasts using polyethylene-glycol (see Figure 4). A selectable plasmid is typically cotransformed with the prion particles to allow for the

determination of total transformation efficiency, as prion protein preparations have variable infectivities. The transformed spheroplasts are allowed to recover in soft agar and then analyzed for the prion phenotype. The putative prion particles to be used for protein transformations can be obtained either from [*PRION*+] cells or from recombinant protein that has been allowed to aggregate *in vitro*.

When using crude extracts, care must be taken to eliminate all viable non-lysed cells remaining in the extract (e.g. by centrifugation or filtration), as they may appear as false positive transformants otherwise. For this reason, we recommend performing control transformations without recipient cells to verify that there are no contaminating cells in the extract. Extracts from [*PRION*+] yeast can be generated either by spheroplasting (e.g. Section 2.1.3) or by glass bead lysis (Brachmann *et al.* 2005; King *et al.* 2006). Cleared lysates resulting from either of these procedures may be adequate for transformations without further manipulations in some cases. However, there are also a number of techniques that can be used to enrich prion particles relative to other cellular components and thereby improve transformation efficiencies. We direct the reader to the corresponding references concerning these procedures, which include: partial purification of aggregated protein by sedimentation (Tanaka and Weissman 2006), sedimentation followed by affinity purification of the prion protein (King *et al.* 2006), and amplification of prion particles in cell extracts by seeding the conversion of exogenously added recombinant prion protein (Brachmann *et al.* 2005; King *et al.* 2006).

The most rigorous proof that a protein is a prion is to transform cells to the [*PRION*+] state using purely recombinant protein that has been converted to the putative prion form *in vitro*. This procedure avoids potential confounding factors that are present in yeast extracts, and also allows one to easily generate highly concentrated infectious preparations without the need for labor-intensive enrichment of prion particles from [*PRION*+] yeast cells.

We routinely purify yeast PrDs from *E. coli* and convert them to amyloid fibers in a near-physiological buffer (Alberti *et al.* 2009). To form infectious amyloids *in vitro,* denatured proteins are diluted from a GdnHCl stock to a final concentration of 10 μM in 1 ml assembly buffer (5 mM K2HPO4, pH 6.6; 150 mM NaCl; 5 mM EDTA; 2 mM TCEP) and rotated end-over-end for at least 24 hrs at room temperature. The formation of amyloid is most easily monitored by thioflavin T fluorescence (450 nm absorption, 482 nm excitation), added at 20 fold molar excess, to aliquots taken from the assembly reaction. Following amyloid conversion, the reaction is centrifuged at maximum speed (20,000 rcf) in a table top centrifuge for 30 min at room temperature, and the pellet of aggregated protein resuspended in 200 μl PBS. The protein is then sonicated with a tip sonicator at the lowest setting for 10 seconds. Sonication shears amyloid fibers into smaller pieces, thereby greatly enhancing their infectivity.

Proper negative controls are essential for interpreting protein transformations. Prions arise spontaneously at a low frequency and this frequency increases after cells are exposed to stress (Tyedmers *et al.* 2008). Consequently, the efficiency of transformation to [*PRION*+] must be normalized

against mock transformations, such as freshly diluted (soluble) prion protein, amyloid fibers of other prions, or [*prion*-] cell extract.

Recipient cells are prepared for protein transformations by a gentle enzymatic removal of the cell wall (spheroplasting), using a protocol adapted from Tanaka *et al*., 2006. Many prions have an increased rate of appearance in yeast cells harboring the [*PIN*+] prion. For this reason, we recommend using [*pin*-] yeast to reduce background from the spontaneous appearance of the prion state of interest. Yeast are grown to an OD of 0.5 in YPD, harvested by centrifugation, and washed twice in sterile distilled water. The cells are then washed with 1 ml SCE (1 M sorbitol, 10 mM EDTA, 10 mM DTT (added just before use), 100 mM sodium citrate pH 5.8) and then resuspended in 1 ml SCE. 60 µL lyticase solution (4.2 mg/ml lyticase (Sigma); 50 mM sodium citrate, pH 5.8) is added and the cells are incubated for 20 to 30 min at 30°C while shaking at 300 rpm. It is very important that this step not be allowed to proceed for too long, or cells will lose viability. We recommend standardizing this step using identical aliquots of lyticase solution prepared from a single lot, which are stored at -80°C. The progress of spheroplasting can be monitored during this incubation by placing 2 µl of cells in 20 µl of 1% SDS and observing them under a microscope. Spheroplasts should lyse and be invisible or appear as ghost cells.

The spheroplasts are harvested at 500 rcf for 3 min at room temperature, followed by washing twice with 1 ml of STC buffer (1 M sorbitol; 10 mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5). Finally, the spheroplasts are resuspended in 0.5 ml of STC buffer. Spheroplasts are sensitive to shear forces and consequently must be

handled gently during all manipulations. To resuspend spheroplasts, we use a 1 ml pipette tip that has ~ 1 cm of the tip removed.

We add 100 µl of spheroplasts to 4 µl of 10 mg/ml salmon sperm DNA, 25 µl of 0.1 mg/ml selectable plasmid (e.g. pRS316 for a URA3-marked plasmid) and 33 µl of the protein solution to be transformed. The final protein concentration of amyloid fibers should be ~ 10 µM, or if using yeast extract, 200-400 µg/ml total protein. The samples are tapped gently to mix and incubated for 30 minutes at room temperature. Next, proteins are fused to spheroplasts by adding 1.35 ml PEG-buffer (20 % (w/v) PEG 8000; 10 mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5) and incubating for 30 minutes at room temperature. Note that the optimal concentration and molecular weight of PEG used in this step may vary depending on the transformed protein (Patel and Liebman 2007). Spheroplasts are collected at 500 rcf for 3 min at room temperature, and resuspended in 0.5 ml of SOS buffer (1 M sorbitol; 7 mM $CaCl_2$; 0.25 % yeast extract; 0.5 % bacto-peptone), followed by incubation for 1 hour at 30°C with 300 rpm shaking. Meanwhile, 8 ml aliquots of spheroplast recovery media are prepared in 15 ml tubes and maintained in a 48°C water bath. The spheroplast recovery media needs to be selective for the plasmid (e.g. SD-ura) and for the prion state if desired (see below), and is supplemented with 1 M sorbitol and 2.5% agar. Each transformation reaction is diluted into one aliquot of media, mixed by gentle inversion, and overlayed immediately onto the appropriate selective plates that have been prewarmed to 37°C.

Plates are incubated at 30°C under high humidity until colonies develop (up to one week). Colonies can then be picked out of the soft agar and scored for [*PRION*+] phenotypes. If transformation efficiencies are low, it is especially important that putative [*PRION*+] transformants are verified by secondary assays like SDD-AGE, to distinguish them from genetic revertants or other background colonies.

We and others (Brachmann *et al.* 2005) have found that selecting directly for the [*PRION*+] state of some prions (e.g. [*MOT3*+] and [*URE3*]) during spheroplast recovery increases conversion to the [*PRION*+] state relative to delaying selection until after the cells have recovered. Newly induced prion states are often initially unstable and seem to be lost at a high frequency under non-selective conditions. Consequently, applying an immediate mild selective pressure during the spheroplast recovery step can improve the apparent transformation efficiency by preventing many prion-containing spheroplasts from losing the prion state during colony formation in the sorbitol-containing media. However, stringent selective conditions can also inhibit spheroplast recovery resulting in drastically reduced transformation efficiencies. For instance, [*URE3*] spheroplasts recover at a low frequency when plated directly to USA containing media (Brachmann *et al.* 2005), and we have observed that [*PSI*+] spheroplasts generally recover poorly in adenine-deficient media. For each prion and selection scheme, there is likely to be an optimum window of selection stringency that maximizes the number of [*PRION*+] transformants recovered.

3. Concluding remarks

Aggregation has been suggested to be a generic property of proteins (Chiti and Dobson 2006), but most proteins aggregate only under conditions that fall outside of the normal physiological range. Studies of proteins that aggregate under non-physiological experimental conditions have provided important insights into general aspects of protein aggregation. Yet proteins that aggregate under physiological conditions are much more interesting from a biological point of view. Recent studies show that misfolding and aggregation propensities are likely to be a dominant force in the evolution of protein sequences (Chen and Dokholyan 2008; Drummond and Wilke 2008). This hypothesis is further underscored by the presence of complex quality control mechanisms that govern the abundance and structure of protein aggregates. Studies that identify large numbers of aggregation-prone proteins under physiological conditions will be necessary to understand how aggregation propensities shape the sequence and structure of proteins and the composition of proteomes. These studies will also allow us to generate comprehensive inventories of proteins that are capable of forming functional or pathological aggregates. Such inventories will be an important asset, as their analysis will facilitate the identification of sequence determinants that drive aggregation behavior. A growing toolbox of scalable and adaptable protein aggregation assays now enables us to rapidly identify and characterize the repertoire of aggregation-prone proteins in yeast. Once more, yeast is found at the vanguard of new technological developments that could turn

out to have a tremendous impact on our understanding of fundamental aspects of organismal biology.

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Figure legends

Figure 1: Diverse biochemical assays used to detect protein aggregation in yeast cell lysates. (A) Fluorescence microscopy was used to identify cellular aggregation patterns of proteins that are expressed as fusions to GFP. GFP alone (left) is equally distributed throughout the cytosol and nucleus. Two aggregation-prone proteins show annular or punctate fluorescent foci, respectively, resulting from the tight packing of amyloid fibrils in the cytosol. (B) Lysates of yeast cells expressing a GFP-tagged prion protein were analyzed by SDD-AGE and Western blotting. The prion protein was detected by immunoblotting with an anti-GFP antibody. (C) [*prion*-] and [*PRION*+] cell lysates were subjected to a filter retardation assay. Aggregates retained on the membrane were detected by immunoblotting.

Figure 2: Using Sup35p to detect phenotype switching behavior. (A) Schematic representation of the Sup35p-based prion assay. The PrD of Sup35p is replaced with a candidate PrD and the resulting strains are tested for the presence of prion phenotypes, such as the switching between a red and a white colony color. (B) Relationship between the cellular concentration of Sup35p, the colony color and the ability to grow on synthetic medium lacking adenine.

Figure 3: Using Ure2 to detect phenotype switching. (A) Schematic representation of a prion detection assay based on Ure2p. See text for further

details. (B) A chimera between the PrD of Sup35p and the C-terminal region of Ure2p shows switching behavior in [*RNQ+*] cells. (C) SDD-AGE of different colony color isolates from a Sup35PrD-Ure2C strain. (D) A chimera between the Rnq1 PrD and Ure2C displays colony color switching in [*RNQ+*] cells. (E) SDD-AGE of different colony color isolated from the plates in (D).

Figure 1

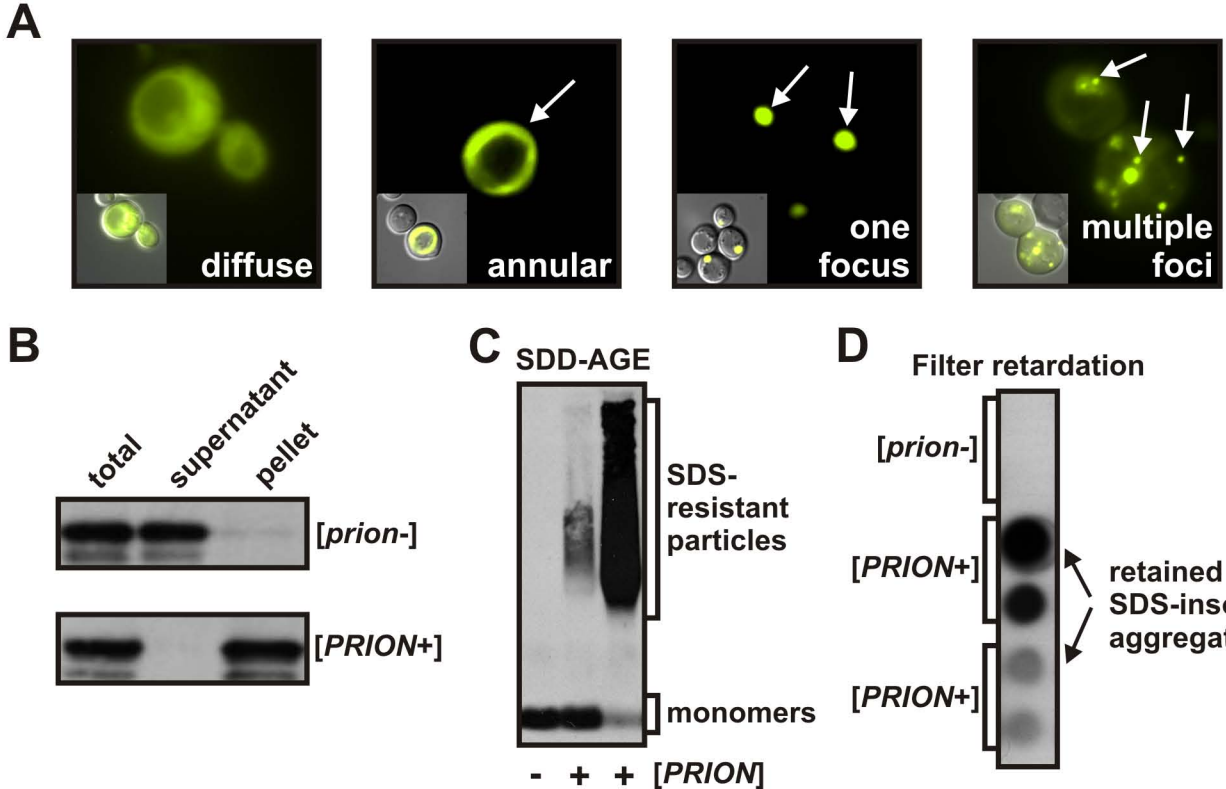
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Figure 2

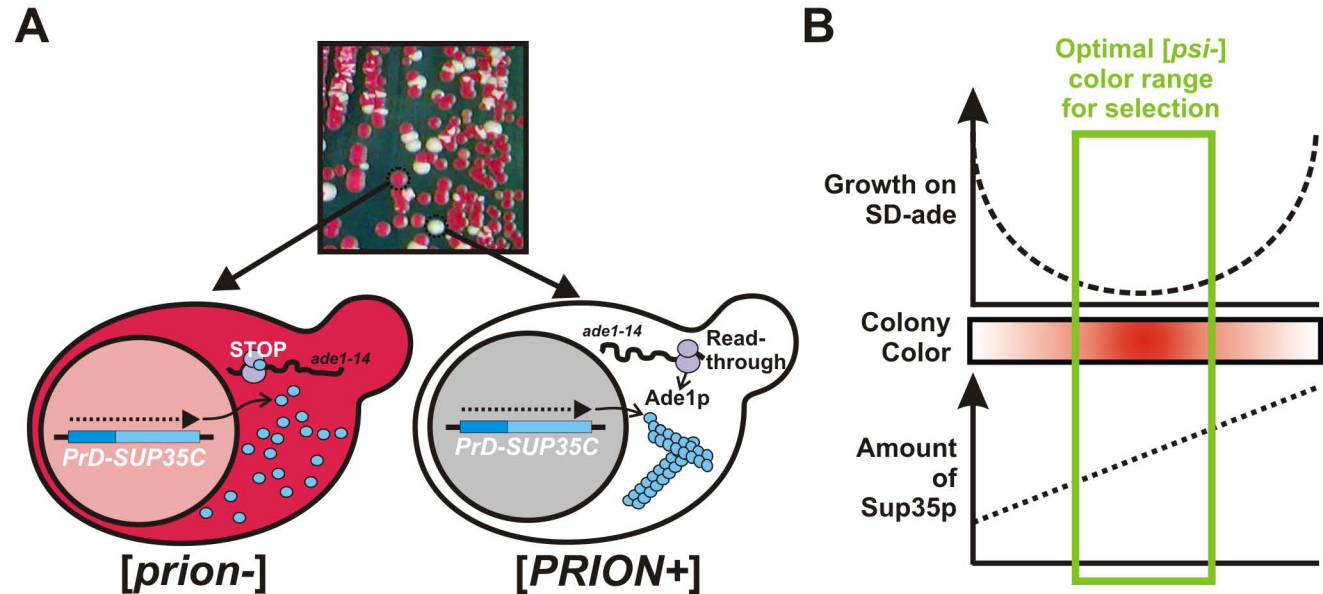
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Figure 3

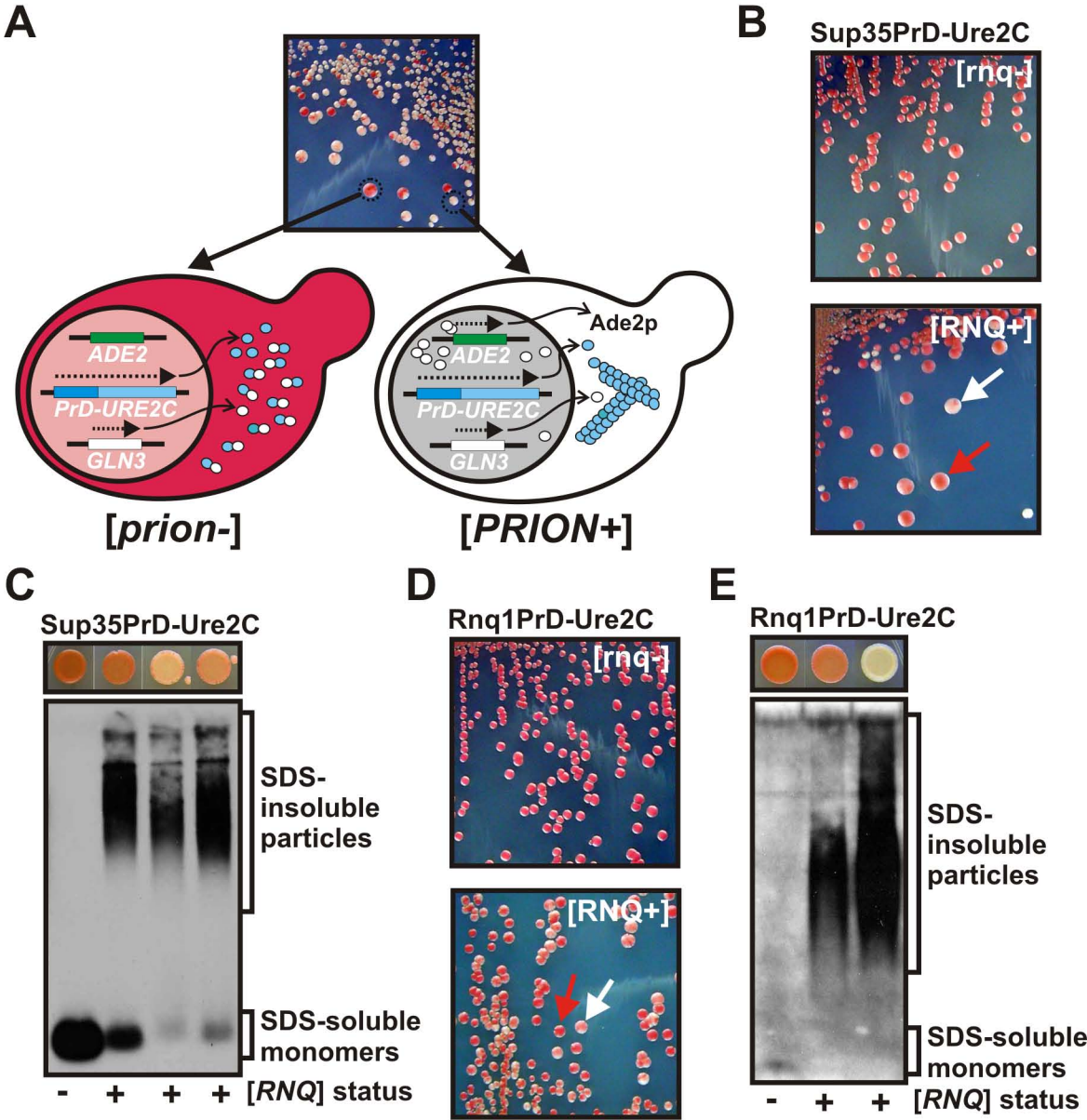
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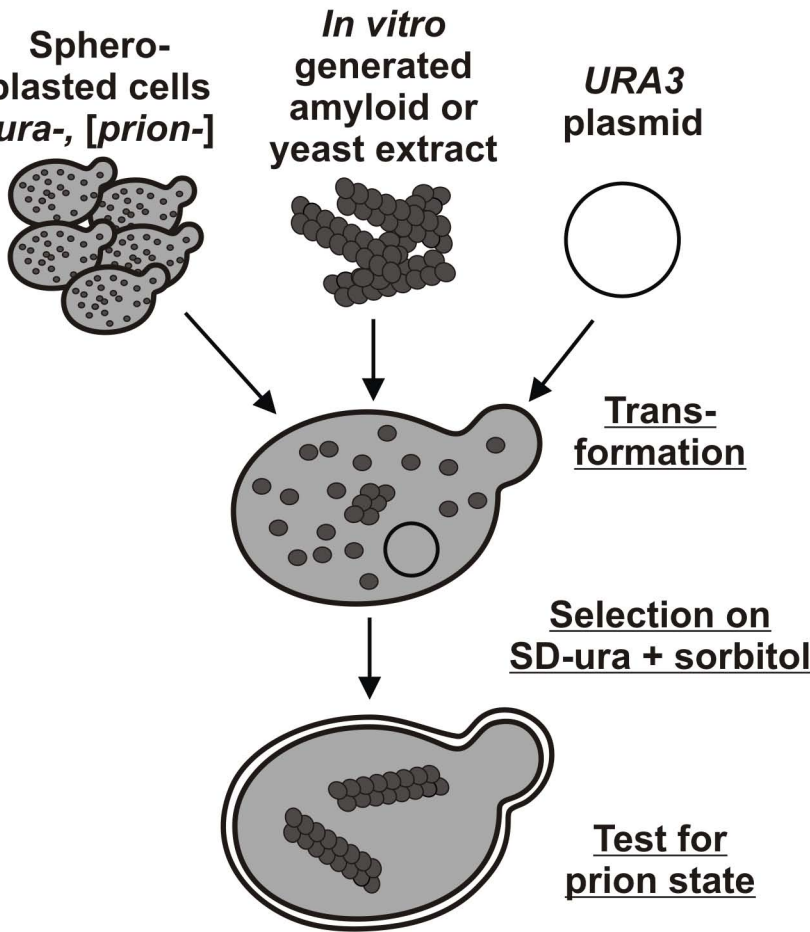
Figure 4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