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Study of amyloids using yeast

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Summary

Saccharomyces cerevisiae has been a useful model organism in such fields as the cell cycle, regulation of transcription, protein trafficking and cell biology, primarily because of its ease of genetic manipulation. This is no less so in the area of amyloid studies. The endogenous yeast amyloids described to date include prions, infectious proteins (Table 1), and some cell wall proteins (1). and amyloids of humans and a fungal prion have also been studied using the yeast system. Accordingly, the emphasis of this chapter will be on genetic, biochemical, cell biological and physical methods particularly useful in the study of yeast prions and other amyloids studied in yeast. We limit our description of these methods to those aspects which have been most useful in studying yeast prions, citing more detailed expositions in the literature. Volumes on yeast genetics methods (2–4), and on amyloids and prions (5, 6) are useful, and Masison has edited a volume of *Methods* on “Identification, analysis and characterization of fungal prions” which covers some of this territory (7). We also outline some useful physical methods, pointing the reader to more extensive and authoritative descriptions.

1. How to identify a new prion

Several approaches have been used to find new prions. The first two yeast prions found were non-chromosomal genetic elements, [URE3] (8, 9) and [PSI+] (10), which were found to have three properties that could not be explained as a nucleic acid replicon, but which were expected of a prion (11).

1.1 Genetic criteria for a yeast prion

Reversible curability—Various nucleic acids may be cured by certain treatments: mitochondrial DNA is eliminated by growth on ethidium bromide (12) and the L-A and M dsRNA viruses are cured by growth at 42C (13, 14), but once cured these elements will not arise again *de novo*. In contrast, the prions [URE3] and [PSI+] can be cured by growth in the presence of millimolar concentrations of guanidine HCl and high osmotic strength medium, respectively (11, 15), but having been cured, they can arise again *de novo* in the cured strain (11, 16).

Overproduction of the prion protein induces prion formation—The more prion protein available, the more likely that a conversion event will occur, and having occurred, it should take over the population of molecules because it is fundamentally a positive feedback event (11). Overproduction of Ure2p induces [URE3] formation (11) and overproduction of Sup35p induces [PSI+] (17). This is best done with transient overproduction, using a *GALI* (galactose-induced) or *CUP1* (copper-induced) promoter and showing that the appearance of the infectious genetic element (prion) is induced *de novo* at increased frequency.

Phenotypes of prion and gene encoding the prion protein—The phenotype of *ure2* mutants is similar to that due to carrying the [URE3] prion, and *ure2* is required for [URE3] prion propagation (9, 11). This is easily understood if [URE3] is a prion of Ure2p, but incomprehensible otherwise (11). Likewise, for the similarity of phenotype of *sup35* mutants and the [PSI⁺] prion and *SUP35* being required for [PSI⁺] prion propagation (11).

1.2 How to find prion candidates

Like [URE3] and [PSI⁺], the [Het-s] prion was long known as a non-chromosomal gene (18), but using the same genetic criteria as for the former prions, along with biochemical evidence of aggregation, [Het-s] was shown to be a prion of the HET-s protein (19).

[PIN⁺] was found as a non-chromosomal genetic element necessary for the induction of [PSI⁺] by overexpression of Sup35p (20). Later, evidence that Rnq1p could be heritably aggregated was presented (21), and finally, Rnq1p was identified as the prion protein underlying [PIN⁺] (22). However, it was shown that overproduction of many proteins could have a Pin-like effect, allowing induction of [PSI⁺] by overproduction of Sup35p, and all of these proteins had Q/N-rich domains (22, 23), similar to the prion domains of Ure2p (24) and Sup35p (25). Although the Pin effect did not require these proteins to be in a prion form, they became candidates for prions nonetheless, and Swi1p (26) and Cyc8p (27) were shown to be capable of prion formation, by the genetic criteria above.

[MOT3⁺] was identified as a prion of Mot3p, a transcription regulator, by screening a group of proteins having Q/N rich domains by fusing the domains to the non-prion part of *SUP35* (29).

There are now quite a few yeast amyloid-based prions to use as a guide to which other proteins might also be prions. Ross et al. showed that, at least for Ure2p and Sup35p, the amino acid composition is more important than the sequence of the prion domain in determining prion-forming ability (30, 31). Now an algorithm that correlates amino acid composition with prion forming ability promises to detect further yeast prions (32).

1.2 Manifestations of a prion domain: prion-inducing, prion propagation, interference

There are a variety of properties of prions that could be used to screen for new prions, but which do not constitute evidence for a prion. Aggregation is certainly a prion property, but any overproduced protein may aggregate and not be a prion. All of the amyloid-based prions form amyloid in vitro, but it has been suggested that any protein can be induced to form amyloid under some condition (33). Of course, not all aggregation is amyloid: proteins may aggregate as a consequence of oxidation or denaturation without forming the ordered filamentous structure that is amyloid.

The prion domain of a prion protein can propagate the prion in the absence of the remainder of the protein (25, 34). In addition, prion domains, when overproduced, are particularly good inducers of formation of the corresponding prion (24, 35). However, as exemplified by the Pin phenomenon described by Derkatch and Liebman (20, 22), not all proteins whose overproduction induces a prion is a prion domain. Overproduction of parts of a prion protein may also specifically interfere with the propagation of the corresponding prion (e.g., (36)).

2 Genetic methods for studying yeast prions

2.1 Materials

Replicapating blocks, velveteens, and dissecting needles (Cora Styles Needles 'N Blocks, CS@CoraStyles.com), 1/8"×6" wooden applicator sticks (sterilize in large glass tubes for streaking for single colonies),

Sources of strains, k/o's, plasmids, libraries: American Type Culture Collection (atcc.org); Open Biosystems (openbiosystems.com). Most yeast workers make their strains and plasmids freely available.

General yeast genetic methods: (37);

Guide to yeast genetics and molecular cell biology C. Guthrie & G. R. Fink volumes of Methods in Enzymol. Vol. 194 & 350.

Masison's vol. on yeast prion methods (7)

Chemicals: G418 (Cellgro), hygromycin B (Calbiochem), zymolyase (Seikagaku).

2.2 Prion phenotypes

Many yeast prions produce a phenotype that reflects deficiency of the normal active form of the prion protein. Others have a phenotype reflecting an activity of the prion amyloid.

2.2.1[URE3] phenotypes—Ure2p is a negative regulator of transcription of genes encoding enzymes and transporters for the utilization of poor nitrogen sources (38, 39). When Ure2p becomes an amyloid prion, it loses activity and the expression of these nitrogen catabolism genes is stuck in the 'on' position (8, 11). The gene most strongly regulated by Ure2p is *DAL5*, encoding allantoin/ureidosuccinate permease (40, 41). Since the product of Ure2p (aspartate transcarbamylase) is ureidosuccinate, the [URE3] prion, by inactivating Ure2p, derepresses *DAL5* transcription and makes *ura2* cells able to grow on ureidosuccinate (USA) in place of uracil in spite of the presence of a good nitrogen source, such as ammonia.

Materials

1. Strains: 3687 (*MATa kar1 leu2 ura2 his- [URE3]*) (11)
1065 (*ura2/ura2 [ure-o]* diploid)
2. Media: SD (Synthetic Dextrose): 6.7 g/l Yeast Nitrogen Base without amino acids (Difco), 20 g/l dextrose, 20 g/l agar.
3. L-ureidosuccinate (L-carbamoylaspartic acid, USA) (Fluka)

Procedures

2.2.1.1 Ureidosuccinate uptake test

1. In place of uracil, spread 1 ml of 1mg/ml ureidosuccinate on a slightly dry SD plate along with the other supplements needed by the strain(s) being tested (see Note 1).
2. Place small streaks of the strains to be tested (including control [URE3] and [ure-o] strains) on the plate or replica plate colonies. Leave at least 3 mm between streaks to prevent cross-feeding.
3. After 2 days at 30C, check for growth (see Note 2).

2.2.1.2 *DAL5:ADE2 fusion gene test:* In place of the USA uptake test, a *DAL5:ADE2* fusion allows using adenine prototrophy in spite of the presence of a good nitrogen source (ammonia) as a measure of Ure2p activity. The red-white color assay (see the [PSI⁺] assay below) may be used to assess *DAL5* transcription, and hence the presence of [URE3]. In this construct, 500 bp upstream of the *ADE2* open reading frame is replaced by 568 bp of the *DAL5* promoter (42). This test has the advantage that there is no significant cross - feeding, and the red-white colony color assay avoids replica plating. The results of the *DAL5::ADE2* based assay are not necessarily exactly the same as the USA uptake test, perhaps as a result of different turnover numbers of the Ade2p and Dal5p, but there is a general consistency of results.

2.2.1.3 *Uracil secretion test:* The presence of [URE3] can be checked also in prototrophic strains. A lawn of $\sim 10^6$ diploid *ura2/ura2* cells are seeded on an SD plate having 100 $\mu\text{g/ml}$ of ureidosuccinate, and small streaks of the strains to be tested are made. A [URE3] strain will take up an excess of USA, convert it to uracil, and secrete it from the cells, producing a halo of growth of the lawn around the patch of the [URE3] strain. The use of 30 $\mu\text{g/ml}$ ureidosuccinate in the USA uptake assay reduces the amount of cross feeding, but does not entirely eliminate it.

1. In place of uracil, spread 1 ml of 3 mg/ml ureidosuccinate on a slightly dry SD plate along with a lawn of $\sim 10^6$ cells of a *MATa/MATa ura2/ura2* diploid strain.
2. When the plate is dry, make small streaks of the strains to be tested along with [URE3] and [ure-o] controls.
3. After ~ 2 days at 30C, examine plates looking for a halo of growth of the lawn around the strain to be tested.

Note 1. The USA test is sensitive to the presence of various nitrogen sources in the medium. Leucine is particularly able to repress USA uptake even in [URE3] strains. Only required amino acids should be added to the plates with USA. The test cannot be done on -Ura dropout plates. Note 2. When selecting USA⁺ clones as a reflection of [URE3] generation, the colony count is generally not linear with cells plated, but is generally sigmoidal, perhaps due to cross-feeding.

2.2.2 [PSI⁺] phenotype—The assay for [PSI⁺], developed by Cox (10), is a general nonsense-suppression assay. Sup35p encodes a subunit of the translation termination factor (Sup45p is the other subunit). In [PSI⁺] cells most of the Sup35p is tied up in amyloid filaments and cannot efficiently terminate translation. Frequent read-through of premature termination codons is assessed using the nonsense mutation *ade2-1* and the weak serine-inserting suppressor *SUQ5*, or just the easily suppressed *ade1-14* mutation. Mutants in *ade1* and *ade2*, when grown on adenine-limiting media, accumulate a precursor (phosphoribosyl - aminoimidazole) that gradually converts to a red pigment. The intensity of the red color is an indirect indicator of the fraction of soluble Sup35p. Cells lacking the prion ([psi⁻]) are bright red, while [PSI⁺] cells are a shade of pink or white. Note that the loss of respiratory capacity, due to mutation of the mitochondrial DNA or a nuclear *pet* gene, greatly reduces the accumulation of the red pigment. In addition, mutations earlier in the adenine pathway prevent the accumulation of the precursor, and so are white. The red pigment is toxic, so these mutations are selected over time. A high level of adenine in the medium represses the adenine biosynthetic pathway and makes colonies be white. Plates of 1/2 YPD (below) contain enough adenine to allow growth of [psi⁻] cells, but not enough to repress adenine biosynthesis. These plates are often used for visualizing the [PSI⁺] or [psi⁻] state of a strain. Yeast grow best at 30C, but the color develops better by leaving the plates at room temperature for a few days after colonies have grown.

Strains: 74-D694 (MATa ade1-14 ura3-52 leu2-2,112 his3-200 trp1-289 [psi-] [PIN⁺]) (43) 5V-H19 (MATa ura3-52 leu2 ade2-1 SUQ5 can1-100 [PSI⁺])

Media: 1/2YPD: 5 g/l Yeast Extract, 20 g/l Peptone, 20 g/l dextrose, 20 g/l agar.
Synthetic Complete minus adenine (SC-Ade) (37)

2.3 Cytoduction

Infectivity is a central defining feature of a prion. In yeast, as in other organisms, vertical transmission—from parents to offspring—is distinguished from horizontal transmission—from one individual to a neighbor. Horizontal transmission of yeast plasmids and viruses is only known via the cell mating process. No infectious element is known to leave one cell and enter another. The same is true for prions.

Laboratory yeast strains are generally haploid. Yeast has two mating types, **a** and **α**, controlled by a single locus on chromosome III. Mixing two haploid strains of opposite mating type on rich medium results in mating with cell fusion occurring within a few hours. Normally diploids are formed which will grow and remain diploid. Meiosis is induced by transfer to 1% potassium acetate medium containing a small amount of required nutrients (if any). Having a *kar1* mutation in one (or both) of the mating strains prevents the nuclear fusion (karyogamy) step that is part of the mating process and follows cell fusion (44). The *kar1* cells then mate, fusing their cytoplasms, but not their nuclei. At the next cell division, the nuclei separate into separate daughter cells, each of which gets a mixture of the cytoplasms of the two parents. This is fundamentally a symmetrical process, but one is usually interested in the transfer of cytoplasm from strain A to strain B. The transfer of cytoplasm is indicated by showing the transfer of some known cytoplasmic genetic element present in strain A but not in B, usually the mitochondrial genome (mitDNA or ρ), or occasionally the killer trait, determined by M₁ dsRNA. Strain B is cured of mitochondrial DNA by growth to single colonies on rich plates containing 30 μ g/ml ethidium bromide. The now ρ^0 strain B is then grown on rich dextrose medium to dilute out any remaining ethidium.

Cytoduction Procedure

1. About 50 μ l of strain A (a large dab from a plate) is mixed with about 25 μ l of strain B (a smaller dab) in ~100 μ g of water. The exact amount of cells is not critical, but having a roughly two-fold excess of donor (strain A) over recipient (strain B) is desirable to insure that all strain B cells mate. Also, having a high cell density insures cells find a mate quickly.
2. The suspension is placed on a slightly dry YPAD plate and allowed to dry, so that the cells are brought into contact, insuring rapid mating. The plate is placed at 30C for ~7 hours.
3. The mating mixture is then streaked for single colonies on media selecting against growth of the donor strain A. The colonies formed will include diploids (few because of the *kar1* mutation), unmated recipient strain B (few because of the modest excess of strain A in the mating mixture) and cytoductants (having the nuclear markers of the recipient strain B and the mitochondrial genome (as shown by ability to grow on a carbon source such as glycerol that requires respiration for its utilization). Alternatively, one can use recessive selectable markers, such as *can1* (canavanine-resistance) or *cyh2* (cycloheximide-resistance), in the recipient to select against diploids and donors.
4. Replica plate colonies to YPG (only diploids and cytoductants will grow), media selective for diploids, and a plate that scores for the phenotype of the prion.

Cytoductants are those colonies that grow on YPG but not on the plate selective for diploids.

Sample cytoduction—*MATa leu2 ura2* [URE3] ρ^+ $\rightarrow$ *MATa his3 ura2* [ure-o] ρ^0 .

Streak cytoduction mix on synthetic complete - Leu plates. When colonies are grown, replica plate to YPG, SD+uracil, and SD+USA (30 μ g/ml). Clones growing on YPG but not SD+uracil are cytoductants, and their acquisition of [URE3] is indicated by their growth on SD+USA. *Note 1.* A cytoduction is denoted, “A $\rightarrow$ B” where A is the donor strain and B is the recipient. A standard mating with nuclear fusion is denoted “A $\times$ B”.

2.4 Curing prions with guanidine

Guanidine hydrochloride, at concentrations of 1 to 5 mM is a surprisingly specific inhibitor of Hsp104 (45–47), and growth to single colonies on rich medium in the presence of ~3 mM guanidine is routinely used to cure yeast prions. If an *ADE2* reporter is being used, then growth on 1/2 YPD with guanidine allows direct detection of cured colonies. [PIN+] is slightly resistant to curing by guanidine (49). The kinetics of guanidine curing have been used to measure prion seed number (50).

3. Fluorescent proteins and yeast expression vectors

3.1 Introduction

The *Saccharomyces cerevisiae* prion proteins capable of forming amyloid *in vitro* also form aggregates *in vivo*, which are readily detected by fluorescence microscopy. Immunofluorescence has been used in yeast (51) but its application has been mostly limited to the study of yeast prion proteins in mammalian cells (52–55). In yeast, prion forming proteins have been tagged with variants of green fluorescent protein (derived from *Aequorea Victoria* jellyfish) although monomeric red fluorescent protein (derived from *Discosoma coral*; (56)) has also been used. Green fluorescent protein with enhanced brightness and containing codons optimized for expression in yeast has been created (57). Using this yeast optimized green fluorescent protein, variants emitting in the cyan and yellow spectral regions have been engineered (58). Plasmids have been created that allow the fusion of a fluorescent protein tag to open reading frames in *Saccharomyces cerevisiae* (Yeast Resource Center <http://depts.washington.edu/yeastrc/pages/plasmids.html>; (58)). However, most experiments have utilized yeast expression vectors containing prion forming domains fused to fluorescent proteins. Sup35p prion formation has also been studied by embedding green fluorescence protein between the N and M domains (59, 60). A collection of yeast vectors can be found at Stanford Genomic Resources (http://genome-www.stanford.edu/vectordb/vector_pages/Yeast.html). Vectors used to study aggregates utilize a centromeric origin of replication ensuring the presence of 1–2 plasmids per cell. The use of a high copy 2 micron origin of replication results in artificial differences in expression levels between cells. The expression of the fusion proteins can be directed by the native promoter of the prion forming protein or by inducible promoters like *GAL1* and *CUP1*.

In order to measure the presence of [PSI+] directly, a glutathione synthetase ORF containing a UGA stop codon was placed directly upstream of red fluorescent protein. Only in the presence of [PSI+] will the GST-DsRed fusion protein be formed (60). A nuclear localization signal engineered upstream of red fluorescent protein but separated from it by a stop codon allows more sensitive detection as the fluorescence signal concentrates in the nucleus (61).

3.2 Microscopy and sample preparation

Yeast cells expressing fluorescent proteins can be observed using a standard fluorescent microscope with the appropriate filter sets using 60x or 100x objective lenses. However, a confocal microscope is often used to reduce light scattering from out of focus aggregates. An alternative is to use a microscope equipped with an ApoTome. In order to minimize movement of the yeast cells it is important to place them at relatively high concentration (clearly turbid) in a minimal volume (2 microliters) on a microscope slide. Slightly tapping the cover slip with a pipette tip after it has been applied but before the slide is placed on the microscope also reduces movement of the cells. To further reduce movement microscope slides can be coated with poly l-lysine (Sigma P0425) or the lectin concanavalin A, which both bind the yeast cell wall. Cover slips cleaned by soaking in 1 M NaOH and rinsed with distilled water can be coated with a 2 mg/ml solution of concanavalin A (Sigma C-5275) for 10 minutes. The slips are air dried, rinsed with distilled water and left to dry again. To firmly immobilize yeast cells an agarose pad can be applied to the microscope slide. If needed, nutrients can be added to the agarose as yeast nitrogen based media has no autofluorescence. The pads are prepared by placing two strips of Scotch Tape on either end of a microscope slide. 30 microliter 1% SeaPlaque GTG Agarose is pipetted on a warm taped slide and a second slide is placed immediately on top. The slides are place on a metal block that has been cooled in ice. After a minute gentle pressure is applied to separate the two slides with a sliding motion. The agarose pad will stick to the warmer top slide. For long-term observations yeast cells can be grown in Lab-Tek™ Chamber Slides (Thermo Fisher Scientific) which allow for direct microscopic observation. To prevent the cells from moving in the chamber during observation the chambers can be coated with concanavalin A as described earlier. Unbound cells are washed away with media prior to microscopic observation. In order to study prion aggregates by fluorescence microscopy yeast cells do not need to be fixed.

3.3 Microscopic observations

In the absence of prions, fusion of prion forming domains to fluorescent proteins results in a diffuse signal present throughout most of the yeast cell. However, in prion containing cells the fluorescence signal is punctate. As has been shown for [PSI] and [URE3] there can be one or multiple foci in a cell (36, 62). However, care has to be taken as expression of some fusions cures cells of the prion. In addition, overexpression of some fusions results in aggregate formation without the presence of the prion (36, 63). Expression of the NM domain of Sup35 fused to green fluorescent protein in [psi-] cells gives a diffuse fluorescence signal. However, continued expression of this fusion protein results in the formation of ring and line shaped aggregates (64). Daughter cells that bud from these ring and line containing mother cells will contain punctate fluorescence signals (65). It should be noted that not all the cells in a population contain aggregates visible by fluorescence microscopy although they all contain the prion. Moreover, foci observed after transient expression of Sup35-GFP disappear after the induction is stopped without the fusion protein being degraded (61). Nevertheless, the presence of fluorescent puncta in a substantial number of cells in the population is indicative of the presence of the prion. It has been shown by electron microscopy that a large filamentous network formed by Ure2p is present in cells that contain [URE3] (66). A similar filamentous network, formed by Sup35p, has been observed in [PSI+] containing cells (67). In this later case the puncta observed by fluorescence microscopy corresponded to the filamentous network observed by electron microscopy.

When two prions are present within a cell the individual prion forming proteins have been labeled with cyan and yellow emitting variants of green fluorescent protein. The aggregates formed by the two prion proteins can be found in different parts of the cell or can colocalize

(68, 69). Likewise, the association of prions with other proteins has been studied using green fluorescent protein and red fluorescent protein (70, 71).

In order to measure prion aggregate size in live yeast cells fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) have been used. The former measures the recovery rate of the fluorescence signal after a limited region of the cell has been photobleached. The latter measures fluorescence intensity fluctuations in a small cell volume. FRAP analysis showed that a yeast mutant that weakens the [PSI⁺] phenotype also results in the presence of larger prion aggregates (59). Using a modified FRAP technique in which a whole budding daughter cell is photobleached (fluorescence loss in photobleaching; FLIP) it was observed that a weaker [PSI⁺] variant forms larger aggregates than a strong [PSI⁺] variant (72). Increase in aggregate size has also been observed after inhibition of Hsp104 (73–75). FCS measurements indicated that in addition to large fluorescence foci Sup35NM-GFP molecules also form smaller diffusible aggregates in [PSI⁺] cells. These smaller diffusible aggregates are also present in the newly forming buds (61).

4. Protein expression, purification, amyloid formation and proteinase K treatment

A huge advantage in using yeast prions to study amyloid is that the biologically-active amyloid state can be produced *in vitro* from recombinant yeast proteins. Yeast prion proteins can be produced using *E. coli* expression systems, and once purified, these proteins will readily form infectious amyloid after incubation in common laboratory buffers. This enables the production of large quantities of prion amyloid and greatly facilitates the biophysical characterization of the biologically-relevant state. A combination of methods such as Proteinase K digestion coupled with LC-MS (Liquid Chromatography Mass Spectrometry) has aided in defining the amyloid core associated with many disease related amyloids. This will be discussed below.

4.1. Materials

4.1.1 His-tagged protein expression/Purification and amyloid formation

1. BL21(DE3)* chemically competent cells (e.g. Stratagene)
2. SDS-PAGE + reagents (commercially available e.g. Invitrogen)
3. Plasmid containing gene of interest
4. Luria broth (LB) media
5. Antibiotic (e.g. Ampicillin)
6. Isopropyl-beta-D-thiogalactopyranoside (IPTG) (MP Biomedicals)
7. Ni-NTA Agarose (Qiagen)
8. Polypropylene columns (5 ml) (Qiagen)
9. Lysis buffer (8M GuHCl, 10 mM imidazole, 0.1 M phosphate buffer, pH 7.4, 0.1 M NaCl, protease inhibitors (e.g. Complete™ (Roche))
10. Wash buffer (8M GuHCl, 20 mM imidazole, 0.1 M phosphate buffer, pH 7.4, 0.1 M NaCl)
11. Elution buffer (8M GuHCl, 250 mM imidazole, 0.1M phosphate buffer, pH 7.4, 0.1 M NaCl)

12. Slide-A-Lyzer Dialysis Cassette (3–20 K MW cut-off, 3–12 ml) (Thermo Scientific)
13. Fibrilization buffer (100 mM Phosphate buffer, pH 7.4, 0.1 M NaCl). The best buffer to induce fiber formation will vary with the protein.

***Note** The BL21(DE3) competent cells are an all-purpose strain for high-level protein expression and easy induction. Other competent cell lines may be used when genes being expressed contain rare codons or proteins are toxic to the cell. BL21(DE3) RIPL cells (Stratagene) overexpress Arg, Ile, Pro and Leu tRNAs which are normally poorly expressed in *E. coli* and aid in expression of some eukaryotic proteins.

4.1.2 Proteinase K digestion and LC-MS (mass-spec)

1. Proteinase K (Merck)
2. Fibrilization buffer (100 mM Phosphate buffer, pH 7.4, 0.1 M NaCl (variable))
3. Trichloroacetic acid (TCA)
4. Acetic acid (5%)/Water (95%)

4.2. Methods

4.2.1 His-tagged protein expression/Purification and amyloid formation

Protein expression

- 1 Refer to manufacturer's protocol for transformation guidelines.
- 2 Select for cells containing plasmid of interest by plating (25–100 μ l) on selection media (LB agar + antibiotic) using a sterile spreader and incubate overnight at 37°C.
- 3 Inoculate 10 ml aliquots of LB broth containing the antibiotic required to maintain the expression plasmid with single colonies from the transformation. Shake at 220–250 rpm at 37°C overnight.
- 4 The next morning, harvest culture by spinning at 4,000 rpm for 10 min and re-suspend pellet in fresh LB (10 ml). Add re-suspended culture to 1 L LB (1% v/v) and incubate at 37°C with agitation on an orbital shaker (220–250 rpm).

Note: If preparing ^{13}C - or ^{15}N -labeled protein for solid-state NMR experiments, a minimal medium or synthetic complete medium should be used with the appropriate labeled amino acid(s) or $^{15}\text{NH}_4\text{Cl}$ or U- ^{13}C -glucose (76).

- 5 Monitor cell growth (O.D_{600}) and add IPTG (1.0 mM) when cells reach an O.D_{600} of approx. 0.5. Continue incubating for a further 4h at 37°C with agitation.

Note: These values for IPTG concentration and induction time are starting values only and may require optimization depending on the gene expressed.

SDS-PAGE analysis

- 6 Check protein expression by SDS-PAGE. Pipette 20 μ l of the induced cultures (and uninduced control) into clean microcentrifuge tubes. Add 20 μ l of 2 $\times$ SDS gel sample buffer to each microcentrifuge tube.
- 7 Heat tubes to 95°C for 5 minutes. Load the associated non-induced (control) and induced sample in adjacent lanes for analysis by SDS-PAGE. Stain the protein

gel with CoomassieR Brilliant Blue stain. Check that the protein of interest has expressed before proceeding.

Protein purification

- 8 Harvest cells by centrifugation at 10,000 rpm for 20 min and wash cell pellet with 100 mM phosphate buffer, pH 7.4. Re-suspend pellet in lysis buffer (40 ml) and incubate at 4°C for 1 h with gentle agitation.
- 9 Spin cell suspension at 30,000 rpm for 45 min and retain lysate (contains his-tagged protein). Add 4 ml NiNTA to 40 ml lysate and gently agitate at 4°C for 2 h. Pour lysate over polypropylene column (5 ml) and wash NiNTA with 10 column volumes of wash buffer. Elute bound protein with 10 ml elution buffer.
- 10 Filter purified protein through YM-100 filter unit (molecular weight cutoff 100 kD, Milipore) and exchange into fibrilization buffer using a dialysis cassette. Incubate overnight at RT.

Amyloid formation

- 11 for amyloid formation incubate dialyzed protein at RT for several days with gentle agitation. A typical concentration for amyloid formation is 1.0 mg/ml.
- 12 Spin amyloid solution at 50,000 rpm for 45 min at 10°C and retain pellet.

4.2.2 Proteinase K digestion and LC-MS (Liquid Chromatography Mass Spectrometry)

1. Prepare serial dilutions of proteinase K in 100 mM phosphate buffer, pH 7.4. A typical concentration range is 1–100 µg/ml.
2. Add 0.5 mg amyloid to serial dilutions of proteinase K giving a final volume of 1.0 ml and incubate overnight at RT with gentle agitation.
3. Terminate reaction by adding 4% (v/v) TCA. Lyophilize sample by freeze-drying or speed-vac.
4. Re-suspend lyophilized material in 285 µl dH₂O and 15 µl acetic acid (5% v/v) for LC-MS analysis.
5. For LC-MS analysis, 100µl of sample is injected onto a C18 column running at 500 nl/min.
6. A long gradient of 95 minutes is used in order to obtain good peptide separation. Buffer A consists of 98% water, 2% acetonitrile, 0.1% acetic acid, and 0.01% TFA. Buffer B contains 80% acetonitrile, 20% water, 0.09% acetic acid and 0.01% TFA.
7. After analysis; the MS data reveals average masses corresponding to peptide fragments generated after Proteinase K digestion, which can be used along with the known protein sequence to identify the sequence of these fragments. Software from ExPASy proteomics tools can be used to analyze the data.

5. Protein Transformation

5.1. Introduction

The prion hypothesis implies that a single protein can misfold into multiple distinct infectious forms, which can be maintained during cell proliferation. The direct proof of this hypothesis can be made by protein transformation, e.g. by introducing in vitro prepared prion aggregates into living organisms that would result in stable prion propagation. This

was first achieved for the [Het-s] prion of *Podospora anserina* (77), and was developed recently for [PSI⁺] (78, 79) and applied successfully for other known yeast prions. Remarkably, introduction of Sup35p amyloid aggregates of different structures led to appearance of different prion variants in yeast cells, suggesting that the prion strain phenomenon results from conformational variations in the underlying amyloid structure. Here, we present a protein transformation protocol based on the original technique of Tanaka et al. (79) as modified by us (42).

5.2 Materials and equipment

To prepare yeast prion material:

- 1 YPD medium (1% yeast extract, 2% bactopectone, 2% dextrose); autoclave before use.
- 2 buffer A (25mM Tris HCl, pH 7.4, 150mM NaCl, 1mM dithiothreitol, 10 mM phenyl methyl sulphonyl fluoride (PMSF) and 1X complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN))
- 3 glass beads (0.5 mm diameter, Biospec products, #11079–105)
- 4 Vortex Genie 2 (Daigger) to break yeast cells
- 5 BCA reagent (Pierce, Rockford, IL)
- 6 Branson Sonifier 250 (Branson Scientific) or Sonic Dismembrator (Fisher Scientific) at lower settings (10–20% intensity)
- 7 20% Triton X-100 solution
- 8 30% (w/v) sucrose solution in buffer A
- 9 1 M lithium acetate with 1X complete protease inhibitor cocktail.
- 10 Optima L-90K ultracentrifuge equipped with SW55 rotor (Beckman Coulter).
- 11 buffer B (5 mM potassium phosphate buffer, 150 mM NaCl)

All buffers for transformation were prepared in advance with ultrapure water, sterile filtered (SFCA filter unit, Nalgene, 0.2 µl pore size) and stored at room temperature. Most reagents were obtained from Sigma.

- 12 ST buffer (1M sorbitol, 10mM Tris HCl, pH 7.5).
- 13 STC buffer (1M sorbitol, 10mM Tris HCl, 10mM CaCl₂, pH 7.5).
- 14 PTC buffer (20% (w/v) polyethylene glycol (PEG) 8000 (MP Biomedicals, 194839), 10mM Tris HCl, 10mM CaCl₂, pH 7.5)
- 15 SOS buffer (1M sorbitol, 7mM CaCl₂, 0.25% yeast extract, 0.5% peptone)

Other reagents:

- 16 lyticase (10U/ml in 20% glycerol; Sigma, L-5263)
- 17 salmon sperm DNA (2 mg/ml; Sigma, D-1626),
- 18 plasmid pRS425 (0.5 mg/ml) or another yeast plasmid for initial clone selection
- 19 Sorbitol agar medium (1x complete amino acid mix lacking leucine and adenine (see 20), 0.67% Yeast nitrogen base, 2% glucose, 2.5% agar, 1M Sorbitol). May contain either 5 mg/l adenine (full amount) or 0.1 mg/l adenine (limited amount for prion selection: for-LEU-0.02ADE plates)

- 20 10x complete amino acid mix, per liter, autoclave before use (200 mg methionine, 500 mg tyrosine, 500 mg isoleucine, 500 mg phenylalanine, 1000 mg glutamic acid, 2000 mg threonine, 1000 mg aspartic acid, 1500 mg valine, 4000 mg serine, 200 mg arginine, 200 mg histidine, 300 mg lysine, 300 mg tryptophan, 200 mg uracil). Alternatively, complete synthetic dropout mix (–LEU) can be used (US Biological).
- 21 –LEU-ADE plates (1x complete amino acid mix lacking leucine and adenine, 0.67% Yeast nitrogen base, 2% glucose, 2% agar)
- 22 ½ YPD medium (0.5% yeast extract, 2% bactopectone, 2% dextrose, 2% agar) (Quality Biologics)

5.3. METHODS

5.3.1. Preparation of prion material—Both crude cellular extracts of prion-containing cells and amyloid fibrils formed by yeast prion proteins *in vitro* can be used for prion protein transformation. Preparation of amyloid fibers is described in section 4 above. To obtain yeast extracts suitable for transformation, grow [PRION+] yeast cells in liquid YPD medium to optical density at 600 nm (OD₆₀₀) of 1.5. After harvesting by centrifugation (5000 g, 5 min) wash yeast cells with buffer A. Mix 1 volume of yeast cell pellet with 2 volumes of buffer A and 2 volumes of glass beads. Vortex tubes at top speed for a total of 4 min with cooling on ice for 30 s after each minute to break the cells. Remove cell debris by centrifugation at 5000 g for 10 min (see Note 1). Protein levels can be measured at this stage with BCA reagent according to manufacturer's protocol. Sonicate protein extracts on ice for 10–20 s at lower settings (10–20% intensity) before use for protein transformation.

Partial purification of prion aggregates can significantly improve transformation efficiency. To this end, treat protein extracts with 0.5% Triton X-100 for 5 min on ice, spin at 5000 g for 10 min, layer the supernatant on the top of a 0.5 ml 30% sucrose pad prepared in buffer A and do ultracentrifugation at 150,000 g for 45 min (SW55 rotor, Beckman). Resuspend the pellet with 1 M lithium acetate with 1X complete protease inhibitor cocktail, incubate on ice for 30 min with gentle agitation and spin again through a 0.5 ml 30% sucrose pad at 150,000 g for 45 min. Resuspend the pellet with buffer B, determine protein concentration with BCA reagent, sonicate on ice for 10–20 s at lower settings (10–20% intensity) immediately before use for protein transformation.

5.3.2. Protein transformation—Although the technique was reported to be general and has been successfully applied for most known yeast prions, we observed that the efficiency of protein transformation is greatly dependent on yeast strain background. Since the factors that determine the success are not clear, one may need to compare available strains experimentally. We recommend 74D-694 [*psi⁻*] (43) for Sup35 prion transformation and BY241 [*ure-o*] (42) for Ure2 prion transformation. In contrast to *de novo* prion induction, the transformation efficiency does not depend on the presence of the [*PIN⁺*] prion (79). In order to select “competent” yeast cells that have taken up prion aggregates, a yeast vector is usually used during the transformation procedure together with the protein preparation.

Grow yeast strains in 50 ml YPD at 30°C with constant shaking at 250 rpm to OD₆₀₀ of 0.6; spin cells at 1500 g for 5 min at room temperature, and wash twice with 25 ml ST buffer. Resuspend cells in 5ml ST buffer, add 10 µl lyticase (10U/ml), shake gently and incubate for 40 min at 30°C while shaking at 200 rpm to digest the yeast cell wall (see Note 2). Pellet spheroplasts at 250 g for 5 min at room temperature, wash twice with 10 ml ST buffer, and then resuspend in 0.5 ml STC buffer (see Note 3). Mix a 100µl portion of the spheroplast suspension with 5 µl of salmon sperm DNA (ssDNA), 1 µl of 0.5 mg/ml selectable plasmid

(pRS425), and 5–10 μ l solution containing prion particles, either in vitro-formed filaments or from cell extracts (see Note 4). The final protein concentration of amyloid fibers should be about 5 μ M; or 0.2 mg/ml total protein for yeast prion extracts. Incubate the mixture for 10 min at room temperature and then induce fusion by addition of 900 μ l PTC buffer. Mix gently and incubate for 20 min at room temperature. Collect spheroplasts by centrifugation at 500 g for 5 min at room temperature, resuspend in 200 μ l SOS buffer and incubated for 30 min at 30°C. Gently mix the transformation reactions with 10 ml liquid sorbitol agar medium at 50°C (see Note 5) and immediately pour onto Petri plates with 20 ml of solidified sorbitol agar medium, selective for the presence of the plasmid (e.g. LEU) and (optional) for the prion state (–LEU + 0.02 ADE) (see Note 6). Incubate plates at 30°C for 5–7 days. Colonies can be picked from agar using sterile toothpicks and streaked to single colonies on –LEU-ADE plates or ½ YPD plates to detect and verify [PRION+] appearance (see Note 7). To determine the efficiency of transformation the number of growing transformants on –LEU+0.02 ADE plates (that can grow further on –LEU-ADE plates) should be divided by the number of transformants on –LEU plates.

5.3.3. Notes

1. During centrifugation all viable (unbroken) cells should be removed to avoid false positives.
2. The progress of spheroplasting can be monitored during this incubation by placing 2 μ l of cells in 5 μ l of 0.5% SDS solution followed by microscopic analysis. Spheroplasts with SDS should appear invisible or as ghost cells.
3. Spheroplasts are sensitive to pressure and must be handled gently. To resuspend spheroplasts, use a 1 ml plastic pipette tip with the narrow tip opening cut off.
4. To increase efficiency of transformation, boil ssDNA at 100°C for 2 min and put immediately on ice just before addition. Also, it is very important to sonicate prion material before addition. Sonication breaks amyloid filaments into smaller pieces, enhancing their infectivity. However, over-sonication may decrease transformation efficiency.
5. Prepare sorbitol agar medium in advance and keep at 50°C (to prevent solidification) to be ready to mix with the spheroplast suspension. Mixing can be done in pre-heated (50°C) sterile 50 ml falcon tubes (Corning).
6. Sorbitol agar medium may contain either 5 mg/l adenine or 0.1 mg/l adenine to select directly for the [PRION+] state of prions, if the yeast strain used for transformation contains an adenine-based genetic system (*ade1-14* or *ade2-1 SUQ5* for [PSI+]; *PDAL5-ADE2* for [URE3]). Such selective pressure during recovery and growth of transformants often improves transformation efficiency likely due to instability of newly arisen prions. Complete absence of adenine may however inhibit recovery of transformants (Brachmann et. al., 2005). Selection stringency may be optimized depending on the particular prion and selection scheme.
7. As a negative control for the transformation, [prion–] cell extract may be used. In our experience, spontaneous prion generation during transformation procedure is hardly detectable. Results from the negative control should be counted when determining the efficiency of transformation.

6. SDS treatment and SDD-AGE

Amyloid-based yeast prions form high-molecular weight complexes *in vivo*, which consist of a prion protein and associated proteins (80, 81). The interaction between prion protein molecules is strong enough to resist the treatment with the strong anionic detergent sodium

dodecyl sulphate (SDS) at room temperature (82, 83). The insolubility in SDS distinguishes prion polymers from the majority of protein-protein complexes in a yeast lysate and allows their purification and analysis. Importantly, SDS treatment does not reduce, but instead modestly improves the infectivity of prion material extracted from yeast lysates (for infectivity assay see Protein transformation section above) (83), providing the evidence that SDS does not disrupt the intrinsic prion structure. Purification of SDS-resistant prion polymers from yeast cell lysates is done by ultracentrifugation and was described in detail in (84). Moreover, high molecular weight prion polymers can be analyzed by semidenaturing detergent agarose gel electrophoresis (SDD AGE) (82, 83). This technique is widely used to provide an alternative confirmation for the presence of a prion in yeast cells; in addition, different prion variants can be distinguished by different migration in agarose gels (82, 85). This method can be applied to characterize different amyloids (83, 86) and for large-scale screening for new prions (29). SDD-AGE is described in detail in Part II, 15 in this book.

7. Electron Microscopy

7.1. Introduction

A combination of methods in electron microscopy and solid-state NMR has made possible the unraveling of the structural elements that underlie infectious amyloid.

7.2. Materials

7.2.1 Transmission Electron Microscopy (TEM)

1. Negative Stain: 1–2% uranyl acetate in water (filtered). Should be protected from light to prevent precipitation.
2. Sample Support: Carbon-coated copper grids (commercially available, eg. Ted Pella Inc. or Electron Microscopy Sciences).

7.2.2 Electron Diffraction

1. Diffraction/atomic spacing control: Thallous chloride crystals (commercially available from Electron Microscopy Sciences).
2. Sample Support: Carbon-coated copper grids.

7.2.3 Mass-per-Length Measurements by Tilted-Beam TEM

1. Internal mass standard: Tobacco mosaic virus
2. Sample Support: Ultra-thin carbon-coated copper grids (commercially available)

7.3. METHODS

7.3.1 Transmission Electron Microscopy (TEM) using Negatively-Stained Amyloid

1. Prepare serial dilutions of amyloid samples in water or the same buffer that is used during fibrillization. A typical concentration range is 0.01–1.0 mg/ml.
2. Apply approximately 10 μ l of each amyloid sample to a carbon-coated copper mesh grid (Figure XA). (*carbon-coated grids are sometimes glow-discharged to increase their hydrophilicity, see Aeby (87))
3. After 2–3 minutes, use absorbent paper to blot off the sample from the grid and quickly add 10 μ l of water to the grid surface.
4. Immediately blot the water away with absorbent paper and quickly add 10 μ l of 1–2% uranyl acetate negative stain to the grid.

5. After 1–2 minutes, blot away the stain with absorbent paper and leave the grid to air dry for a few minutes. Once the grid is completely dry it is ready for examination by TEM, or can be stored for subsequent examination.
6. Negatively-stained amyloid samples are generally visualized with the electron microscope operating at 80kV.

7.3.2 Electron Diffraction

1. To achieve strong electron diffraction from amyloid, a greater amount of sample is usually used than would be for a negatively-stained sample viewed by TEM. As described above, first prepare samples with negative stain and check for concentrations of the amyloid that yield larger aggregates or a thin film of amyloid on the grid surface. Incubation time can also be increased to get more sample adherence to the grid surface.
2. Once the desired concentration and incubation time is determined, prepare the grid as would be done for negative staining, but without adding the stain.
3. At low magnification, find a region of the grid that clearly contains sample aggregates.
4. Put the EM in diffraction mode and use the smallest condenser aperture and remove the objective aperture.
5. Minimize the beam dose to avoid destroying the amyloid sample; use low intensity and heating settings, and reduce beam spot size.
6. Use the pointer to block the center of the beam before putting CCD camera in (Figure XC, D).
7. Adjust camera settings to quickly acquire images before electron diffraction diminishes (biological samples are damaged by the electron beam).
8. Scan grid for strong electron diffraction signal (Figure XC).
9. After recording an electron diffraction signal, the microscope can be set to regular mode to visualize diffracting species, although resolution is lower due to lack of negative stain.
10. Diffraction distances and atomic spacing will ultimately be calibrated using the electron diffraction of a known crystal, like thallos chloride, under identical conditions (Figure XD). Before checking the diffraction of the amyloid sample, it may be easier to adjust the initial settings using a grid with thallos chloride crystals, which are not destroyed by the constant electron beam.
11. Electron diffraction images from the thallos chloride control and the amyloid sample can be viewed by image-processing software such as ImageJ (freeware). The atomic spacing of the amyloid diffraction can be determined from the known spacing and diffraction radii of the thallos chloride crystals.

7.3.3 Mass-per-Length Measurements by Tilted-Beam TEM

1. Make serial dilutions each of Tobacco Mosaic Virus (TMV) and the amyloid sample in water or buffer. TMV concentrations around 0.1 mg/ml are in the typical working range.
2. Apply the samples to EM grids and stain with uranyl acetate, as described above.

3. By TEM, determine the proper concentrations and ratios of TMV and amyloid that will yield an even and equal distribution of both amyloid fibrils and virus particles in the microscope field of view. At a magnification of 56,000X, the desired field should have a few individual virus particles along with a few individual amyloid fibrils, without clumping or crowding (Figure XE).
4. After the ideal sample concentrations have been determined, prepare the EM grid with amyloid and TMV, but without negative stain. It is best to use a copper grid with an ultra-thin carbon coating (~5 nm) to avoid a high background of electron scatter in the dark field.
5. After application of the sample to the EM grid, wash the grid several times with 10 μ l aliquots of water in order to reduce electron scatter from residual salts.
6. Operate the EM at 80 kV in the dark field, or tilted beam, mode at 56,000 X magnification. Use a beam tilt angle of at least 1.2 to prevent the measuring of unscattered electrons.
7. Before viewing the sample, adjust the beam with the condenser stigmators to produce a uniform beam spread over the field of view.
8. Once the initial settings have been adjusted, scan the sample-containing EM grid. Adjust intensity settings and ensure the beam is centered with no visible intensity gradients in the field of view.
9. When pairs of TMV and amyloid fibrils are found in the field of view, quickly adjust focus and acquire an image as a 16-bit TIF file with threshold and tail set to zero. Adjusting the camera to multiple image acquisitions may be necessary to obtain quality pictures. However, it is important to not prolong the exposure of the samples in the electron beam, which can lead to mass loss of the sample.
10. Once sufficient numbers of images have been acquired, image-processing software such as ImageJ can be used to determine the relative intensities of the scattered electrons from TMV particles and amyloid fibrils (see boxes in Figure XE). Using the known mass-per-length of TMV (131 kDa/nm), the mass of the amyloid fibril can be determined. Because of the variability of any single measurement, it is typical to make scores of measurements and present the data as a histogram (**Borrowed from Chen et al, PNAS. Figure XF**).

7.3.4. Notes—4.Y The electron diffraction method described here is for quickly checking if the amyloid sample contains a repeated spacing, such as the 4.6 angstrom spacing seen for all amyloids. To determine cross- β structure, the amyloid fibrils must be laterally aligned to get the additional directional information. Also, the orientation of the aligned fibers with respect to the diffraction pattern must be determined.

8. Solid-state NMR

Because amyloid is neither soluble, nor highly mobile, X-ray crystallography and solution NMR are both of little use in studying its structure. Solid-state NMR (ssNMR) has the potential to determine detailed structures of amyloids, as has been done for A β peptide (88–90) and the HET-s prion protein (91–93). The application of ssNMR to amyloid structure determination is reviewed by Tycko (94) and by Baldus (95). An excellent introduction to NMR is Levitt's book (96). An excellent explanation of dipolar recoupling by R. Tycko, a pioneer in this method, is available at <http://fbml.scripts.mit.edu/Conferences/>. We will not attempt to present detailed NMR methods but simply emphasize the importance of this approach in achieving an understanding of amyloid structure.

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Figure 1. Strain 74D-694 [*psi*⁻] was transformed with amyloid fibrils formed *in vitro* from recombinant Sup35 prion protein mixed with pRS425 (carrying *LEU2*). Transformants were selected on -Leu plates containing 2% of the normal amount of adenine. [*PSI*⁺] transformants are large, Ade⁺ and white, while [*psi*⁻] transformants are small, Ade⁻ and red.

Figure 2.

A, The application of a 10 μ l amyloid suspension to a carbon-coated copper grid. B, MCA prion domain fibers stained with uranyl acetate. C, Typical amyloid electron diffraction of unaligned fibers (mouse Pmel rpt). D, electron diffraction of thallos chloride crystals. E, TMV and Sup35NM fibers viewed in the darkfield mode (tilted beam). F, histogram representation of Sup35NM mass-per-length measurements (from Chen et al(97)).

Table 1Prions of *Saccharomyces cerevisiae* and *Podospora anserina*

Prion	Protein	Normal function	Pathology	Reference
[URE3]	Ure2	nitrogen catabolite repression	slow growth; NCR stuck in “on” position	(11)
[PSI ⁺]	Sup35	translation termination	translation termination read-through	(11)
[PIN ⁺]	Rnq1	unknown	rare seeding of other prions	(22)
[β]	Prb1	vacuolar protease B	a normal non-amyloid prion: active form of Prb1p	(98)
[SWI ⁺]	Swi1	chromatin remodeling complex subunit	unable to use ethanol, galactose, sucrose; mating type switch - deficient	(26)
[OCT ⁺]	Cyc8	transcription repressor subunit	reduced fermentation	(27)
[MOT ⁺]	Mot3	transcription factor		(29)
[ISP ⁺]	Sfp1	transcription factor for ribosome components	translational antisuppression	(99)
[Het-s]	HET-s	prion functions in heterokaryon incompatibility and in a meiotic drive system.		(19)