GPI anchoring facilitates propagation and spread of misfolded Sup35 aggregates in mammalian cells

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Prion diseases differ from other amyloid-associated protein misfolding diseases (e.g. Alzheimer's) because they are naturally transmitted between individuals and involve spread of protein aggregation between tissues. Factors underlying these features of prion diseases are poorly understood. Of all protein misfolding disorders, only prion diseases involve the misfolding of a glycosylphosphatidylinositol (GPI)-anchored protein. To test whether GPI anchoring can modulate the propagation and spread of protein aggregates, a GPI-anchored version of the amyloidogenic yeast protein Sup35NM (Sup35^{GPI}) was expressed in neuronal cells. Treatment of cells with Sup35NM fibrils induced the GPI anchor-dependent formation of self-propagating, detergent-insoluble, protease-resistant, prion-like aggregates of Sup35^{GPI}. Live-cell imaging showed intercellular spread of Sup35^{GPI} aggregation to involve contact between aggregate-positive and aggregate-negative cells and transfer of Sup35^{GPI} from aggregate-positive cells. These data demonstrate GPI anchoring facilitates the propagation and spread of protein aggregation and thus may enhance the transmissibility and pathogenesis of prion diseases relative to other protein misfolding diseases.

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Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, include Creutzfeldt–Jakob disease and Gerstmann–Sträussler–Scheinker syndrome in humans, chronic wasting disease in cervids, scrapie in sheep, and bovine spongiform encephalopathy in cattle. These diseases are fatal infectious neurodegenerative diseases characterized by the conformational conversion of the normal monomeric cell-associated prion protein (PrP^C) into an abnormally folded form called

PrP^{Sc}. PrP^C is a protease-sensitive glycoprotein attached to cell surfaces and intracellular membranes by a glycosylphosphatidylinositol (GPI) anchor. PrP^{Sc}, like other proteins associated with protein misfolding diseases, exhibits greater protease resistance and exists in β -sheet-rich multimeric aggregates of varying size, including amyloid fibrils. One key difference between prion diseases and other protein misfolding diseases is that prion diseases are infectious, with PrP^{Sc}, perhaps in association with other cofactors, suspected as being the infectious agent/prion. It remains unclear why only prion diseases, among all protein misfolding diseases, are infectious under natural circumstances (Caughey *et al*, 2009).

Processes that are critical for a misfolded protein aggregate to behave as a naturally transmissible prion in mammals include: (i) aggregate-induced conversion of the non-aggregated precursor protein to the prion-associated conformation; (ii) a net positive growth rate versus clearance for the aggregates; (iii) fragmentation of aggregates to generate new seeding entities; and (iv) horizontal spread of aggregates between cells and tissues. The inherent capacity of different protein aggregates to undergo each of these processes likely varies between proteins. The sum of these factors could determine whether a given protein aggregate can be a prion and influence the phenotype associated with the prion state. It follows that modulation of these factors could make otherwise non-infectious protein aggregates behave as prions. Experimental evidence from yeast prion and polyglutamine protein systems supports these concepts (Borchsenius *et al*, 2001; Osherovich *et al*, 2004; Tanaka *et al*, 2006; Immel *et al*, 2007).

Yeast prion replication has many features in common with mammalian prion replication (Chien *et al*, 2004; Ross *et al*, 2005). One yeast prion, [PSI⁺], was identified as a non-Mendelian element of inheritance caused by self-propagating aggregates of the translation termination factor Sup35p (Cox, 1965; Wickner, 1994; Patino *et al*, 1996; Paushkin *et al*, 1996). The prion-forming activity of Sup35p is controlled by the N-terminal NM domains of the protein. Using recombinant amyloid fibrils of the NM domains of Sup35p (Sup35NM) investigators have shown that, like mammalian prions, Sup35NM prions exhibit strain (Tanaka *et al*, 2004) and species barrier (Tanaka *et al*, 2005) properties that are dependent on Sup35NM aggregate conformation.

A unique feature of PrP^C relative to yeast prion proteins and amyloidogenic proteins associated with human protein misfolding disorders is that PrP^C is tethered to membranes via a GPI anchor. This membrane anchor could affect the biochemical, biophysical, and neurotoxic properties of PrP molecules, and the efficiency of PrP^{Sc} formation and propagation (Caughey *et al*, 2009). This is consistent with data obtained from prion infections of transgenic mice expressing anchorless PrP^C (Chesebro *et al*, 2005) and other studies (Gabizon *et al*, 1987, 1988; McKinley *et al*, 1991; Baron *et al*, 2002, 2006; Fevrier *et al*, 2004; Silveira *et al*, 2005). Furthermore, there are cellular mechanisms (e.g. exosomes, membrane microparticles, membrane nanotubes) that

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mediate the exchange of GPI-anchored and other membrane-bound proteins between cells (Mack *et al*, 2000; Fevrier *et al*, 2004; Onfelt *et al*, 2004; Sherer *et al*, 2007; Sowinski *et al*, 2008). This includes a very recent report of intercellular spread of PrP^{Sc} via tunnelling nanotubes (Gousset *et al*, 2009). These exchange processes could provide a mechanism for the efficient spread of membrane-bound protein aggregates to new host cells and tissues, that is, transmitting the 'infection', at least as defined by the presence of self-propagating protein aggregates.

Cell-cell spread of GPI-anchored protein aggregation is particularly relevant in cases of peripheral mammalian prion infection, the most common natural route. Intercellular transmission of mammalian prions occurs during the neuroinvasion and spread of prions (and PrP^{Sc}) along defined neural pathways after peripheral prion infection (Mabbott and MacPherson, 2006). In infected animals at terminal stages of disease, PrP^{Sc} is distributed throughout the central nervous system (CNS), with lymphoid tissues involved to varying extents (Mabbott and MacPherson, 2006). This neuroinvasive capacity of PrP^{Sc} is a distinctive feature that is an important element of TSE disease transmission between hosts. Such horizontal spreading activity is crucial for a protein aggregate to act as a naturally transmissible prion in mammals, particularly for aggregates whose pathogenic effects require spread to, and within, the CNS. *In vitro* studies suggest that transmission of PrP^{Sc} and infection from dendritic cells to neurons through TNTs may contribute to neuroinvasion (Gousset and Zurzolo, 2009; Gousset *et al*, 2009). Altogether, this raises important questions about the mechanisms of neuroinvasion and spread of prions within mammals, and the factors controlling these features that appear to be specific to prions among mammalian protein misfolding diseases.

Consideration of these issues prompted the current study to investigate whether membrane anchoring could modulate the induction and intercellular transmission of protein aggregation when applied to another protein capable of forming inducible, misfolded aggregates. Although other modes of membrane anchoring (e.g. transmembrane) may confer this activity, in this initial study we focused on the effect of GPI anchoring because this is the form of membrane anchoring associated with the lone naturally transmissible protein misfolding disorder (i.e. prion diseases). To explore this question, we expressed GPI-anchored forms of the NM domains of Sup35p (Sup35^{GPI}) in N2a mouse neuroblastoma cells. Sup35NM offers several advantages for our investigation. The aggregation properties of Sup35NM are well-characterized and it can aggregate when fused to other proteins (Patino *et al*, 1996; Li and Lindquist, 2000). It has no mammalian orthologues and would not be expected to participate in physiological interactions between mammalian amyloidogenic proteins and other molecules. This allows us to specifically assess the effects of GPI anchoring and explore questions of broad interest such as whether GPI-anchored, membrane-bound aggregates are directly neurotoxic. In addition, its prion behaviour in yeast is dependent on the appropriately tuned expression of the cytoplasmic chaperone protein Hsp104 (Chernoff *et al*, 1995), which facilitates [PSI⁺] prion replication by fragmentation of large aggregates into smaller aggregation-inducing seeds (Chernoff *et al*, 1995; Cox *et al*, 2003; Kryndushkin *et al*, 2003; Shorter and Lindquist, 2006; Satpute-Krishnan *et al*, 2007). Hsp104 has

no known mammalian orthologue with ATP-dependent protein disaggregase activity or equivalent biological activity localized to the cell surface/extracellular environment (Doyle and Wickner, 2009). A very recent report described the yeast prion-like vertical propagation of Sup35NM aggregates in the cytoplasm of mammalian cells, suggesting that Hsp104-like activity may be present in this specific subcellular compartment (Krammer *et al*, 2009). Thus in the context of our experiments, Sup35NM would not be expected *a priori* to behave as a prion unless GPI anchoring could somehow compensate for the factors necessary to propagate Sup35p prion aggregates in the yeast cytoplasm. Using this new model we show that self-propagating, detergent-insoluble, protease-resistant aggregates of Sup35^{GPI} can be induced in N2a cells expressing Sup35^{GPI} by treatment with exogenous Sup35NM aggregates. Moreover, spread of aggregation to separate cells can occur through cell-to-cell contact and aggregate shearing. Collectively, our data demonstrate that GPI anchoring can modulate the properties of Sup35NM protein aggregates to allow their replication as prions when expressed, like PrP^C, on the surface of mammalian cells. These observations may help explain why among all protein misfolding diseases only prion diseases are recognized as naturally transmissible.

Results

Recombinant Sup35NM fibrils induce acute aggregation of Sup35-GFP^{GPI}

To test the role of GPI anchoring as one mode of membrane association that could modulate protein aggregation, N2a cells stably transfected with either GFP^{GPI} or Sup35-GFP^{GPI} (Figure 1A) were generated. Cells expressing either construct exhibited GFP fluorescence on the cell surface and in a perinuclear region. Immunolabelling of non-permeabilized Sup35-GFP^{GPI} cells using an anti-Sup35 N-domain antibody (anti-Sup35 N) suggested that full-length Sup35-GFP^{GPI} was expressed on the cell surface (Figure 1B, top row). Immunolabelling of permeabilized Sup35-GFP^{GPI} cells with anti-Sup35 N showed labelling of the intracellular population with a comparable efficiency to that observed using anti-GFP antibody (Figure 1B, third row). Together, these data verified that the cells expressed the GFP fusion proteins with a distribution typical of GPI-anchored proteins.

To induce aggregation of Sup35-GFP^{GPI}, we prepared fibrils of recombinant Sup35NM labelled with Alexa Fluor-568 (AF568) (Supplementary Figure S1A and B). We also prepared AF568-labelled recombinant fibrils of the prion domain (residues 1–80) of the yeast prion protein Ure2p (Ure2p⁸⁰) as a specificity control (Supplementary Figure S1C and D). Sup35-GFP^{GPI}-expressing cells were treated with either PBS or equimolar amounts of AF568-labelled Sup35NM or Ure2p⁸⁰ fibrils (Figure 2A). Two days after treatment, more AF568-positive cells were present in cultures treated with Sup35NM-AF568 than in the Ure2p⁸⁰ control culture (Figure 2B and C). Sup35NM-AF568 fibril binding to GFP^{GPI} control cells was negligible (Figure 2B and C). These data suggested that Sup35-GFP^{GPI} specifically bound the Sup35NM-AF568 fibrils, although we cannot ascertain the subcellular localization based on these images. Punctate accumulations of GFP appeared only in Sup35NM fibril-treated cells (arrow; Figure 2B), suggesting that Sup35NM fibril treatment induced

Figure 1 Plasmids and the expression of Sup35-GFP^{GPI}. (A) Plasmids used to generate stably transfected cell lines. (B) Immunofluorescence using anti-Sup35 N or anti-GFP antibody. Top two rows: Cell-surface labelling of non-permeabilized cells. Bottom three rows: Labelling of permeabilized cells. Merge image: GFP (green) and anti-Sup35 N or anti-GFP (magenta). Colocalized areas appear white. Bars, 10 μ m.

acute aggregation of Sup35-GFP^{GPI}. These differences did not result from any toxic effects from the fibril treatments (Figure 2D).

Biochemical characterization of self-propagating Sup35-GFP^{GPI} aggregates induced by Sup35NM fibrils

To confirm that Sup35NM fibrils induced Sup35-GFP^{GPI} aggregation, cells were analysed by detergent-insolubility and protease resistance assays after extended passage post-treatment to allow removal of the input Sup35NM fibrils by dilution. After six 10-fold serial passages, only cells treated with Sup35NM fibrils exhibited GFP aggregates (Figure 3A) and detergent (sarkosyl)-insoluble Sup35-GFP^{GPI} (Figure 3B). The level of induced Sup35-GFP^{GPI} aggregation was dependent on the fibril dose (Figure 3C).

To rigorously assess the stability of the Sup35-GFP^{GPI} aggregates, detergent-insolubility assays were performed in a filter-trap format using a strong denaturing detergent (SDS). Others have shown that Sup35NM aggregates and infectious polymers resist solubilization in SDS (Serio *et al*, 1999; Bagriantsev *et al*, 2008). SDS-insoluble Sup35-GFP^{GPI} was only detected in cells treated with Sup35NM fibrils (Figure 3D). Cells treated with Alzheimer's amyloid β -peptide (1–42) fibrils (A β 42) also did not produce Sup35-GFP^{GPI} aggregates (Figure 3E). Together, these data indicated that treatment of Sup35-GFP^{GPI}-expressing cells with Sup35NM fibrils specifically induced the production of aggregated Sup35-GFP^{GPI}.

Protease resistance was assessed by chymotrypsin digestion. The N domain forms the core of Sup35p aggregates and acquires resistance to chymotrypsin digestion after incorporation into Sup35NM fibrils (Serio *et al*, 2000). Because the M domain lacks chymotrypsin-cleavage sites, the development of chymotrypsin-resistant full-length Sup35NM species is characteristic of aggregated forms of Sup35NM. Only lysates from Sup35NM fibril-treated cells contained chymotrypsin-resistant, full-length Sup35-GFP^{GPI} (Figure 3F). The protease-resistant and detergent-insoluble material observed in the Sup35NM fibril-treated cells was not from residual Sup35NM fibrils because no AF568-labelled material was detectable by fluorescence microscopy (Figure 3A) and on western blots Sup35NM could be distinguished from Sup35-GFP^{GPI} based on apparent molecular mass.

In cell culture models of prion infection, acute induction of PrP^{Sc} formation does not necessarily lead to persistent PrP^{Sc} formation (i.e. infection) (Vorberg *et al*, 2004). To be considered stably infected, cultures must maintain PrP^{Sc} propagation for multiple passages (usually at least 5–6, which also removes the inoculum by dilution). With Sup35NM fibril-treated Sup35-GFP^{GPI} cells, maintenance of Sup35-GFP^{GPI} aggregates was observed for many passages (Figure 3B and F, and Supplementary Figure S3) and as high as 23 (unpublished data). Similar results were obtained using multiple Sup35-GFP^{GPI} cloned cell lines with different Sup35-GFP^{GPI} expression levels (unpublished data). Collectively, these data indicate that induced Sup35-GFP^{GPI} aggregates were self-propagating.

Sup35-GFP^{GPI} aggregates induced by intercellular contact

To assess whether GPI-anchored Sup35 aggregates could induce Sup35^{GPI} aggregation in separate cells, Sup35-GFP^{GPI} cells were co-cultured with cells stably expressing a GPI-anchored Sup35NM domain fusion with mCherry red-fluorescent protein (Sup35-mC^{GPI}) and analysed by live-cell confocal microscopy. The Sup35-mC^{GPI} cells persistently produce aggregates, which are often visualized as cell-surface sheet-like accumulations (arrowhead in Figure 4A and Supplementary Figure S4), are detected by filter-trap assay (Figure 4B), and are biochemically indistinguishable from induced Sup35-GFP^{GPI} aggregates. Although we cannot exclude the possibility that a small fraction of the aggregate population may be intracellular, the punctate cell-surface accumulations that we call aggregates invariably correlate with the detection of aggregated Sup35-mC^{GPI} in our biochemical assays. Because mCherry oligomerization can be detected under certain conditions with sensitive assays

Figure 2 Sup35NM fibrils bind specifically to Sup35-GFP^{GPI} cells. (A) Fluorescence SDS-PAGE of AF568-labelled fibrils. Laddering in lanes 2 and 3 corresponds to SDS-insoluble oligomers. Lower apparent molecular mass material (~13 kDa) in lane 3 and to a lesser extent in lane 2 is free dye, which does not detectably associate with cells (Supplementary Figure S2). (B) Wide-field fluorescence microscopy of cells 2 days after adding fibrils. Arrow (third row) indicates newly induced Sup35-GFP^{GPI} aggregate colocalized with Sup35NM-AF568 fibrils. Merge images: GFP (green), AF568 (magenta) and colocalization (white). Bars, 50 μ m. (C) Percentage of Sup35-GFP^{GPI} cells associated with AF568-labelled fibrils 2 days after treatment with PBS, Ure2p⁸⁰-AF568 fibrils, or Sup35NM-AF568 fibrils. For an additional specificity control, the rightmost bar indicates the percentage of cells expressing GFP^{GPI} associated with Sup35NM-AF568 fibrils. Data are mean $\pm$ standard deviation (s.d.) of $n = 5316$ (PBS), $n = 5107$ (Ure2p⁸⁰-AF568), $n = 4746$ (Sup35NM-AF568), and $n = 6388$ (GFP^{GPI} cells + Sup35NM-AF568) cells counted from six different fields of view for each treatment ($*P < 0.0001$; unpaired *t*-test). (D) Cell viability was determined 2 days after fibril treatments and is expressed as a percentage versus untreated controls (mean $\pm$ s.d., $n = 12$). Staurosporine (STS), positive control. All data are representative of at least two independent experiments performed in triplicate.

such as FRET (Piston and Rizzo, 2008; Snapp, 2009), it is possible that GPI-anchored mCherry has a natural tendency to interact with itself to form small oligomeric species undetectable in our assays that can facilitate spontaneous nucleation of associated Sup35NM moieties by bringing them into close proximity. These aggregates were not seen in cells stably expressing either GPI-anchored mCherry protein alone (mC^{GPI}) or secreted GPI-anchorless Sup35NM-mCherry (Sup35-mC), which were used as negative controls (Figure 4A, middle and right panels, respectively, and Figure 4B). After 3 days, GFP aggregates appeared specifically in co-cultures with aggregate-positive Sup35-mC^{GPI} cells (Figure 4A, left panels). Overall cell-surface labelling was

decreased for cells making Sup35-GFP^{GPI} aggregates, suggesting that a substantial portion of the cell-surface Sup35-GFP^{GPI} was sequestered in the aggregates (compare GFP distribution in Figure 4A). Interestingly, most of the GFP aggregates (we estimate >90%) colocalized with Sup35-mC^{GPI} material (Figure 4A, arrows), suggesting that Sup35-GFP^{GPI} aggregation was associated with intercellular transfer of Sup35-mC^{GPI} aggregates.

It appeared that more Sup35-mC^{GPI} was transferred to Sup35-GFP^{GPI} cells than either of the control co-cultures (unpublished data). Such transfer was apparent where cell-surface GFP aggregates colocalized with Sup35-mC^{GPI} (Figure 4A, arrows) and in areas containing intracellular

Figure 3 Sup35NM fibrils induce aggregation. (A) Wide-field fluorescence images of fibril-treated Sup35-GFP^{GPI} cells at passage-6 following fibril addition. Arrows: GFP punctae indicative of Sup35-GFP^{GPI} aggregates. Merge: GFP (green) and AF568 (magenta). Insets: Magnified view of the boxed areas. Bar, 200 μ m. (B) Western blots of detergent insolubility assays from fibril-treated cell lysates at passage-6 and 15. Ure2p⁸⁰ fibril-treated samples shown are from the same blot with irrelevant lanes removed. Well number indicates cells were treated in triplicate. (C) Dose-dependent induction of Sup35-GFP^{GPI} aggregation by Sup35NM fibrils. Western blot (anti-Sup35 N antibody) of insoluble and soluble fractions separated by sedimentation of 1% sarkosyl lysates of Sup35-GFP^{GPI} cells treated with serial dilutions of Sup35NM fibrils nine passages after adding fibrils. The relative amounts of fibrils are indicated with '1x' corresponding to the amount of fibrils used for fibril addition experiments as described under Materials and methods. Lanes shown are from the same blot with intensity range settings for the soluble lanes 10-fold higher (i.e. less sensitive) than the settings for the insoluble lanes. (D) Filter-trap assay immunoblot at passage-10 showing SDS-insoluble Sup35-GFP^{GPI} present only in Sup35NM-AF568 fibril-treated samples. The total protein amounts filtered per well for each row are indicated. 'Buffer' wells were loaded with 4% SDS buffer then treated identically to other wells. Wells shown are from the same blot with irrelevant wells removed. (E) Heterologous amyloid fibrils do not induce Sup35-GFP^{GPI} aggregation. Filter-trap assay of Sup35-GFP^{GPI} cells at passage-12 after treatment (in triplicate wells) with AF568-labelled amyloid fibrils of A β (1-42) (A β 42), Ure2p⁸⁰, or Sup35NM. Total protein filtered per well was 5 μ g. (F) Western blots of chymotrypsin-treated cell lysates at passage-7 and 16. All data are representative of two independent experiments performed in triplicate.

Figure 4 Acute induction of aggregates on Sup35-GFP^{GPI} cells by co-culture with live Sup35-mC^{GPI} aggregate-positive cells. **(A)** Live-cell confocal microscopy images of Sup35-GFP^{GPI} cells co-cultured with Sup35-mC^{GPI} aggregate-positive cells for 3 days showed induction of Sup35-GFP^{GPI} aggregates (left column, arrows). Sup35-GFP^{GPI} aggregates were not induced by co-culture with mC^{GPI} or Sup35-mC control cells (middle and right panels). Arrowhead: Cell-surface sheet aggregate of Sup35-mC^{GPI}. Asterisks: Sup35-GFP^{GPI} cells with intracellular Sup35-mC^{GPI}. Images represent single Z planes selected from the middle of cells. DIC: Differential interference contrast microscopy. For panels A and D: Merge: GFP (green), mCherry (magenta), and GFP/mCherry colocalization (white). Bars, 10 μm. **(B)** Filter-trap assay of FACS-sorted co-cultures of cells at passage-6 post FACS. Immunoblotting was performed using anti-GFP (left three columns) to detect Sup35-GFP^{GPI} aggregates and anti-RFP (right three columns) to demonstrate that Sup35-mC^{GPI} cells were removed by FACS and there were no detectable Sup35-mC^{GPI} aggregates in the co-culture. Rows 2–4 represent control samples not subjected to FACS. Wells shown are from the same respective blot with irrelevant wells removed. **(C)** Percentage of Sup35-GFP^{GPI}-recipient cells positive for mCherry as determined by flow cytometry. Cells were co-cultured as in panel A prior to analysis. The first four samples represent cells cultured alone to demonstrate the sorting efficiency. The values represent the percentage of GFP-positive cells that are also positive for mCherry. Data are representative of at least three independent experiments in which at least 100 000 events were recorded (**P* < 0.05, ***P* < 0.001, one-way ANOVA with Bonferroni's post test). **(D)** Time-lapse imaging of Sup35-GFP^{GPI} (green) and Sup35-mC^{GPI} (magenta) co-culture. Panels were selected from the time-lapse experiment shown in Supplementary Video 1. The elapsed times (hours:minutes) relative to Supplementary Video 1 are indicated. Panels are maximum intensity Z-projection images. Both GFP and mC channels were smoothed by median filtering and gamma-adjusted.

Sup35-mC^{GPI}, which usually colocalized with Sup35-GFP^{GPI} (Figure 4A, asterisks). Flow cytometry confirmed this observation showing a higher percentage of mCherry-positive recipient Sup35-GFP^{GPI} cells when co-cultured with Sup35-mC^{GPI} cells as opposed to control cells (Figure 4C). Relative to Sup35-mC^{GPI} cells, both Sup35-mC cells and mC^{GPI} cells released higher amounts (~20-fold and >2-fold, respectively) of mCherry to the culture media (Supplementary Figure S5A). Since detection of transferred mCherry was

limited to co-cultures with cells expressing GPI-anchored mCherry proteins, these data suggest that both GPI anchoring and the Sup35NM domains contribute to efficient intercellular transfer of mCherry. The Sup35-GFP^{GPI}/Sup35-mC co-cultures also provide evidence that exposure to high levels of soluble Sup35NM is not sufficient to induce aggregation of Sup35-GFP^{GPI}.

To assess whether co-culture-induced Sup35-GFP^{GPI} aggregation was self-propagating, Sup35-GFP^{GPI}-expressing cells

were isolated from co-cultures by fluorescence-activated cell sorting (FACS). Self-propagating Sup35-GFP^{GPI} aggregates were present only in Sup35-GFP^{GPI} cells that had been co-cultured with aggregate-positive Sup35-mC^{GPI} cells (Figure 4B). Together these data show that Sup35-mC^{GPI} aggregates induced self-propagating Sup35-GFP^{GPI} aggregates in Sup35-GFP^{GPI} cells in association with intercellular transfer of Sup35-mC^{GPI} protein.

Live-cell imaging of Sup35^{GPI} aggregation and spread

Time-lapse imaging of cultures over 1–3 days was used to determine the mechanisms of Sup35-GFP^{GPI} aggregate formation in Sup35-GFP^{GPI}/Sup35-mC^{GPI} co-cultures. Examples of two classes of aggregation-related events observed over the course of six different experiments are shown in Supplementary Videos 1 and 2, with selected frames from Supplementary Video 1 shown in Figure 4D. In the initial frames of Supplementary Video 1, a Sup35-GFP^{GPI} and a Sup35-mC^{GPI} cell shared an aggregate containing GFP and mCherry (Figure 4D, $t = 02:20$, white arrow). Aggregate shearing was mediated by ‘tug of war’-like events due to the divergent movement of cells sharing aggregates (Figure 4D, $t = 02:55$), leaving a portion of the original co-aggregate on each cell (Figure 4D, arrow and arrowhead, $t > 02:20$). It is difficult to determine whether this represented shearing of a single large aggregate versus subdivision of a cluster of smaller aggregates, but in either case these events generated at least two new surfaces for interaction with other cells to initiate new seeding events. In a typical experiment we may observe at least four independent aggregate-shearing events (between different cells). This observation provided a potential mechanism for aggregate fragmentation, a process essential for efficient propagation of prions (Borchsenius *et al*, 2001; Ness *et al*, 2002; Kryndushkin *et al*, 2003; Castilla *et al*, 2005; Shorter and Lindquist, 2006; Tanaka *et al*, 2006; Deleault *et al*, 2007; Satpute-Krishnan *et al*, 2007). In other cases, as shown at later time points in Figure 4D and Supplementary Video 1, similar interactions resulted in the transfer of entire aggregates between cells. For example, in Supplementary Video 1 an aggregate (Figure 4D and Supplementary Video 1, $t > 02:55$, white arrow) initially associated with cell G₁ is transferred to cell G₂ and is shared by both G₂ and G₃ by the end of the time lapse. Altogether, this suggests that Sup35^{GPI} aggregates can exhibit strong attachment to cell surfaces as might be expected due to the multi-valent membrane association of a GPI-anchored polymer.

Sheared aggregates exhibited seeding activity, as shown during subsequent induction of Sup35-GFP^{GPI} aggregation by a freshly sheared Sup35-mC^{GPI} aggregate (Figure 4D, $t = 08:10$ – $10:30$, arrowhead, and Supplementary Video 1). Remarkably, the kinetics of induced Sup35-GFP^{GPI} aggregation were very rapid with initial aggregation usually apparent within 35–70 min after initial cell–cell contact. In some instances, the first detectable intercellular contact appeared to be mediated by fine filopodial or membrane nanotube-like projections through space (Figure 4D, $t = 08:45$, bent arrow), structurally akin to those involved in retroviral and PrP^{Sc} spread (Sherer *et al*, 2007; Sowinski *et al*, 2008; Gousset *et al*, 2009). Analysis of 3D volume projection images of Sup35-mC^{GPI}/Sup35-GFP^{GPI} co-aggregates indicated that induction of Sup35-GFP^{GPI} aggregation occurred at points of intimate intercellular contact (Supplementary

Video 2). In a typical experiment we may observe at least 17 aggregate formation events. These data indicate that intercellular spread of GPI-anchored Sup35 aggregation can occur through direct cell–cell interactions involving contact and either complete or partial transfer of GPI-anchored aggregates. Collectively, these experiments visualize the fundamental processes important for *in vivo* propagation and spread of PrP^{Sc}: formation of new seeding entities via fragmentation of large aggregates; transfer of aggregates between cells; and induction of aggregation in separate cells, thereby resulting in intercellular spread of self-propagating protein aggregation.

Inefficient induction of persistent Sup35-GFP^{GPI} aggregation by fixed Sup35-mC^{GPI} cells

The association between intercellular transfer of Sup35-mC^{GPI} protein to Sup35-GFP^{GPI} cells and induction of Sup35-GFP^{GPI} aggregation raised the question of whether physical transfer of Sup35-mC^{GPI} to recipient cells was necessary. To investigate this issue, we employed a co-culture model analogous to one previously used to study mouse prion infections (Kanu *et al*, 2002). This involved co-culture of fixed Sup35-mC^{GPI} ‘donor’ cells with live Sup35-GFP^{GPI} recipient cells to inhibit transfer of Sup35-mC^{GPI} aggregates. Over 3–4 days of co-culture, new Sup35-GFP^{GPI} aggregates were readily detected in live Sup35-GFP^{GPI} co-cultures with fixed Sup35-mC^{GPI} cells but not in control cultures (Figure 5A). In most cases, GFP aggregates were localized either on Sup35-mC^{GPI} cells or at interfaces between Sup35-GFP^{GPI} and Sup35-mC^{GPI} cells (Figure 5A). After prolonged co-culture (3–4 days), only rare examples of Sup35-GFP^{GPI} cells in distinct possession of GFP aggregates lacking detectable mCherry fluorescence were found (unpublished data). Sup35-GFP^{GPI} cells that contained intracellular Sup35-mC^{GPI} protein were also rare, showing that fixation greatly reduced but did not completely prevent protein transfer from Sup35-mC^{GPI} cells (Supplementary Video 3, $t = 5:15$ – $6:00$). Subsequent passage of the co-cultures was used to eliminate the fixed cells and to assess whether persistent propagation of Sup35-GFP^{GPI} aggregation was induced. Over six passages, only rare examples of aggregate-positive Sup35-GFP^{GPI} cells were found (i.e. at most one Sup35-GFP^{GPI} aggregate might be visualized in a scan of every field of view in a 25-cm² culture flask) and aggregates from such cultures were near the limit of biochemical detection (Figure 5B). These observations established that fixed Sup35-mC^{GPI} aggregates efficiently induced acute but not persistent Sup35-GFP^{GPI} aggregation.

Time-lapse imaging of Sup35-GFP^{GPI} aggregate formation revealed that new Sup35-GFP^{GPI} aggregates rapidly assembled onto Sup35-mC^{GPI} aggregates after initial cell contact (Figure 5C and Supplementary Video 3). The growth of the GFP-derived portion of newly induced Sup35-GFP^{GPI} aggregates initiated from small regions of interaction with Sup35-mC^{GPI} seeds (Figure 5A and C, compare 1:30 versus 3:00) strongly suggests that Sup35-GFP^{GPI} aggregation is also seeded by the newly induced Sup35-GFP^{GPI} portion of the co-aggregate seen at $t = 1:30$. In Figure 5C, two successive rounds of Sup35-GFP^{GPI} aggregate assembly on a single pre-existing aggregate were observed. Closer examination of the two aggregation events showed that in the second round ($t = 3:45$ – $4:30$) at least some of the Sup35-GFP^{GPI} added onto

Figure 5 Induction of Sup35-GFP^{GPI} aggregation by fixed Sup35-mC^{GPI} cells. **(A)** Confocal microscopy of co-cultures of live Sup35-GFP^{GPI} cells with aldehyde-fixed Sup35-mC^{GPI}, mC^{GPI}, or Sup35-GFP^{GPI} cells. Images were acquired after 3 days of co-culture and correspond to maximum intensity Z-projections. Arrows: Induced Sup35-GFP^{GPI} aggregates and apparent Sup35-mC^{GPI} seeds. Asterisks: Glutaraldehyde fixation-induced green autofluorescence. GFP (green), mCherry (magenta). Bars, 20 μm. **(B)** Filter-trap assay of Sup35-GFP^{GPI} cell lysates six passages after co-culture with fixed cells. Sup35-GFP^{GPI} (Agg +): Sup35-GFP^{GPI} cells stably propagating Sup35-GFP^{GPI} aggregates. Wells shown are from the same blot with irrelevant wells removed. **(C)** Time-lapse imaging of Sup35-GFP^{GPI} aggregate induction by fixed Sup35-mC^{GPI} cell. Sup35-mC^{GPI} cells exhibit mCherry fluorescence (magenta) and diffuse fixation-induced green autofluorescence (asterisks). Arrow: Induced Sup35-GFP^{GPI} aggregate (see insets for magnified view). Panels show 3D volume renderings and the numbers indicate the elapsed time (hours:minutes) relative to Supplementary Video 3. **(D)** Magnified 3D volume side view (see Supplementary Video 4) of induced Sup35-GFP^{GPI} aggregate (arrow) from panel C. All data are representative of two independent experiments.

the new Sup35-GFP^{GPI} aggregate created in the previous round ($t=1:30-3:00$) (Figure 5C, inset; Figure 5D, and Supplementary Video 4). In a typical experiment we may observe at least 26 Sup35-GFP^{GPI} aggregate induction events, with 38% of these corresponding to sequential rounds of aggregate assembly seeded by a Sup35-GFP^{GPI} aggregate. This provides direct evidence of the self-propagating nature of Sup35-GFP^{GPI} aggregation. Surprisingly, new Sup35-GFP^{GPI} aggregates were usually retained by fixed Sup35-mC^{GPI} cells when Sup35-GFP^{GPI} cells ultimately disengaged (Figure 5C and Supplementary Video 3). This provides an explanation for the inefficient induction of persistent protein aggregation in Sup35-GFP^{GPI} cells by fixed Sup35-mC^{GPI} cells. The data suggest that intercellular transfer of Sup35-GPI aggregates is

important for efficient induction of self-propagating Sup35-GPI aggregation in separate cells.

Sup35 aggregate induction and propagation is dependent on GPI anchoring

To establish that GPI anchoring modulates the inducible aggregation of Sup35NM in N2a cells, a GPI-anchorless construct (Sup35-GFP) was generated (Figure 1A). Subcellular localization of the resulting Sup35-GFP protein was consistent with that of secreted GPI-anchorless proteins (unpublished data) such as anchorless PrP^C (Chesebro *et al*, 2005), including release of high levels of protein to the culture media (Supplementary Figure S5B). Cells stably expressing Sup35-GFP were treated with recombinant

Figure 6 Induction of aggregation is dependent on GPI anchor. **(A)** Wide-field fluorescence microscopy of Sup35-GFP (top two rows) and Sup35-GFP^{GPI} positive control (bottom row) cells 2 days after treatment with Sup35NM-AF568 fibrils. Arrows: newly induced Sup35-GFP^{GPI} aggregates. Merge: GFP (green), AF568 (magenta), and colocalization (white). Bars, 50 μ m. **(B)** Detergent insolubility assays of fibril-treated cells at passage-6 and 17. Lanes shown are from the same blot for each passage, with irrelevant lanes removed. Cells were treated in triplicate. **(C)** Filter-trap assay of lysates (5 μ g total protein) of fibril-treated Sup35-GFP cells at passage-6 and 10. '+' control, Sup35NM-AF568 fibril-treated Sup35-GFP^{GPI} cells at equivalent pass post fibril addition; '-' control, 4% SDS buffer. Wells shown are from the same blot with irrelevant wells removed. **(D)** Filter-trap assay of culture supernatants from cells at passage-6 shown in panel C. Culture supernatant volumes were normalized based on total protein from cells and adjusted to 4% SDS prior to filtering. Positive ('+') control as described in panel C. Negative ('-') control is culture medium in 4% SDS. The culture supernatant volume analysed corresponds to approximately six times the cell equivalents loaded in the positive control well. Wells shown are from the same blot with irrelevant wells removed. All data are representative of two independent experiments performed in triplicate.

Sup35NM-AF568 fibrils. Analysis of the cultures 2 days after fibril treatment showed no evidence of Sup35-GFP aggregates contrary to the Sup35-GFP^{GPI} control (Figure 6A). It appeared by fluorescence microscopy that the Sup35-GFP cells bound less fibrils than Sup35-GFP^{GPI} controls and this was confirmed by quantitative analysis (Supplementary Figure S6). These data suggested that expression of GPI-anchored Sup35NM enhanced the binding of exogenously added Sup35NM fibrils.

Extended passage showed that Sup35NM fibril treatment had no effect on Sup35-GFP distribution, suggesting that persistent aggregation was not induced (unpublished data). This observation was confirmed by biochemical analyses, which indicated that there were no detectable Sup35-GFP aggregates in the cell lysates or culture supernatants of Sup35NM fibril-treated cells (Figure 6B–D). Acute co-culture of Sup35-GFP cells with aggregate-positive Sup35-mC^{GPI} cells also failed to induce aggregate formation in Sup35-GFP cells (Supplementary Figure S7). Filter-trap assays verified that Sup35-GFP was capable of specific incorporation into recombinant Sup35NM fibrils (Supplementary Figure S8). These results indicated that, for Sup35NM expressed in the

secretory pathway, GPI anchoring was required for induction of self-propagating Sup35NM aggregation.

Discussion

We have shown that Sup35-GFP^{GPI} cells were induced to generate self-propagating aggregates by exposure to Sup35NM amyloid fibrils or cell-associated, GPI-anchored Sup35NM aggregates. In contrast, cells expressing GPI-anchorless Sup35NM produced no detectable aggregates when exposed to the same treatments (Figure 6 and Supplementary Figure S7). This illustrated the role of GPI anchoring in mediating persistent aggregate propagation and ruled out the possibility that aggregate propagation in Sup35-GFP^{GPI} cells was attributed to GPI anchor-independent mechanisms of cell association. These findings were consistent with the inability of GPI-anchorless PrP^C to support persistent scrapie infection in cell culture (McNally *et al*, 2009). Live-cell imaging revealed that new aggregation occurred through intercellular contact between Sup35-GFP^{GPI} cells and aggregates on the surface of Sup35-mC^{GPI} cells (Figures 4 and 5, and Supplementary Videos 1–3). In addition, inhibition of intercellular aggregate transfer

by chemically fixing the aggregate donor Sup35-mC^{GPI} cells significantly decreased the level of persistent Sup35-GFP^{GPI} aggregate induction (Figure 5B), suggesting that efficient intercellular aggregate propagation requires intercellular aggregate transfer. Together, these data demonstrate that GPI anchoring facilitates the propagation and intercellular spread of Sup35NM aggregation and provide direct evidence of fundamental cell-cell aggregate-spreading mechanisms thought to be required for propagation of PrP^{Sc} in prion diseases.

Efficient propagation of [PSI⁺] and other prions in the yeast cytoplasm requires a complex interplay between Hsp104 and other cytoplasmic chaperone proteins (Shorter and Lindquist, 2008; Tipton *et al*, 2008). The recent report of vertical propagation of cytoplasmic Sup35NM aggregates in N2a cells shows that the mammalian cell cytoplasm contains factors sufficient for propagation of Sup35NM aggregates (Krammer *et al*, 2009), which could conceivably include orthologues of the corresponding yeast chaperone proteins (Doyle and Wickner, 2009). This is consistent with another very recent report of propagation of polyglutamine aggregates inside mammalian cells (Ren *et al*, 2009). In our study, the prion-like propagation of GPI-anchored Sup35NM in mammalian cells apparently occurred in a cell-surface/extracellular compartment lacking these cytoplasmic chaperones. This suggests that GPI anchoring of Sup35NM compensated for the absence of these chaperones, perhaps by altering the properties and/or behaviour of Sup35NM aggregates, such as structure and/or propensity, for intercellular spread as discussed below. It is possible that extracellular chaperone-like molecules such as clusterin (Wilson *et al*, 2008) can assist the propagation and spread of extracellular protein aggregates. However, there are no reports that extracellular chaperones possess the specific aggregate fragmentation activity of Hsp104 required for amplification of seeding-competent particles. Regardless, our findings provide an example of how GPI anchoring can facilitate the replication of a misfolded protein aggregate as a mammalian prion.

Intercellular transfer of PrP^C was demonstrated to be dependent on the GPI anchor and cell-to-cell contact, which raised the question of whether GPI anchoring could mediate the transfer of PrP^{Sc} (Liu *et al*, 2002) and other misfolded protein aggregates. Data presented in Figures 4 and 5, and Supplementary Videos 1–3 show that in our model Sup35 aggregation was induced by cell contact and that stable propagation of new aggregation in a cell depends on the transfer of all or part of the original co-aggregate. Similar to our model, others observed reduced mouse prion infection efficiency initiated by fixed infected cells versus live cells (Kanu *et al*, 2002; Paquet *et al*, 2007). Our data provide an explanation for this result, where we observed that fixed aggregates are highly stabilized and less prone to shearing or transfer, thereby favouring the retention of newly induced aggregates by fixed cells. Shearing within the GFP portion of GFP-tagged aggregates induced by Sup35-mC^{GPI} aggregates is possible, but in our many imaging experiments we rarely observed such an event. This may be because GFP-tagged Sup35 aggregates rarely have an opportunity to grow large enough to create a sufficiently frangible surface on which fracture can occur, the size apparently being dependent upon the length of time for which the two cells are in contact. Altogether, our results demonstrate horizontal intercellular

spread of Sup35^{GPI} aggregation, a process that presumably allows neuroinvasion and spread of PrP^{Sc} that is ultimately essential to transmission of TSE disease between hosts after peripheral infection. By contrast, horizontal spread of cytoplasmic protein aggregates between live cells was either not examined (Krammer *et al*, 2009) or not clearly detected (Ren *et al*, 2009). Consequently, propagation of these cytoplasmic protein aggregates either fails to meet an important practical criterion for a mammalian prion *in vivo* or is much less efficient than mammalian prions. This highlights a key difference between propagation of cytoplasmic and GPI-anchored protein aggregates, and emphasizes the significance of the role of GPI anchoring in aggregate-spreading mechanisms.

Although different mechanisms may be involved, our observation of the importance of cell contact/proximity in intercellular spread of protein aggregates is consistent with previous reports of transmission of mouse prion infection between cells (Kanu *et al*, 2002; Paquet *et al*, 2007; Gousset *et al*, 2009). Furthermore, immunogold electron microscopy studies of scrapie-infected tissues suggest that PrP^{Sc} can spread between adjacent cells *in vivo* (Jeffrey *et al*, 2009). Other mechanisms of intercellular spread of GPI-anchored proteins that may contribute to PrP^{Sc} propagation include membrane painting (Medof *et al*, 1996) and/or transport on intercellular processes such as exosomes, microparticles, filopodia, and tunnelling nanotubes (Fevrier *et al*, 2004; Caughey and Baron, 2006; Sherer *et al*, 2007; Sowinski *et al*, 2008; Caughey *et al*, 2009; Gousset *et al*, 2009). The role of these trafficking pathways in the spread of Sup35^{GPI} aggregates remains to be investigated although we occasionally observed filopodial/membrane nanotube-like projections during initial interactions between aggregate-positive and aggregate-negative cells (Figure 4D).

In addition to effects on intercellular spread, GPI anchoring restricts aggregation to the two-dimensional plane of a membrane thereby impacting aggregate structure. GPI anchoring may limit the formation of large, higher order, highly stable aggregates such as amyloid plaques and promote the formation of smaller oligomeric assemblies that can exhibit enhanced seeding activity, neurotoxic activity, and infectivity (Caughey, 2001; Silveira *et al*, 2005; Chiti and Dobson, 2006; Tanaka *et al*, 2006), and might spread readily through the extracellular milieu. Data from *in vivo* and *in vitro* studies provide evidence of modulation of PrP^{Sc} fibril formation, neurotoxicity, and infectivity via PrP membrane association (Gabizon *et al*, 1987; McKinley *et al*, 1991; Chesebro *et al*, 2005; Silveira *et al*, 2005; Baron *et al*, 2006). Similarly, the specific infectivity ([PSI⁺] induction per mole of Sup35p) and propagation efficiency of Sup35p prion particles in yeast is also higher for smaller particles (Borchsenius *et al*, 2001; Kryndushkin *et al*, 2003; Shorter and Lindquist, 2006; Tanaka *et al*, 2006; Bagriantsev *et al*, 2008). The upper limit of Sup35-GFP^{GPI} and Sup35-mC^{GPI} aggregate size by fluorescence microscopy varied, but none approached the size of typical amyloid plaques, and other analyses (to be described elsewhere) suggest that these aggregates are not amyloid. Live-cell imaging revealed examples of very small Sup35-mC^{GPI} aggregates inducing the formation of much larger Sup35-GFP^{GPI} aggregates, suggesting that small GPI-anchored Sup35 aggregates can efficiently seed new aggregation (unpublished data). Thus, the potential effects of GPI-anchoring

on protein aggregation and transmission of self-propagating aggregates are multifaceted and interrelated.

GPI-anchored PrP^C is critical to TSE disease pathogenesis. When mice expressing GPI-anchorless PrP^C were inoculated intracerebrally (IC) they did not develop typical scrapie disease (Chesebro *et al*, 2005). Although PrP^{Sc} was produced in the anchorless PrP^C mice, the nature and distribution of PrP^{Sc} deposits in the brain was very different from that in wild-type mice. Instead of forming diffuse, widespread membrane-bound accumulations as in wild-type mice, GPI-anchorless PrP^{Sc} formed large, localized amyloid deposits associated with limited pathology, which resembles that associated with other amyloid diseases (e.g. Alzheimer's disease). It is unclear in this model whether aggregate spread due to PrP^{Sc} transport between distant locations is occurring within the CNS, but if it occurs it would seem less pervasive than GPI-anchored PrP^{Sc}. In addition, significantly longer incubation times were required to produce TSE infectivity titres and PrP^{Sc} levels comparable to those in infected wild-type mice, but these and other phenotypes mentioned above were complemented by introducing a single copy of wild-type *prnp* to GPI-anchorless PrP mice. This shows that neurodegenerative pathology, the rate of PrP^{Sc} formation, and dissemination of PrP^{Sc} within the CNS is greatly facilitated by the presence of GPI-anchored PrP^C (Chesebro *et al*, 2005).

There are outstanding questions in regards to scrapie infections of anchorless PrP^C mice. Because all the above mentioned experiments were performed using IC inoculations, the distribution of and neuroinvasion by PrP^{Sc} in these transgenic mice after peripheral infection remains unknown. The data from anchorless PrP^C mice would indicate that with respect to propagation of prions in the context of IC infections, GPI-anchoring may not be essential. However, as discussed in the Introduction, this does not exclude a role for GPI anchoring in modulating the transmissibility of other protein misfolding diseases due to the high likelihood of the multifactorial nature of such processes. It is possible that factors specific to PrP or inherent properties of anchorless PrP^{Sc} fibrils (e.g. interactions with membrane proteins or extracellular matrix components, GPI anchor-independent binding to membranes, high frangibility of fibrils) may partially compensate for the absence of the GPI anchor.

The possibility that Alzheimer's amyloid- β (Meyer-Luehmann *et al*, 2006), polyglutamine (Ren *et al*, 2009), and tau (Clavaguera *et al*, 2009; Frost *et al*, 2009) aggregation are transmissible might raise questions about the argument that GPI anchoring can modulate the transmissibility of protein-misfolding diseases. Despite a lack of epidemiological evidence, this has been noted before in the case of Alzheimer's disease (Goudsmit *et al*, 1980) and is based in part on an investigation which demonstrated that Alzheimer's disease mouse models developed agent-dependent A β -amyloid deposits at accelerated time points after IC inoculation with brain homogenates from Alzheimer's patients or aged Alzheimer's transgenic mice (Meyer-Luehmann *et al*, 2006). In more relevant models, however, no reproducible clinical signs of disease were observed in 240 primates inoculated IC with brain homogenates from patients with either familial or sporadic Alzheimer's disease (Goudsmit *et al*, 1980; Brown *et al*, 1982, 1994). The inability of A β -containing brain material to induce cerebral β -amyloidosis when administered by peripheral routes clearly distin-

guishes this phenomenon from prion transmission (Eisele *et al*, 2009). The cumulative data support the proposition that TSEs are significantly more transmissible, if not uniquely so, relative to other protein deposition diseases even if there is an element of cell-to-cell propagation of pathology in the brain in other diseases. One author has even proposed a separate terminology (prionoid) for these prion-like disorders (Aguzzi, 2009). The reason for this difference has not been established, but we believe GPI anchoring (or at least membrane anchoring) of the associated misfolded polypeptide could play a critical role in enhancing the spread of protein aggregation from the periphery into and within the CNS. Whether any other protein-misfolding disorder is ultimately found transmissible under special circumstances, it nevertheless remains an important question as to whether GPI anchoring might modulate the associated transmissibility and pathology.

Our data are the first, to our knowledge, to directly demonstrate that GPI-anchored protein aggregation can spread between unrelated cells by cell-to-cell contact and transfer of aggregate seeds. While not excluding additional mechanisms of transfer, we propose that GPI anchor-mediated intercellular spread of PrP aggregates is a central component to prion disease pathogenesis. While such transfer would have implications for prion biogenesis and spread in all circumstances of prion disease, it would be particularly critical for infections resulting from peripheral prion exposure where prion neuroinvasion and propagation in different tissue types occurs over the course of infection. Although no natural examples exist, it will also be important to investigate whether other mechanisms of membrane association (e.g. transmembrane) could support the propagation of misfolded protein aggregates as this may not be limited to GPI anchoring.

Materials and methods

Aggregate induction by Sup35NM-AF568 fibrils

Cells were plated at 10% confluence in a 24-well tissue culture plate. After one day of growth, cells were treated with 2.6 pmole of either recombinant Sup35NM or Ure2p⁸⁰ fibrils labelled with AF568. Mock controls were treated with equal volumes of PBS that had been processed through the AF568 labelling protocol to account for the possibility of AF568 carry-over. Sup35-GFP cells were treated with PBS or Sup35NM-AF568 fibrils only. Cells were maintained for 2 more days at which point they were split and re-plated at 10% confluence. Cells were subsequently re-plated at 5 or 10% confluence every 4 or 3 days, respectively.

GFP-tagged Sup35 cell co-cultures with aggregate-producing Sup35-mC^{GPI} cells

For flow cytometric and FACS experiments cells were plated at 5% confluence each, grown for 3 days, and then analysed for GFP and mCherry or sorted by FACS to collect GFP-expressing cells only. FACS-sorted GFP cells were grown to confluence and analysed by wide-field fluorescence microscopy for mCherry-expressing cells. Cells were re-plated at 10% confluence and FACS was repeated until no mCherry cells were visible by fluorescence microscopy. Cells were subsequently re-plated at 5 or 10% confluence every 4 or 3 days, respectively. Flow-cytometric data were analysed using the FlowJo software.

Live-cell confocal microscopy of co-cultured cells was performed on cells each plated at either 5 or 25% confluence for live-cell co-cultures. Fixed cell co-cultures were created as described elsewhere (Kanu *et al*, 2002) with the exception that fixed 'donor' cells were plated at ~30% confluence and fixed 2.5 h after plating while live Sup35-GFP^{GPI} 'recipient' cells were plated at 5% confluence.

Detergent insolubility

Cells were plated at 10% confluence in six-well tissue culture plates and grown to confluence. Cells were washed with phosphate-buffered balanced salts solution (PBBS) (pH 7.4) and lysed with DS buffer (1% sarkosyl, 1 mM EDTA in PBS) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche). Lysates were treated with 25 U of benzonase per millilitre of lysate supplemented with 8 mM MgCl₂ for 30 min. After removing aliquots for BCA total-protein analysis, samples were supplemented with EDTA to 50 mM to prevent precipitation of sarkosyl-magnesium salts. Lysates were centrifuged for 30 min at 350 000 × g to separate detergent-insoluble material. The resulting supernatants were methanol-precipitated. Samples were analysed by SDS-PAGE and Western blotting using anti-Sup35 N and anti-rabbit IgG alkaline phosphatase, loading 100 µg total protein equivalents per lane.

Filter-trap assays were performed based on methods described previously (Scherzinger *et al*, 1997; Xu *et al*, 2002). Cells plated at 10% confluence and grown to confluence in 24-well plates were lysed in FT buffer (1% SDS, 1 mM EDTA in PBS) supplemented with Complete EDTA-free protease inhibitors and treated with benzonase as described above, except using 75 U of benzonase per millilitre of lysates supplemented with 16 mM MgCl₂. EDTA was added to a final concentration of 50 mM after benzonase digestion. Cell lysates were adjusted to 10 and 50 µg/ml protein in 4% SDS with PBS and allowed to incubate at room temperature for 30 min. Culture supernatants were prepared by centrifugation at 3000 × g for 5 min to clear cellular debris. The top 80% of the cleared supernatant was removed and adjusted to 4% SDS in PBS by adding 1 volume of 8% SDS in 2 × PBS. Volumes were normalized for total protein from their respective cell lysates using 4% SDS in PBS, with 50% unconditioned cleared media. All samples were applied to an Immobilon-P 0.45 µm membrane pre-wetted with FT buffer using The Convertible dot blot apparatus (Gibco). Wells were washed five times with 200 µl of FT buffer. Proteins were detected by Western blotting as described above using mouse anti-GFP (Roche) or rabbit anti-RFP (for mCherry; Rockland Immunochemicals) as primary

antibodies, and anti-mouse or anti-rabbit IgG alkaline phosphatase as secondary antibody.

Chymotrypsin digests

Cells were plated at 10% confluence and grown to confluence before being lysed in DS buffer and treated with benzonase as described above. The lysates were treated with chymotrypsin at a 1:250 chymotrypsin (Ct)-to-total protein mass ratio for 15 min at 37°C. The reactions were placed on ice and Pefabloc was added to stop the reactions. Samples were methanol-precipitated at −20°C after adding thyroglobulin. The samples were analysed by SDS-PAGE and Western blotting using anti-Sup35 N with anti-rabbit IgG alkaline phosphatase secondary. All lanes were loaded with 25 µg total protein equivalents.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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