Research Report OTKA K82090

The purpose of our research is to understand the physiological function of the prion protein and the prion protein family, as well as, their role in the pathomechanism of transmissible spongiform encephalopathy and Alzheimer disease. We mainly focus on the neurotoxic and neuroprotective functions of the prion protein family and on its role in neurogenesis. We examine also the structure and the mechanism of formation of conformationally altered variants of the prion protein that are typical in the progression of TSE.

[In order to explain the importance of our results, few introductory words are necessary.] The exact function of neither the prion protein, nor of the two other members of the prion protein family are known, although their participation has been suggested/demonstrated in several processes. It has also not been clarified yet what is their exact role in the neuronal death observed in transmissible spongiform encephalopathies and Alzheimer's. From this point of view, examining N-terminal deletion mutant forms of PrP (ΔCRPrP, Shmerling mutant PrP-s) were promising; overexpression of these variants caused neurodegeneration in mice on a PrP KO background. The co-expression of WT PrP prolonged the lifespan of the mice and/or diminished the neurodegenerative phenotypes. Ex vivo experiments suggest that the expression of ΔCRPrP is associated with neurotoxicity (in primary culture), glutamate induced exitotoxicity, zeocin/G418 hypersensitization and the presence of large inward ion currents detected by patch clamps. More interestingly, all of these effects are diminished by the coexpression of wt PrP. It is not known if these ex vivo observed mechanisms are also effective in vivo in TSE, Alzheimer's or in Schmerling mutant mice.

One of the prion protein family members, the Shadoo protein (shadow of the prion protein) is thought to be a functional analogue of PrP; especially, of the disordered N-terminal parts of PrP that has been showed to mediate neuroprotective effects in a number of settings. Shadoo has been showed to counteract the effect of the neurotoxicity of ΔCRPrP in primary culture, and glutamate induced excitotoxicity in SH-SY5Y cells. Here we examined the effect of Shadoo on the zeocin hypersensitization of ΔCRPrP.

Materials and Methods

→ **Transgenes used**: Wild type mouse prion protein (PrP) or a truncated form, missing the residues 105-125th (PrPDCR), and the mouse Shadoo protein (Sho) were used in these experiments. As a marker for the transgene expression, the coexpression of either the green fluorescent protein (GFP) or mCherry were used.

→ **Establishment of stable transgene expressions in SH-SY5Y human neuroblastoma cell line.**

1. GFP only or GFP with either PrPDCR or PrP were introduced into SH-SY5Y cells, using the Sleeping beauty transposon based gene delivery system[7].

→ **These transfected cells are referred to as GFP, dCR or PrP in captions, respectively.**

2. mCherry alone or with either Sho or PrP were introduced by lentiviral transduction into the **dCR** cells.
   → **These transduced cells are referred to as dCR+mCh, dCR+Sho, dCR+PrP in captions, respectively.**

   Shadoo and mCherry was also expressed in SH-SY5Y cells.
   → **Shadoo and Cherry expressing cells are referred to as Sho in captions.**

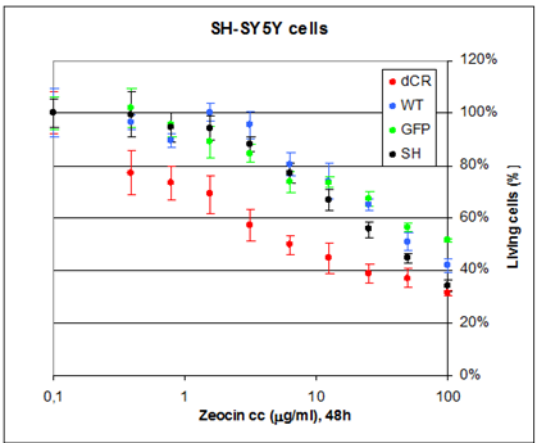
1. GFP only or GFP and PrP coexpression was established in Sho cells by lentiviral transduction
   → **These are referred to as Sho+GFP, Sho+PrP in the captions, respectively.**

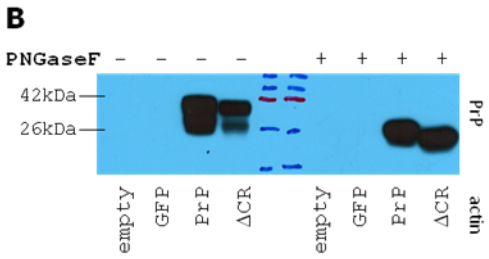
2. GFP and/or mCherry positive cells expressing the respective transgenes were enriched by fluorescence associated cell sorting (FACS).

→ **Immunoblotting**. SAF32 monoclonal anti-prion antibody (Prionics) and Abgent SPRN (C-term) polyclonal anti-Shadoo antibody (Abgent) were used to detect PrP, and the Shadoo protein, respectively. PNGaseF (NEB) was used to probe the glycosylation state of the proteins, and to facilitate the differentiation between the full length and truncated form of PrP. The proper cell surface localization and GPI-anchor attachment were confirmed in case of each proteins by PIPLC treatment (data not shown).

→ **Cell viability assays** were carried out on 96-well plates. After the cell seeding and Zeocin (LifeTechnologies) or G418 (Sigma) treatment their viabilities were determined using PrestoBlue (LifeTechnologies) reagent The viability of drug-treated cells were always normalized to the untreated cells.

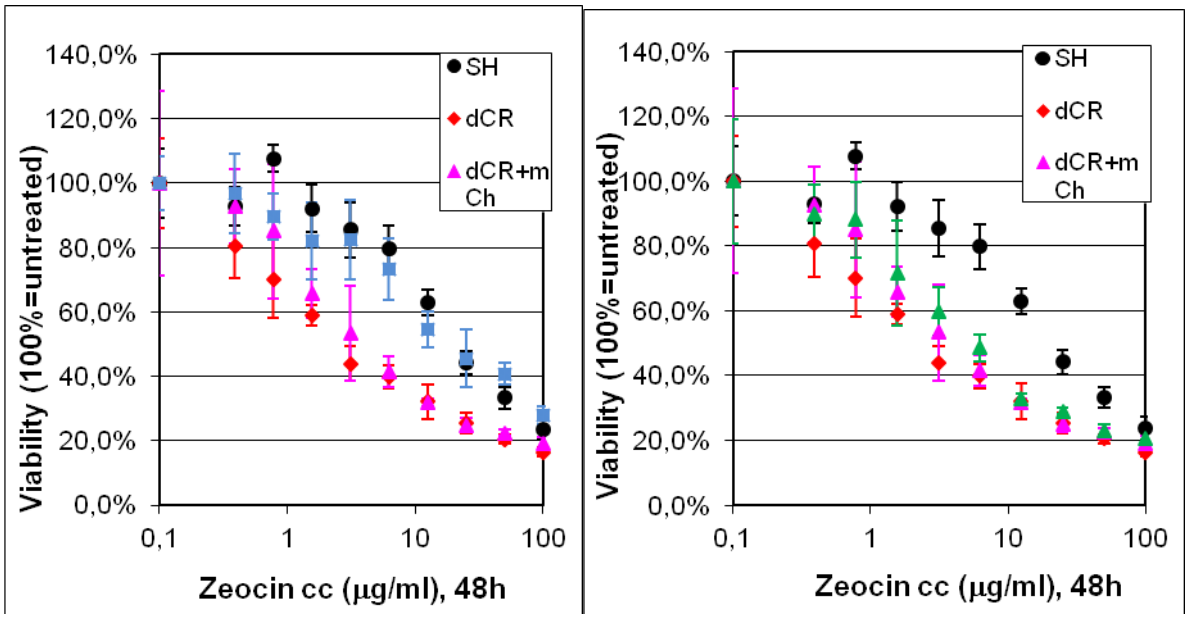
1) PrPDCR exhibits a Zeocin-sensitizing phenotype in SY-SY5Y cells confirming former results[4]

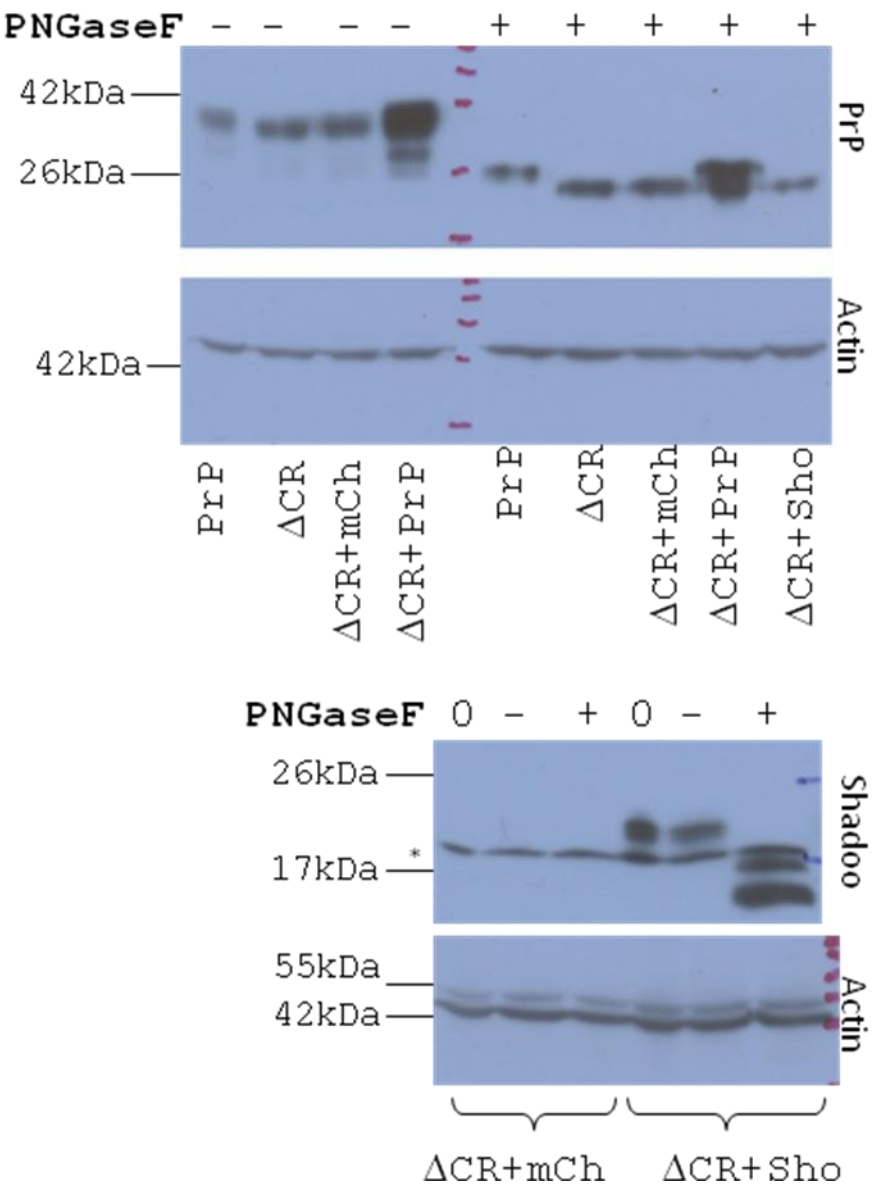




A) Cell viability assay. Untransfected SH-SY5Y (empty), **dCR**, **PrP**, and **GFP** cells were treated with Zeocin at various concentrations for 48 hours. **Cells expressing PrPDCR** (red dots on graph, **dCR**) **show increased sensitivity to the drug.**
B) Immunoblot. Expression of the transgenes was assessed by immunoblotting the lysates of each cell line (conditions) for the respective proteins. PNGaseF treatment of the lysates allows to visualize the mobility difference caused by the deletion of 20 amino acids of the Central Region. Endogenous PrP of the SH-SY5Y cells was detectable at longer exposure times (not shown) but its expression level was significantly lower than the transgenic expression levels.

2) Co-expression of wild type PrP eliminates Zeocin hypersensitivity, but Shadoo doesn't rescue this phenotype…





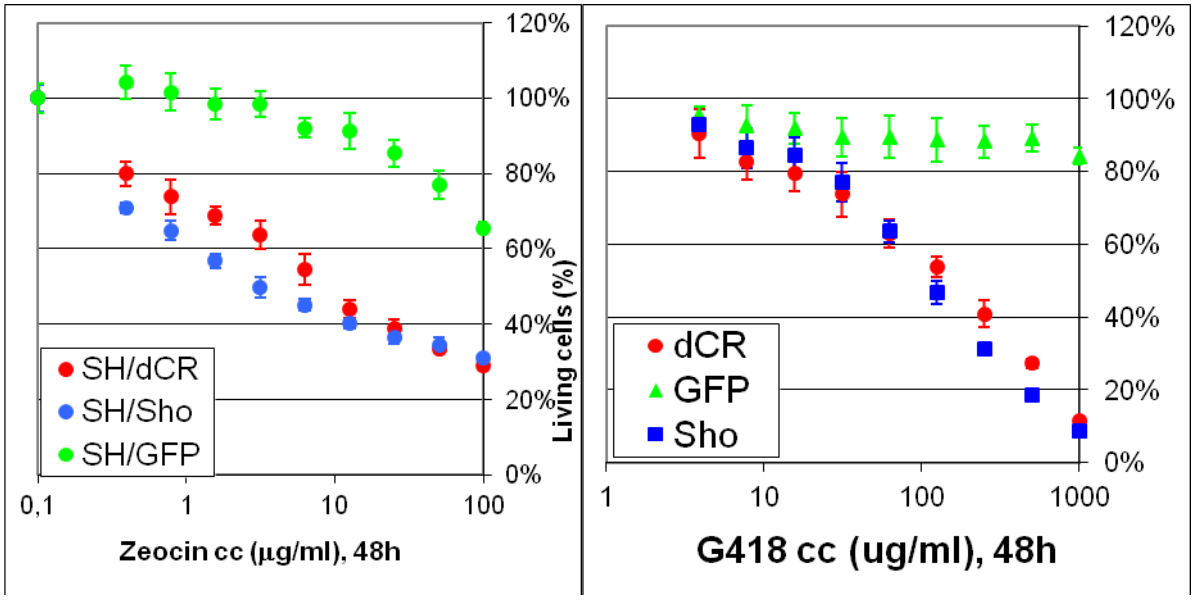
A Cell viability assay. Untransfected SH-SY5Y cells (empty), dCR, dCR+PrP, and dCR+mCh cells were treated with various concentrations of Zeocin for 48 hours. While coexpression of mCherry alone does not alter the sensitivity of PrPDCR cells (pink vs red dots on graph), coexpression of full length PrP diminishes Zeocin hypersensitivity almost completely (blue dots on the graph).

B Cell viability assay. By contrast, Sho coexpression (dCR+Sho) does not diminish Zeocin hypersensitivity (green dots on the graph).

C Immunoblot. PrP expression was examined in the parental and the transduced cells. PNGaseF treatment facilitates separating the full length and the deletion mutant forms and enables us to assess the relative amount of PrP-s.

D Immunoblot. Shadoo expression was examined by immunoblotting. * Marks an unspecific band detected by Sho-antibody in total cell lysates. PNGaseF treatment reveals that the overexpressed Shadoo has a complex glycosylation. Samples marked ”+” or "-" : incubated with or without enzyme added, respectively. "0": non treated sample to see if Shadoo is degraded during the incubation.

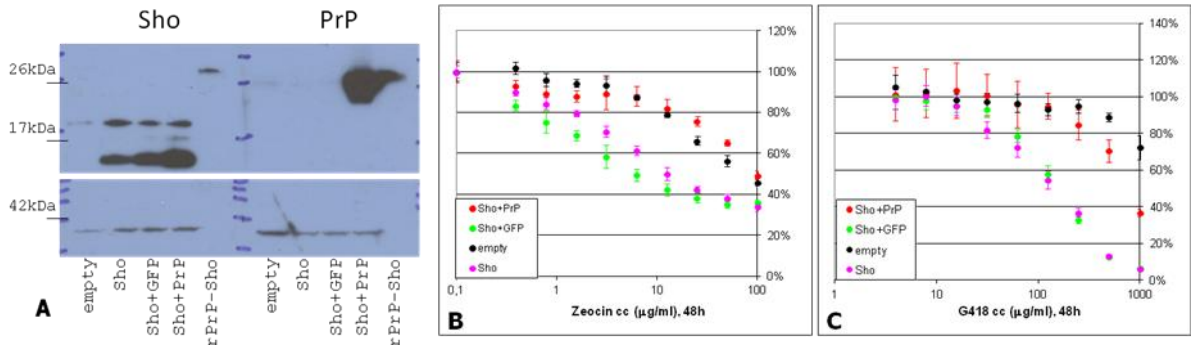
3. …because, Shadoo, just like PrPD105-125, induced Zeocin and G418 hypersensitivity in the cells expressing the protein stably.



A, B Cell viability assay. **Sho**, **dCR** or **GFP** cells were treated with Zeocin (A) or G418 (B). **Shadoo expression** (blue dots in both graphs) **increased the sensitivity of cells to both Zeocin and G418, just like PrPDCR** (red dots in both graphs) **expression does**.

C Cell viability assay. Untransfected SH-SY5Y (**empty**), **Sho** or **dCR** cells were treated with puromycin to examine the specificity of the sensitizing phenotype of Shadoo to G418/zeocin. **Neither Shadoo or PrPDCR expression increased the sensitivity of the cells to puromycin.** The later result is in accord with the literature[3].

4. The co-expression of the wild type PrP was able to eliminate the drug hypersensitivity caused by the Shadoo protein.



A Immunoblot. Shadoo and PrP expression was examined in untransduced (**empty**), **Sho**, **Sho+GFP**, and **Sho+PrP** cells. After PNGaseF treatment, expression levels of Shadoo were compared. Lower blot shows loading control (b-actin). **Cells expressing both Shadoo and PrP had the highest Shadoo expression level, followed by the Sho+GFP and Sho cells.** rPrP-Sho is a recombinant fusion protein having epitopes of both Shadoo and prion proteins. „*" Marks an unspecific band detected by Sho-antibody in total cell lysates.

B, C Cell viability assays. Untransduced (**empty**), **Sho**, **Sho+GFP**, and **Sho+PrP** cells were treated with either Zeocin (B) or G418 (C). **Coexpression of PrP (red dots in both graphs) but not GFP (green dots in both graphs) rescued the sensitizing phenotype of the Shadoo protein.**

We also examined the localization and the traffic of the GPI-anchored shadoo protein in comparison to those of the similarly GPI-anchored prion protein. The prion protein (PrP has been reported to possess two nuclear localization signals and to localize in the nuclei of certain cells in various forms. Although these data are superficially contradictory, it is apparent that nuclear forms of the prion protein can be found in cells in either the healthy or the diseased state. Here we report that Shadoo (Sho) – a member of the prion protein superfamily – is also found in the nucleus of several neural and non-neural cell lines as visualized by using an YFP-Sho construct. This nuclear localization is mediated by the (25-61) fragment of mouse Sho encompassing an $(RXXX)_8$ motif. Bioinformatics analysis shows that the $(RXXX)_n$ motif (n=7-8) is a highly conserved and characteristic part of mammalian Shadoo proteins. Experiments to assess if Sho enters the nucleus by facilitated transport gave no decisive results: the inhibition of active processes that require energy in the cell, abolishes nuclear but not nucleolar accumulation. However, the $(RXXX)_8$ motif is not able to mediate the nuclear transport of large fusion constructs exceeding the size limit of the nuclear pore for passive entry.

These results have been published in BBA . Tracing the journey of various forms of Sho from translation to the nucleus and discerning the potential nuclear function of PrP and Sho requires further studies that is under way in our lab.

One of the proposed functions of the prion protein is a role in embriogenesis and in neurogenesis. What extent the two proteins have complementary roles is a long debated issue, raised by some experiments suggesting that PrP and Shadoo have overlapping function while others showing opposite results. We have a collaboration with Dr Emilia Madarász (KOKI) to study neurogenesis and assess the expression and localization changes of PrP, Shadoo and members of the retinoic acid biosynthesis and metabolism and their potential role in these processes.

Retinoic acid (RA) presents at sites of neurogenesis in both the embryonic and adult brain. While it is widely accepted that RA signaling is involved in the regulation of neural stem cell differentiation, little is known about vitamin A utilization and biosynthesis of active retinoids in the neurogenic niches or about the details of retinoid metabolism in neural stem cells and differentiating progenies. We provided data on retinoid responsiveness and RA production of distinct neural stem cell/neural progenitor populations. In addition, we demonstrated differentiation-related changes in the expression of genes encoding proteins of the retinoid machinery, including components responsible for uptake (Stra6) and storage (Lrat) of vitamin A, transport of retinoids (Rbp4, CrbpI, CrabpI-II), synthesis (Rdh10, Raldh1-4), degradation of RA (Cyp26a1-c1) and RA signaling (Rara,b,c, Rxra,b,c). We showed that both early embryonic neuroectodermal (NE-4C) stem cells and late embryonic or adult derived radial glia like progenitors (RGl cells) are capable to produce bioactive retinoids but respond differently to retinoid signals. However, while neuronal differentiation of RGl cells can not be induced by RA, neuron formation by NE-4C cells is initiated by both RA and RA-precursors (retinol or retinyl acetate). The data indicate that endogenous RA production, at least in some neural stem cell populations, may result in autocrine regulation of neuronal differentiation. We have already published the first paper based on the results of this cooperation in Stem Cells Dev.

We have attempted a few times to generate anti-Shadoo antibodies (eight times altogether) without any success. Since no appropriate commercial antishadoo antibody is available for performing immunocytochemistry, preparation of tagged versions of the protein are underway to monitor the changes of the localization and expression pattern of Shadoo during the life span of mice.

2.

In order to get structural insight into the formation of the protease resistant variant of PrP, PrP(res), we incorporated non-natural amino acids into the sequence of the prion protein. Basically we followed two approaches: intein based protein ligation is used to couple bacterially expressed fragments of PrP to peptides generated by solid phase peptide synthesis that contain the non-natural amino acids. Alternatively, we incorporated genetically encoded non-natural amino acids into bacterially-expressed prion protein.

In order to prepare the ligation-competent *prionfragment-intein* fusion polypeptides the NEB IMPACT expression vector system needs to be used. These bacterial vectors permit the cloning of the coding DNA of a protein to be expressed to an NcoI site which restricts the

possible second amino acids to codons starting with a G. In order to facilitate the expression of the various prion protein forms without adding extra amino acids to its N-terminal, a new method was developed that allows the generation of overhangs of a restriction enzyme without using the given enzyme for digestion.

The procedure described here allows the cloning of PCR fragments containing a recognition site of the restriction endonuclease (Type IIP) used for cloning in the sequence of the insert. A Type IIS endonuclease - a Body Double of the Type IIP enzyme - is used to generate the same protruding palindrome. Thus, the insert can be cloned to the Type IIP site of the vector without digesting the PCR product with the same Type IIP enzyme. We achieve this by incorporating the recognition site of a Type IIS restriction enzyme that cleaves the DNA outside of its recognition site in the PCR primer in such a way that the cutting positions straddle the desired overhang sequence. Digestion of the PCR product by the Body Double generates the required overhang. Hitherto the use of Type IIS restriction enzymes in cloning reactions has only been used for special applications, the approach presented here makes Type IIS enzymes as useful as Type IIP enzymes for general cloning purposes. To assist in finding Body Double enzymes, we summarized the available Type IIS enzymes which are potentially useful for Body Double cloning and created an online program (http://group.szbk.u-szeged.hu/welkergr/body_double/index.html) for the selection of suitable Body Double enzymes and the design of the appropriate primer.

This work has been published in PLOS ONE.

We successfully expressed the protein and we collaborated with a peptide chemist, Thömböly Csaba (SZBK) in the protein ligation step who set up a protein ligation approach in his lab that is able to couple polypeptides at positions other than cysteine based on literature protocols.

Since we proceeded faster with the alternative approach: the incorporation of genetically encoded non-natural amino acids, later we focused on this second approach while continued to establishes the semisynthetic preparation of PrP. The non-natural amino acid, a parabenzoyl phenylalanine (pBpa) has been inserted to a number of position (over 30) mainly at the N-terminal part of PrP. Using mass spectrometry we confirmed the incorporation of pBpa and the lack of tyrosine at the positions. The effect of the mutation on the stability of all protein variant were assessed and the proteins were cross-linked by UV light under various condition. These crosslinking experiments revealed a dimerization interface on the protein molecule.

In order to assess if this dimer formation takes place in living cells as well, a GPI-anchor mimetic was coupled to the molecule to facilitate its incorporation to the cell membrane of mammalian cells. The synthesis of this GPI-anchor mimetic was carried out in a scientific collaboration with Csaba Thömböly, a peptide chemist, and methods developed for the delivery of the recombinant proteins to the cell membrane of mammalian cells was published in Bioconjugate Chem.

The conformational conversion of the recombinant pBpa-containing proteins in the cell membrane of living cells is under way to assess if the same dimer intermediate exist in a more relevant live-cell PrP conversion process.

References:

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