Video Article

Isolation of Soluble and Insoluble PrP Oligomers in the Normal Human Brain

Xiangzhu Xiao[1], Jue Yuan[1], Wen-Quan Zou[1,2]

[1]Department of Pathology, National Prion Disease Pathology Surveillance Center, Case Western Reserve University School of Medicine

[2]Department of Neurology, National Prion Disease Pathology Surveillance Center, Case Western Reserve University School of Medicine

Correspondence to: Wen-Quan Zou at Wenquan.zou@case.edu

URL: https://www.jove.com/video/3788
DOI: doi:10.3791/3788

Keywords: Medicine, Issue 68, Neuroscience, Physiology, Anatomy, Prion protein, brain, prion disease, insoluble prion protein, oligomer, ultracentrifugation, Western blotting, Sucrose gradient sedimentation, gel filtration

Date Published: 10/3/2012

Citation: Xiao, X., Yuan, J., Zou, W.Q. Isolation of Soluble and Insoluble PrP Oligomers in the Normal Human Brain. *J. Vis. Exp.* (68), e3788, doi:10.3791/3788 (2012).

Abstract

The central event in the pathogenesis of prion diseases involves a conversion of the host-encoded cellular prion protein PrP^{C} into its pathogenic isoform PrP^{Sc} [1]. PrP^{C} is detergent-soluble and sensitive to proteinase K (PK)-digestion, whereas PrP^{Sc} forms detergent-insoluble aggregates and is partially resistant to PK[2-6]. The conversion of PrP^{C} to PrP^{Sc} is known to involve a conformational transition of α-helical to β-sheet structures of the protein. However, the *in vivo* pathway is still poorly understood. A tentative endogenous PrP^{Sc}, intermediate PrP* or "silent prion", has yet to be identified in the uninfected brain[7].

Using a combination of biophysical and biochemical approaches, we identified insoluble PrP^{C} aggregates (designated $iPrP^{C}$) from uninfected mammalian brains and cultured neuronal cells[8, 9]. Here, we describe detailed procedures of these methods, including ultracentrifugation in detergent buffer, sucrose step gradient sedimentation, size exclusion chromatography, iPrP enrichment by gene 5 protein (g5p) that specifically bind to structurally altered PrP forms[10], and PK-treatment. The combination of these approaches isolates not only insoluble PrP^{Sc} and PrP^{C} aggregates but also soluble PrP^{C} oligomers from the normal human brain. Since the protocols described here have been used to isolate both PrP^{Sc} from infected brains and $iPrP^{C}$ from uninfected brains, they provide us with an opportunity to compare differences in physicochemical features, neurotoxicity, and infectivity between the two isoforms. Such a study will greatly improve our understanding of the infectious proteinaceous pathogens. The physiology and pathophysiology of $iPrP^{C}$ are unclear at present. Notably, in a newly-identified human prion disease termed variably protease-sensitive prionopathy, we found a new PrP^{Sc} that shares the immunoreactive behavior and fragmentation with $iPrP^{C}$ [11, 12]. Moreover, we recently demonstrated that $iPrP^{C}$ is the main species that interacts with amyloid-β protein in Alzheimer disease[13]. In the same study, these methods were used to isolate Abeta aggregates and oligomers in Alzheimer's disease[13], suggesting their application to non-prion protein aggregates involved in other neurodegenerative disorders.

Video Link

The video component of this article can be found at https://www.jove.com/video/3788/

Protocol

1. Preparation of Brain Homogenate and Detergent -Soluble (S2) and -Insoluble (P2) Fractions

1. Take 100 mg frozen human brain tissue and add 100 µl lysis buffer (10 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40), 0.5% EDTA, pH 7.4).
2. Homogenize it on ice using pestle driven by a cordless motor, freeze it in dry ice for 5 min and homogenize it again using pestle driven by hands first and then by motor, and add 300 µl or 800 µl lysis buffer (this is either the 20% or 10% total brain homogenate, respectively).
3. Centrifuge the 20% or 10% total brain homogenate at 1,000 x g for 10 min at 4 °C to collect supernatant (S1) using a benchtop centrifuge.
4. Ultracentrifuge 10% S1 at 35,000 rpm (100,000 x g) in an SW55 rotor (Beckman Coulter, Fullerton, CA) for 1 hr at 4 °C. Transfer the supernatants (S2) that contain the detergent-soluble fraction into a clean tube. Resuspend the pellets that contain detergent-insoluble fraction (P2) in 100 µl or 200 µl the lysis buffer to make 10- or 5-fold concentrated preparation.
5. For PK-digestion, incubate the sample with the same amounts of PK at 50 µg/ml at 37 °C for 1 hr for both S2 and P2 fractions. Add phenylmethylsulfonyl fluoride at the final concentration of 5 mM and equal volumes of SDS sample buffer (3% SDS, 2 mM EDTA, 4% β-mercaptoethanol, 10% glycerol, 50 mM Tris, pH 6.8) to terminate the PK digestion. Boil the samples for 10 min and cool them at the room temperature for 5 min. They are ready for Western blotting.

2. Velocity Sedimentation in Sucrose Step Gradients

1. Incubate 450 µl 20% S1 with an equal volume of 2% sarkosyl in H_2O for 30 min on ice.

2. Add 0.5 ml each of 60%, 30%, 25%, 20%, 15%, and 10% sucrose into a Beckman tube with a maximal 5 ml capacity.
3. Load 0.4 ml of S1 onto the top of 10-60% sucrose step gradients.
4. Centrifuge at 48,000 rpm (200,000 x g, SW55 rotor) for 1 hr, at 4 °C.
5. Collect 283 µl fraction each from top of the tube and obtain 12 fractions in total.
6. Take 20 µl of each fraction to a new Eppendorf tube, add 20 µl sample buffer, and boil for 10 min.
7. Cool samples for 4 min in hood, centrifuge at 1,000 x g for 1 min and vortex them. Load samples onto a pre-cast gel.

3. Size Exclusion Chromatography

1. Use Superdex 200 HR beads (Pharmacia, Uppsala, Sweden) in a 1 x 30 cm column to determine the oligomeric state of PrP molecules.
2. Seven molecular mass markers (Sigma, St. Louis, MI) including Dextran blue (2,000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa) are loaded independently at the concentrations recommended by Sigma in 200 µl sample volumes.
3. The elution volume of blue dextran is used to determine the void volume (V_0 = 8.45 ml) and the total volume (V_t = 24 ml) is provided by the product instruction. The peak elution volumes (V_e) are calculated from the chromatogram and fractional retentions. K_{av}, the partition coefficient (defining sample behavior), is calculated using the equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$. The calibration curve is determined by plotting the K_{av} of the protein standards against the log MW of the standards[8].
4. The molecular weight (MW) of the various PrP species recovered in different FPLC fractions is evaluated according to a calibration curve generated with the gel filtration of various standards.
5. Inject 200 µl S1 in lysis buffer containing 1% sarkosyl into the column for each run.
6. Chromatography is performed in an FPLC system (GE Healthcare) at a flow rate of 0.25 ml/min and fractions of 0.25 ml each are collected using a fraction collector (Amersham Biosciences, RediFrac).
7. Incubate 0.125 ml each fraction with 0.5 ml pre-chilled methanol at -20 °C for 2 hr.
8. Centrifuge samples at 13,000 x g for 30 min at 4 °C in a benchtop microcentrifuge. Discard the supernatant and resuspend the pellet in 20 µl SDS sample buffer as mentioned above, boiling for 10 min, cool to room temperature, load them onto a pre-cast gel.

4. Capture of PrP by g5p

1. The g5p molecule (100 µg) (kindly provided by Drs. Geoff Kneale and John McGeehan from the University of Portsmouth, UK) is conjugated to 7 x 10^8 tosyl activated magnetic beads by incubating 100 µg g5p and 350 µl g5p beads in 1 ml of phosphate-buffered saline (PBS) at 37 °C for 20 hr. Attract g5p-beads to the sidewall of Eppendorf tubes by external magnetic force and then remove all solution. Wash the beads with 1 ml of PBS containing 0.1 % bovine serum albumin (BSA) for three times.
2. Incubate the g5p-conjugated beads in 1 ml of 0.2 M Tris-HCl, pH 7.4 containing 0.1 % BSA at 37 °C for 5 hr to block non-specific binding and then wash the beads with 1 ml of PBS containing 0.1% BSA for three times as mentioned above. Remove the solution and add 1 ml of PBS into the beads. The prepared g5p beads are stable for at least 3 months at 4 °C.
3. The capture of PrP by g5p is performed by incubating 100 µl S1 fractions or P2 with 60 µl g5p conjugated beads (10 µg protein/6 X 10^7 beads) in 1 ml of binding buffer (3% Tween-20, 3% NP-40 in PBS, pH 7.5).
4. After incubation with constant rotation for 3 hr at room temperature, the PrP-containing g5p beads are attracted to the sidewall of Eppendorf tubes by external magnetic force, allowing for easy removal of all unbound molecules in the solution.
5. Following three washes in wash buffer (2% Tween-20 and 2% NP-40 in PBS, pH 7.5), g5p beads are collected and heated at 95 °C for 5 min in the SDS sample buffer (3% SDS, 2 mM EDTA, 10% glycerol, 50 mM Tris-HCl, pH 6.8).
6. After cooling samples for 5 min at room temperature, the samples are centrifuged at 1,000 x g for 5 min at room temperature. The supernatants are ready for Western blotting.

5. Western Blotting

1. Load samples boiled in the SDS sample buffer onto 15% Tris-HCl Criterion pre-cast gels (Bio-Rad) at 150 V for ~80 min.
2. The proteins on the gels are transferred to PVDF for 2 hr at 70V.
3. After blocking membranes in 5% milk in Tris-buffered saline containing 0.01% Tween-20 (TBS-T), the membranes are incubated for 2 hr at room temperature with primary monoclonal or polyclonal antibody 3F4 (1:40,000), 1E4 (1:1,000), anti-N[8] (1:6,000), or anti-C[8] (1:6,000) for probing the PrP molecule.
4. Following incubation with HRP-conjugated sheep anti-mouse IgG at 1:3000, or donkey anti-rabbit IgG at 1:3,000 the PrP bands are visualized on Kodak film using ECL Plus, in accordance with the manufacturer's protocol.

6. Representative Results

Compared to sporadic CJD samples, a small amount of $iPrP^C$ was detected in the P2 fraction in normal brains although most of PrP^C was recovered in the S2 fraction (**Figure 1**). As indicated previously[8], iPrP accounts for approximately 5-25% of total PrP including full-length and N-terminally truncated species.

Analyses using sucrose step gradient sedimentation revealed that while most of PrP^C from non-CJD brains was recovered in the top fractions 1-3, small amounts of PrP were also detected in the bottom fractions 9-11 that normally contain large aggregates[8] (**Figure 2**).

A variety of PrP^{Sc} species ranging from monomers, small oligomers to larger aggregates were isolated by gel filtration in the brain with Creutzfeldt-Jakob disease (**Figure 3A**). However, a small amount of larger aggregates with molecular weight greater than 2,000 kDa was also detected in insoluble fractions of normal brains (**Figure 3C**). Moreover, dimers and tetramers of PrP^C were not only detected in insoluble fractions but also in soluble fractions (**Figure 3B** and **3C**).

After PK and PNGase treatment, the PrP captured by g5p was detected with the 1E4 antibody against PrP97-105 [8]. Three PK-resistant core fragments termed PrP^{*20}, PrP^{*19}, and PrP^{*7} were detected, migrating at ~20 kDa, ~19 kDa and ~7 kDa, respectively (**Figure 4**, left panel). However, no PrP was detected when the 1E4 antibody was pre-incubated with a synthetic peptide that has a sequence identical to the 1E4 epitope (**Figure 4**, middle panel), indicating that bands detected by 1E4 are PrP fragments. Moreover, the anti-C antibody revealed two different PrP fragments migrating at ~18 kDa (PrP^{*18}) and ~12-13 kDa (PrP-CTF12/13), in addition to PrP^{*20} (**Figure 4**, right panel).

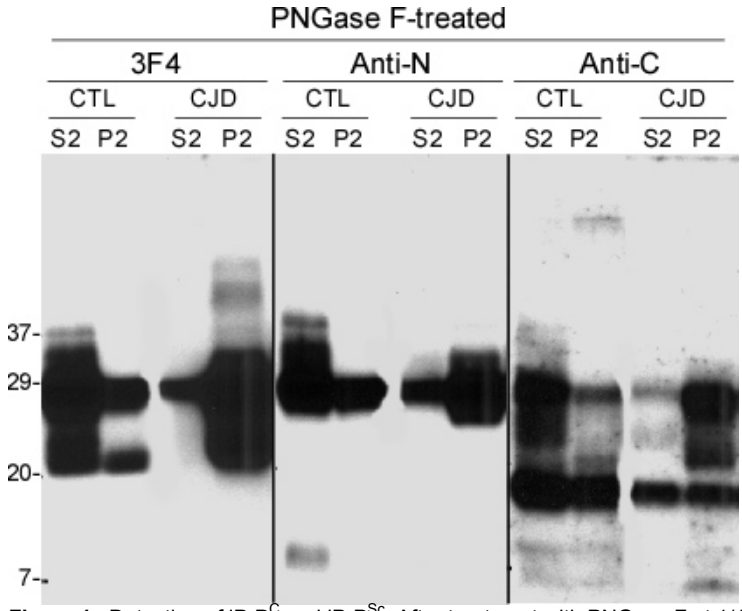

Figure 1. Detection of $iPrP^{C}$ and $iPrP^{Sc}$. After treatment with PNGase F at 1/10 of the total reaction volume at 37 °C for 1 hr to remove glycans from the protein, full-length or N-terminally truncated PrP species in the soluble and insoluble fractions (S2 and P2) isolated by ultracentrifugation in brain samples from normal control (CTL) and sporadic CJD (sCJD) were detected with 3F4 against PrP106-112 (left panel), anti-N against PrP23-40 (middle panel), and anti-C against PrP220-231 (right panel). In CTL samples, a small amount of PrP is detected in P2, whereas a large amount is present in S2. In contrast, more PrP is detected in P2 than in S2 in sCJD samples.

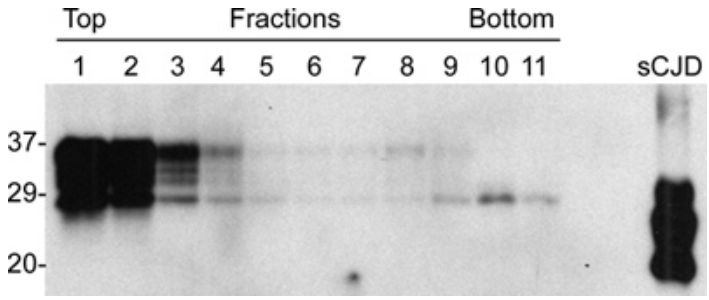

Figure 2. Sedimentation of PrP in sucrose step gradients. PrP in individual fractions from 1 to 11 of non-CJD brain sample S1 was detected by Western blotting with 3F4. Although most of PrP^{C} was detected in top fractions 1-3, small amounts of PrP were also observed in bottom fractions 9-11. Moreover, the banding pattern of PrP from top and bottom is different: PrP recovered in the top fractions has a dominant upper band while PrP recovered in the bottom fractions has a dominant lower band. A PK-treated PrP^{Sc} was loaded as a control on the right side of blot.

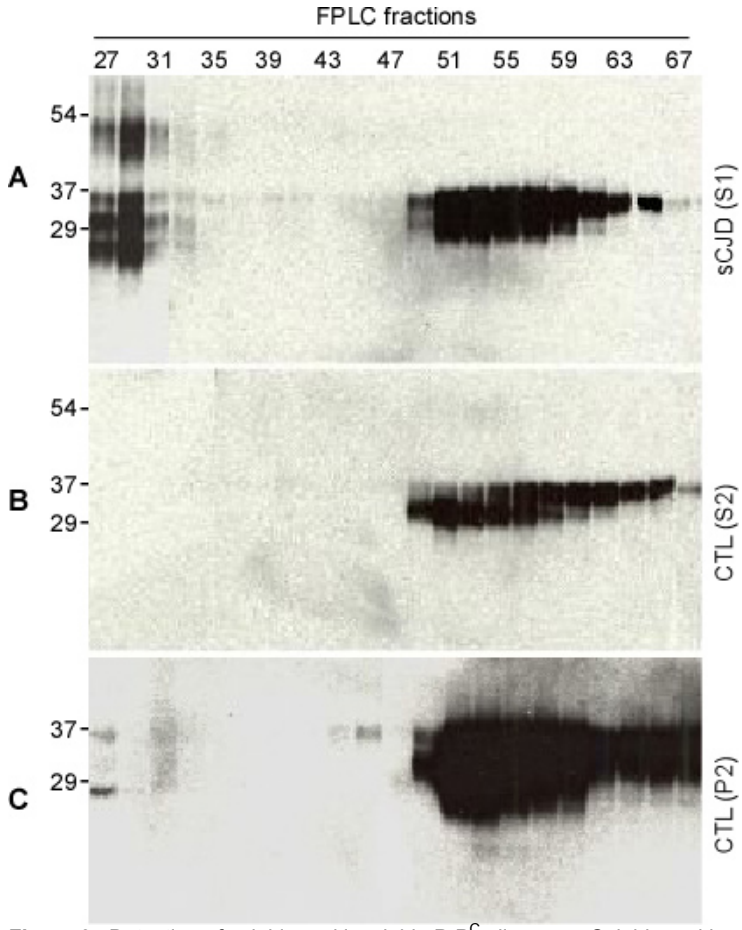

Figure 3. Detection of soluble and insoluble PrP^C oligomers. Soluble and insoluble PrP^C from normal human brains were separated by ultracentrifugation and then subjected to gel filtration, respectively. The molecular sizes of individual fractions were measured by running a group of molecular mass markers. (***A)*** PrP^{Sc} species from sCJD brain samples. Two populations of PrP species were detected: gel filtration fractions 49 - 65 contain monomers and small oligomers, whereas fractions 27-33 contain large aggregates. The PrP^C species from soluble fraction (S2) (***B***) and insoluble fraction (P2) (***C***) of normal controls were detected. PrP was probed with the 3F4 antibody. Dimers (fraction 55) and tetramers (fraction 51) of PrP were detected not only in P2 but also in S2 of normal brain samples (*B* and *C*). Large aggregates were only detected in P2 of normal samples (*C*).

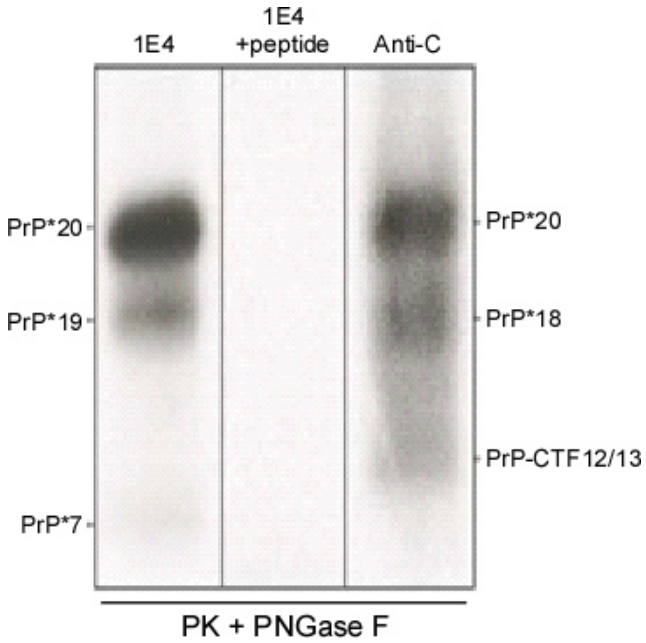

Figure 4. Detection of various PK-resistant iPrP fragments in g5p-enriched preparations from normal human brains. Samples enriched by g5p were treated with PK and PNGase F prior to Western blotting probing with 1E4 (left panel), 1E4 pre-incubated with a synthetic peptide that had a sequence identical to the 1E4 epitope (middle panel), and anti-C (right panel). 1E4 detected three PK-resistant PrP fragments termed PrP^{*20}, PrP^{*19}, and PrP^{*7} (left panel). After blocking of 1E4 with the peptide, no PrP^{res} were detected (middle panel), indicating that the bands detected with 1E4 are PrP fragments. Anti-C revealed two addition PK-resistant PrP fragments termed PrP^{*18} and PrP-CTF12/13, in addition to PrP^{*20}.

Discussion

The combination of approaches reported here isolates consistently insoluble PrP^{C} aggregates and soluble PrP^{C} oligomers from the normal human brain. Ultracentrifugation at 100,000 x g for one hour is a classic method that has been widely used for the separation of the insoluble PrP^{Sc} from the soluble PrP^{C} [14]. While it is efficient, one of the cautions is to avoid contamination during pulling the supernatant (S2) after centrifugation. Since the gel profile of $iPrP^{C}$ is distinct from that of PrP^{C}, it is unlikely that the PrP detected in the P2 fraction resulted from contamination of S2. The sucrose gradient sedimentation assay has been used to separate various PrP^{Sc} species based on their densities, sizes and shapes[15]. We have noticed that the fraction 12 often contains some particle that may make this fraction unaccountable. Fraction 10 often is the one that contains the greatest amounts of PrP aggregates among the fractions collected[8]. The molecular weights (MW) of various PrP^{C} conformers were further characterized using gel filtration (also called size exclusion chromatography). We first generated a calibration curve with seven molecular mass markers (data not shown) and then examined the MW of PrP from brains of normal controls and sCJD patients. We noted that a small amount of PrP aggregates may be precipitated along with cellular debris during the preparation of S1 fraction. To increase the recovery rate, brain tissues should be homogenized well and the incubation of brain homogenate with the sarkosyl solution should be extended to half an hour or an hour at 4 °C. Although there is no volume limitation for g5p capture of $iPrP^{C}$, we recommend using 60 to 80 µl of beads and 100 to 200 µl samples for each experiment.

Disclosures

No conflicts of interest declared.

Acknowledgements

The authors are grateful to the Human Brain and Spinal Fluid Resource Center (Los Angeles, CA) for providing normal brain samples. This study was supported by the National Institutes of Health (NIH) R01NS062787, the CJD Foundation, Alliance BioSecure, as well as the University Center on Aging and Health with the support of the McGregor Foundation and the President's Discretionary Fund (Case Western Reserve University).

References

1. Prusiner, S.B. Prions. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13363-13383 (1998).
2. Oesch, B., Westaway, D., Wälchli, M., McKinley, M.P., Kent, S.B.H., Aebersold, R., Barry, R.A., Tempst, P., Teplow, D.B., Hood, L.E., Prusiner, S.B., & Weissmann, C. A cellular gene encodes scrapie PrP 27-30 protein. *Cell.* **40**, 735-746 (1985).
3. Bolton, D.C., McKinley, M.P., & Prusiner, S.B. Identification of a protein that purifies with the scrapie prion. *Science.* **218**, 1309-1311 (1982).
4. Prusiner, S.B., McKinley, M.P., Bowman, K.A., Bolton, D.C., Bendheim, P.E., Groth, D.F., & Glenner, G.G. Scrapie prions aggregate to form amyloid-like birefringent rods. *Cell.* **35**, 349-358 (1983).

5. Hope, J., Morton, L.J., Farquhar, C.F., Multhaup, G., Beyreuther, K., & Kimberlin, R.H. The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). *EMBO J.* **5**, 2591-2597 (1986).
6. Prusiner, S.B., Groth, D.F., Bolton, D.C., Kent, S.B., & Hood, L.E. Purification and structural studies of a major scrapie prion protein. *Cell*. **38**, 127-134 (1984).
7. Hall, D. & Edskes, H. Silent prions lying in wait: a two-hit model of prion/amyloid formation and infection. *J. Mol. Biol.* **336**, 775-786 (2004).
8. Yuan, J., Xiao, X., McGeehan, J., Dong, Z., Cali, I., Fujioka, H., Kong, Q., Kneale, G., Gambetti, P., & Zou, W.Q. Insoluble aggregates and protease-resistant conformers of prion protein in uninfected human brains. *J. Biol. Chem.* **281**, 34848-34858 (2006).
9. Yuan, J., Dong, Z., Guo, J.P., McGeehan, J., Xiao, X., Wang, J., Cali, I., McGeer, P.L., Cashman, N.R., Bessen, R., Surewicz, W.K., Kneale, G., Petersen, R.B., Gambetti, P., & Zou, W.Q. Accessibility of a critical prion protein region involved in strain recognition and its implications for the early detection of prions. *Cell. Mol. Life Sci.* **65**, 631-643 (2008).
10. Zou, W.Q., Zheng, J., Gray, D.M., Gambetti, P., & Chen, S.G. Antibody to DNA detects scrapie but not normal prion protein. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 1380-1385 (2004).
11. Gambetti, P., Dong, Z., Yuan, J., Xiao, X., Zheng, M., Alshekhlee, A., Castellani, R., Cohen, M., Barria, M.A., Gonzalez-Romero, D., Belay, E.D., Schonberger, L.B., Marder, K., Harris, C., Burke, J.R., Montine, T., Wisniewski, T., Dickson, D.W., Soto, C., Hulette, C.M., Mastrianni, J.A., Kong, Q., & Zou, W.Q. A novel human disease with abnormal prion protein sensitive to protease. *Ann. Neurol.* **63**, 697-708 (2008).
12. Zou, W.Q., Puoti, G., Xiao, X., Yuan, J., Qing, L., Cali, I., Shimoji, M., Langeveld, J.P., Castellani, R., Notari, S., Crain, B., Schmidt, R.E., Geschwind, M., Dearmond, S.J., Cairns, N.J., Dickson, D., Honig, L., Torres, J.M., Mastrianni, J., Capellari, S., Giaccone, G., Belay, E.D., Schonberger, L.B., Cohen, M., Perry, G., Kong, Q., Parchi, P., Tagliavini, F., & Gambetti, P. Variably protease-sensitive prionopathy: A new sporadic disease of the prion protein. *Ann. Neurol.* **68**, 162-172 (2010).
13. Zou, W.Q., Xiao, X., Yuan, J., Puoti, G., Fujioka, H., Wang, X., Richardson, S., Zhou, X., Zou, R., Li, S., Zhu, X., McGeer, P.L., McGeehan, J., Kneale, G., Rincon-Limas, D.E., Fernandez-Funez, P., Lee, H.G., Smith, M.A., Petersen, R.B., & Guo, J.P. Amyloid-beta42 interacts mainly with insoluble prion protein in the Alzheimer brain. *J. Biol. Chem.* **286**, 15095-15105 (2011).
14. Zou, W.Q., Colucci, M., Gambetti, P., & Chen, S.G. Characterization of prion proteins. In: Methods in Molecular Biology-Neurogenetics: Methods and Protocols., Potter, N.T., ed., Humana Press Inc., Totowa, 305-314 (2002).
15. Tzaban, S., Friedlander, G., Schonberger, O., Horonchik, L., Yedidia, Y., Shaked, G., Gabizon, R., & Taraboulos, A. Protease-sensitive scrapie prion protein in aggregates of heterogeneous sizes. *Biochemistry*. **41**, 12868-12875 (2002).

