# Supporting Information

## Karapetyan et al. 10.1073/pnas.1303510110

### SI Materials and Methods

**Cell Culture.** LD9 cells (a subclone of L929 fibroblasts) (1) and neuroblastoma cells (N2a or PK1; ref. 2) were cultured in 15-cm tissue culture dishes in OptiMEM medium supplemented with 5% (vol/vol) fetal bovine serum and 1% (vol/vol) Pen/Strep (Invitrogen). They were trypsinized and split 1:10 every 3–4 d.

**Primary Assay: Prion Protein–FRET-Enabled High Throughput Assay.** LD9 cells were plated in the wells of 96- or low-volume 384-well microtiter plates at the desired density. The 384-well format prion protein–FRET-enabled high throughput assay (PrP-FEHTA): One hundred nanoliters of compound (US Drug Collection, MicroSource Discovery Systems) at 20 μM (final, a classical drug screening concentration) or brefeldine A (pharmacological control, Epicentre Biotechnologies) at 6 μg/mL (final) in 0.6% DMSO were added to 384-well plates. Cells were cultured for 24 h at 37 °C, a time period long enough to reveal a compound's effect, given the PrP half-life of approximately 3–6 h. One microliter of PrP antibodies at twice the concentration optimal for the 96-well format, namely 0.33 μg/mL (D18-d2) and 0.036 μg/mL (SAF32-Tb), respectively, were added, and the cells were kept at room temperature for 3 h before readout. The SAF32 antibody directed against the N-terminal octapeptide repeat region of PrP was purchased from Cayman Chemical. The D18 antibody directed against epitope 133–157 was produced in-house from a hybridoma (a generous gift of D. Burton and A. Williamson, The Scripps Research Institute, La Jolla, CA). Antibodies were labeled with the donor (Terbium) and acceptor fluorophore (d2) by Cisbio for time-resolved FRET (TR-FRET). Fifty-microliter aliquots of labeled antibodies were stored frozen at −80 °C.

The Hpl3-4 $PrP^{0/0}$ neuronal cell line (3) was used as negative control for assay development and optimization.

**TR-FRET Data Analysis.** Readings were performed at 665 and 620 nm with an Envision plate reader (PerkinElmer). Ratios ($R$) of the 665 nm to the 620 nm measurements were calculated, to take into account nonspecific absorption of 620-nm light by the assay matrix. The value for the specific signal of the sample or, in this example, the positive control is given by $\triangle F\% = [(R_{C+} - R_{C-})/R_{C-}] \times 100$, where $R_{C+}$ and $R_{C-}$ are the 665/620 ratios of the positive and negative controls.

**Assay Performance Analysis.** We used the Z′ factor to assess the quality of the assay throughout development (4). The Z′ factor integrates the assay signal dynamic range (difference between the mean of the positive controls and the mean of the negative controls) and the statistical variability of the signals. The higher the Z′ value, the greater is the assay robustness, with values equal to or higher than 0.5 indicating an excellent assay. In our assay the equation is as follows: $Z' = 1 - [3 \times (SD_{C+} + SD_{C-})/(Mean_{C+} - Mean_{C-})]$. $SD_{C+}$ = SD of the positive control (maximum signal, LD9 cells or LD9 cells + compound solvant); $SD_{C-}$ = SD of the negative control (minimum signal, $PrP^{0/0}$ cells or LD9 cells + pharmacological control); $Mean_{C+}$ = mean $\triangle F\%$ of the positive control; $Mean_{C-}$ = mean $\triangle F\%$ of the negative control.

**Cell Viability.** Cell viability was measured by using the luminescent CellTiter-Glo assay according to the manufacturer's instructions (Promega). Briefly, the addition of the CellTiter-Glo reagent results in cell lysis and generation of a luminescent signal at 610 nm proportional to the amount of ATP, reflecting the number of living cells in culture.

**Orthogonal Assays: Immunocytochemistry and High-Content Analysis.** PrP was labeled at the surface of living neuroblastoma cells (N2a or PK1) at +4 °C with mAb D18 and fixed with 4% PFA before the addition of the Alexa-488 labeled secondary antibody. Cells were examined on a Nikon Eclipse TE 2000-U inverted microscope with bright field and epifluorescence. High-content quantification of PrP immunolabeling was performed by IN Cell Analyzer 1000 with Developer software.

**Biotinylation of Cell Surface Proteins.** Cells were rinsed three times in cold PBS. Cells were surface-biotinylated by using the EZ-Link Sulfo-NHS-LC-LC-Biotin reagent (Pierce) at +4 °C for 30 min. Cells were rinsed three times with 0.1 M glycine/PBS solution, once in PBS, and lysed. Biotinylated cell surface proteins were pulled down on streptavidin-coated beads. Both cell surface PrP (biotinylated fraction) and intracellular PrP (unbiotinylated fraction) were analyzed by Western blot. Gel loading was standardized by cell number.

**Western Blot Analysis.** Cells were lysed in lysis buffer (50 mM Tris·HCl at pH 8, 0.5% Na deoxycholate, 0.5% Triton X-100, and Roche Protease inhibitor mixture) and centrifuged at 13,400 × $g$ for 10 min. Postnuclear cell lysates were normalized for cell number and fractionated by SDS-polyacrylamide gel electrophoresis, electroblotted onto a PVDF membrane (Millipore), and probed with either D18 antibody at 0.03 μg/mL, LC3 antibody (NB100-2220, Novus Biologicals) at 2 μg/mL, or GAPDH antibody (Epitomics) at 1:1,000, followed by a murine HRP-conjugated anti human IgG antibody at a 1:15,000 dilution (Southern Biotech). Immunoreactive bands were visualized by incubation with West Pico (Pierce) and imaged by using a BioSpectrum Imaging System (UVP).

**RT-PCR Analysis.** Messenger RNAs were extracted by using the *mir*Vana Kit (Ambion) and reverse-transcribed by using random hexamers (Applied Biosystems). PrP and GAPDH cDNAs were amplified and detected by using 6-fluorescein amidite (FAM)-labeled probe/primer sets from TaqMan PrP and GAPDH gene expression assays (Applied Biosystems) and quantified on the Applied Biosystems ABI Prism 7900HT. PrP mRNAs were normalized against GAPDH mRNAs.

**Cell Treatments.** PK1 neuroblastoma cells were pretreated for 3 d with the indicated doses of drugs and infected with Rocky Mountain Laboratories (RML) or 22L prions ($10^{-4}$ final dilution of an RML- or 22L-infected mouse brain homogenate). Cells were split every 3 d. Treatment was continued for 12 d after infection (d p.i.). Cells were analyzed for proteinase K-resistant PrP by Western blot 9 and 18 d p.i. (i.e., for the latter 6 d after treatment cessation). PPS (pentosan polysulfate, 16 μg/mL) was used as positive control for treatment efficacy.

**Animal Treatments.** Eight-week-old female C57BL/6 mice were intracerebrally inoculated with 20 μL of 1% RML-infected brain homogenate. Mice were injected intraperitoneally daily from 20 to 50 d.p.i. with 100 μL of drug in 50% DMSO (astemizole, 3 mg/kg; tacrolimus, 1.5 mg/kg) ($n$ = 7). Control mice were injected with vehicle solution alone ($n$ = 5). Mice were monitored for the occurrence of clinical signs (ataxic gait, reduced grooming,

plastic tail, kyphosis, and weight loss) and euthanized at terminal stage of prion disease.

The research was approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute (TSRI), Scripps Florida. TSRI, Scripps Florida maintains a centralized animal care and use program registered by the US Department of Agriculture, assured with the Office of Laboratory Animal Welfare and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Housing and care of animals is consistent with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, The Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and other applicable state and local regulations.

**Statistical Analysis.** Kaplan–Meier survival curves were established by using individual survival data with Prism v5 software. Statistical significance was calculated by using the log-rank test.


1. Mahal SP, et al. (2007) Prion strain discrimination in cell culture: The cell panel assay. *Proc Natl Acad Sci USA* 104(52):20908–20913.
2. Klöhn PC, Stoltze L, Flechsig E, Enari M, Weissmann C (2003) A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions. *Proc Natl Acad Sci USA* 100(20):11666–11671.
3. Sakudo A, Onodera T, Ikuta K (2007) Prion protein gene-deficient cell lines: Powerful tools for prion biology. *Microbiol Immunol* 51(1):1–13.
4. Zhang JH, Chung TD, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 4(2): 67–73.


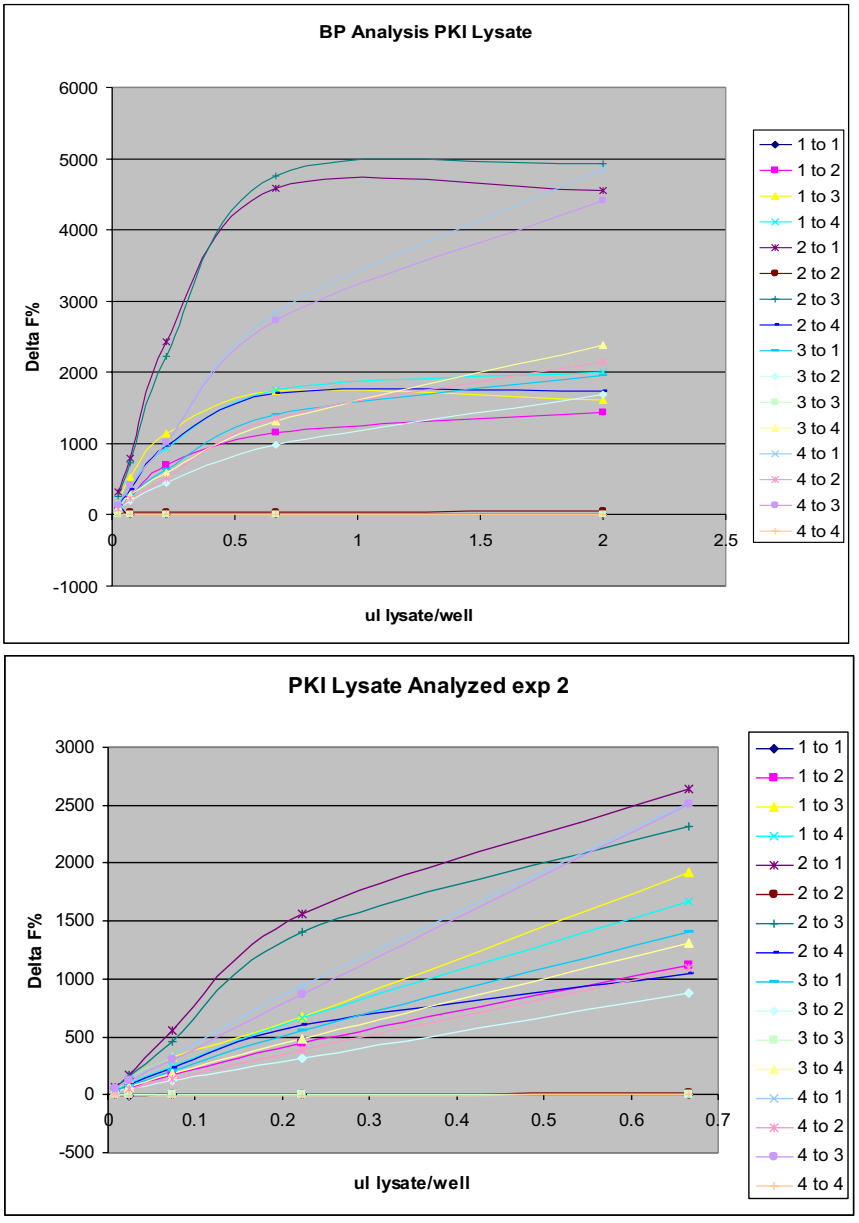


**Fig. S1.** Best pair analysis. Cellular lysates from neuroblastoma PK1 cells were used to determine which PrP antibody pair provides the highest specific FRET signal. Two experiments were performed (*Upper* and *Lower*, respectively). Cell lysates contained 18.6 μg/mL protein. Antibody key: 1, POM19; 2, SAF32; 3, D18; 4, D13. Pairs of antibodies are referred as: Tb labeled (donor fluorophore; left number) to d2 labeled (acceptor fluorophore; right number). Experiments were performed by Cisbio.

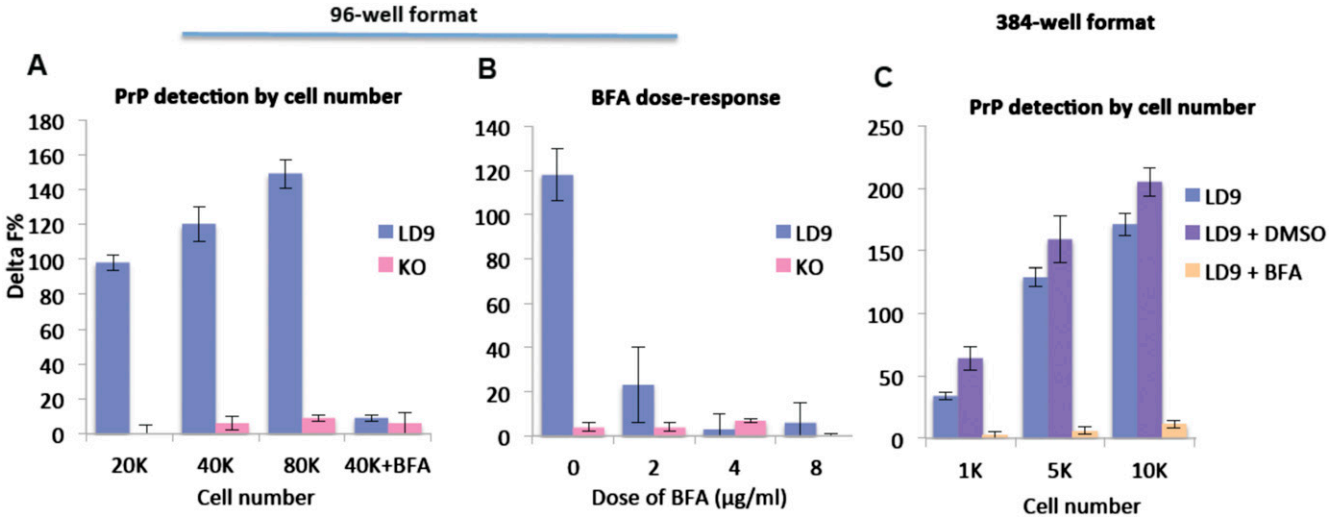


**Fig. S2.** PrP-FEHTA optimization. (*A* and *B*) Optimization of LD9 cell density and pharmacological control brefeldine A (BFA) in the 96-well format. (*A*) Z′ values (defined in *SI Materials and Methods*) were 0.7 for 20,000, 0.6 for 40,000, and 0.8 for 80,000 LD9 cells (LD9 cells providing the maximal, PrP KO cells the minimal signal intensity). Z′ was 0.7 when 40,000 LD9 cells provided the maximal signal and 40,000 BFA-treated LD9 cells the minimal signal (4 μg/mL BFA). (*B*) BFA (4 and 8 μg/mL) reduced the PrP signal to background. Triplicates are shown. (*C*) Optimization of cell density and DMSO tolerance in the 384-well format. Z′ values were calculated for LD9+DMSO versus LD9+BFA and were equal to 0.4 (1K), 0.6 (5K), and 0.8 (10K). Each point was done in triplicate.