Supplementary Methods

Plasmid Constructions

Expression plasmids in the pCDNA vector (Invitrogen) encoding wild-type hamster PrP, Opn-PrP, Prl-PrP, and Ang-PrP have been described (Kim et al., 2002; Rutkowski et al., 2001). The Opn, Prl, and Ang signals were from rat, bovine, and human sequences, respectively. Fusions to the PrP mature domain were blunt-ended and did not introduce any linker codons. The expression plasmid for TF (a heterologous Gal4-NFkB transcription factor) driven by a CMV promoter (plasmid pBD-NFkB) and the luciferase reporter plasmid preceded by the Gal4 DNA-binding element (plasmid pLuc) were obtained from Stratagene. To prepare the signal-TF constructs, the TF coding region was first amplified by PCR from plasmid pBD-NFkB and subcloned in frame into a calreticulin expression construct at the unique Not1 site just preceding the C-terminal KDEL ER retention sequence. Using this plasmid as a template, the region coding for TF, together with the KDEL sequence, was then PCR amplified and subcloned into a blunted Pst1 site and a downstream Xba1 site of cassettes containing different signal sequences. Such signal sequence cassettes have been described previously (Kim et al., 2002). Again, fusions between the signal and mature domains were blunt-ended and did not introduce any linker codons. The coding regions of PrP(G123P), PrP(AV3), and PrP(A117V) have been described (Hegde et al., 1998). They were subcloned by standard methods into the pCDNA mammalian expression vector for use in this study. Point mutations to monomerize the CFP and YFP expression plasmids (obtained from Clonetech) were introduced as described previously (Snapp et al., 2003) by site-directed mutagenesis using the Quickchange method (Stratagene). PCR-amplified products of these monomerized versions of CFP or YFP (mCFP and mYFP,

respectively) were inserted into the Bsu36I site of PrP between residues 51 and 52 to generate the tagged versions of PrP and Opn-PrP.

TF sequestration assay

MDCK cells were cultured in DMEM containing 10% fetal bovine serum. Between 5000 and 10000 cells were plated per well of a 96-well plate between 24 and 36 hours prior to transfection, at which time they were ~40-80% confluent. Transfections with Lipofectamine 2000 (Invitrogen) were performed in 96-well plates with a total of 200 ng plasmid DNA per well. Of this 200 ng, the luciferase reporter plasmid (pLuc) was always kept constant at 160 ng. The remaining 40 ng constituted a mixture of varying amounts of the TF-containing plasmid of interest and a EYFP expression plasmid. In this manner, TF expression could be changed systematically without altering other parameters of the experiment, while EYFP expression allowed routine monitoring of transfection efficiency (which remained constant at approximately 70%). In independent experiments, we confirmed that the use of less plasmid for one of the components (while keeping the total amount of DNA constant) results in lower expression of only that component in individual cells. The cells were analyzed 18-24 hours after transfection. For the luciferase assays, the media was aspirated, the cells recovered in 100 ul per well of freshly prepared luciferase reporter assay substrate (Roche), and measured in a tube luminometer. For parallel western blot analysis, the cells in replicate samples were harvested in 40 ul of 2x SDS-PAGE sample buffer, of which 10 ul was analyzed per lane on 12% Tris-tricine mini-gels.

Biochemical analyses

Pulse-chase studies. All experiments analyzing PrP constructs were performed in N2a cells cultured in DMEM containing 10% fetal bovine serum. After transfection with the appropriate plasmids (using Lipofectamine 2000), cells were analyzed between 18 and 24 hours later. Pulse-chase experiments were performed in 6-well dishes on cells pre-incubated for 30 min in media lacking methionine and cysteine. Pulse labeling was for 15 min with methionine/cysteine-free media containing 150 uCi/ml ^{35}S-Translabel (ICN); chase was carried out with unlabeled complete media for 1 h. Proteasome inhibition (5 uM MG132) was initiated 2 h prior to pulse labeling, and continued throughout the pulse and chase incubations. At the time of harvesting, cells in each well were rinsed in PBS, solubilized in 100 ul of 1% SDS, 0.1M Tris, pH 8, denatured by boiling, diluted 10 fold in IP buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 100 mM NaCl), and immunoprecipitated with the 3F4 anti-PrP monoclonal antibody as described (Kim et al., 2002). Gels of the immunoprecipitates were always stained with Coomassie blue to verify equal recovery of the antibody complexes and equal loading prior to their drying and autoradiography.

PrP aggregation assay. Analysis of PrP solubility and aggregation was performed with minor modifications to previously published procedures (Yedidia et al., 2001; Ma and Lindquist, 2001; Ma and Lindquist, 2002). Cells were cultured and transfected in 12-well dishes, and between 18-24 hours after transfection, treated with various manipulations as described in the individual figure legends. At the time of harvesting, cells in each well were washed once in PBS, scraped into 500 ul of ice cold solubilization buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate), dispersed by repeated pipetting, and centrifuged for 10

min at 13,000 x g at 4°C. Proteins in the pellet were dissolved in 50 ul of 1% SDS, 0.1M Tris, pH 8. Proteins in the supernatant were precipitated on ice with trichloroacetic acid (added to 15% w/v), collected by centrifugation at 4° C for 2 min at maximum speed in a microcentrifuge, washed once in ice cold acetone, air dried, and dissolved in 50 ul of 1% SDS, 0.1M Tris, pH 8. Equal aliquots of each fraction (usually 7.5 ul) were analyzed by SDS-PAGE and immunoblotting using 12% Tris-tricine minigels. All blots were stained for total protein with Ponceau S and confirmed to have equal loading in the lanes (as shown in Fig. 2B). The treatment conditions for proteasome inhibition in each experiment are provided in the respective figure legends. The assay for 'self-propagation' of cytosolic PrP aggregates was performed as previously reported (Ma and Lindquist, 2002) and described in the legend to Fig. 3. Briefly, transfected cells were incubated for 2 hours in media containing 5 uM MG132 (a reversible proteasome inhibitor). After washing the cells in PBS, they were returned to normal media lacking MG132 and cultured for an additional 21 hours. Parallel cultures of cells were harvested after the initial MG132 treatment as well as after 'chase' in the absence of MG132, and processed for analysis of PrP solubility and aggregation as described above.

Assay for ^{Ctm}PrP in N2a cells. Detection of ^{Ctm}PrP was performed on microsomes isolated from transfected cells in a 10 cm dish. Cells were first washed in PBS, scraped into ice cold hypotonic buffer (10 mM Tris, pH 7.4), disrupted by ~10 passes through a 25 gauge needle, and adjusted to 100 mM KAc, 2 mM $MgCl_2$. Debris, unbroken cells, and nuclei were removed by centrifugation at 1,000 x g for 10 min at 4°C in a microcentrifuge. The supernatant was transferred to 1.3 ml ultracentrifuge micro-test tubes (Beckman) and centrifuged at 70,000 rpm in a TL100.3 rotor at 4° C to sediment the membranes. This crude membrane fraction was resuspended in 50 ul of 50

mM Hepes, pH 7.4, 100 mM KAc, 2 mM MgAc2, and 250 mM sucrose. Detection of ^{Ctm}PrP in these microsomes utilized the limited protease digestion assay described in Hegde et al. (1998). An aliquot of untreated microsomes were reserved for comparison. Samples after protease digestion were deglycosylated with PNGase F (New England Biolabs) prior to analysis by immunoblotting. In this assay, ^{Ctm}PrP is detected by its selective resistance to digestion under 'mild' conditions (1 h at 4°C with 0.25 mg/ml proteinase K and 1% Triton X-100) to generate a diagnostic 18 kD fragment (after removal of carbohydrates with PNGase). This fragment is lost upon digestion with 'harsh' conditions (1 h at 37°C with 0.1 mg/ml proteinase K, 0.5% Triton X-100, 0.5 % deoxycholate).

Imaging and quantitative analyses

Fluorescence microscopy of N2a cells utilized an LSM510 confocal microscopy system (Zeiss) equipped with a UV laser (for CFP excitation with the 413 nm line), a Kr-Ar laser (for GFP or YFP excitation with the 488 nm and 514 nm lines, respectively), and two He-Ne lasers (for Cy3 and Cy5 excitation with the 543 and 633 lines, respectively). A 40x or 63x 1.4 NA oil immersion objective was used for all imaging. For quantitative analyses and comparisons between multiple samples, images were collected using identical excitation and detection settings. The detector gain settings were chosen to allow imaging of the desired cells within the linear range of the photomultiplier tube without saturating pixels. For quantification of localization (Fig. 4C), randomly chosen fields of cells (as in Fig. 4B) were imaged at two detector gain settings to visualize both dim and bright cells. Total fluorescence (average brightness x number of pixels) was used to derive relative expression levels (Snapp et al., 2003). A correction factor (derived in independent measurements based on the change in detector gain settings) was used to allow

plotting of both bright and dim cells from the two imaging settings on the same graph. Percent surface localization was defined as the fraction of total fluorescence found in the outer rim of each cell. Although this systematically underestimates surface localization (because the top and bottom surfaces of cells are not included), it nonetheless allows relative comparisons to be made. We determined the degree of underestimation to be roughly two-thirds the actual value based on independent experiments using protease digestion of cell surface PrP (which showed ~90% surface localization, compared to ~60-70% estimated by fluorescence). For immunofluorescence and co-localization studies, cells were grown on glass coverslips. At the time of analysis, they were fixed for 10 min at room temperature with 3.7% formaldehyde in PBS, rinsed twice with PBS, and permeabilized for 5 min with 0.5% Triton X-100 in PBS. After removing the permeabilization buffer, cells were incubated for 1 h at room temperature in blocking buffer (PBS containing 10% fetal bovine serum, 0.1% saponin, and 50 ug/ml RNAse A). Incubation with primary antibodies (mouse anti-EEA1 and rabbit anti-beta-COP) diluted into blocking buffer was for 1 hour at room temperature. After washing, cells were incubated for 30 min with Cy5- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch), washed with several changes of PBS, and mounted in Fluoromount (Molecular Probes) prior to imaging.

Cytotoxicity analysis

For Annexin V analysis, cells were co-transfected with GFP and the appropriate PrP plasmids. 24 h after transfection, cells were either left untreated or treated with 5 uM MG132 for 4 h. After rinsing in Annexin buffer, staining was for 15 min with 5 ug/ml Cy5-Annexin V (Molecular Probes) before washing and fixation with 3.7% formaldehyde in PBS. The cells were either mounted and viewed directly, or in some experiments, counterstained with 3F4 anti-PrP and a

Cy3-anti-mouse secondary for quantifying PrP expression levels to confirm equal levels of expression among samples. Random fields of transfected cells (identified by GFP expression) were chosen without visualization of Cy5-Annexin, and images collected in both the GFP and Cy5 channels (and if appropriate, the Cy3 channel to visualize PrP expression). Ten to 20 fields were quantified automatically by a macro written for NIH image to count positive pixels above background in each channel. Uniform transfection efficiency was ensured by identical GFP expression levels among samples. Comparable PrP expression levels were measured by both imaging and in some experiments, immunoblots of parallel samples. The amount of Annexin staining from three independent experiments was averaged to generate the graph in Fig. 8B.