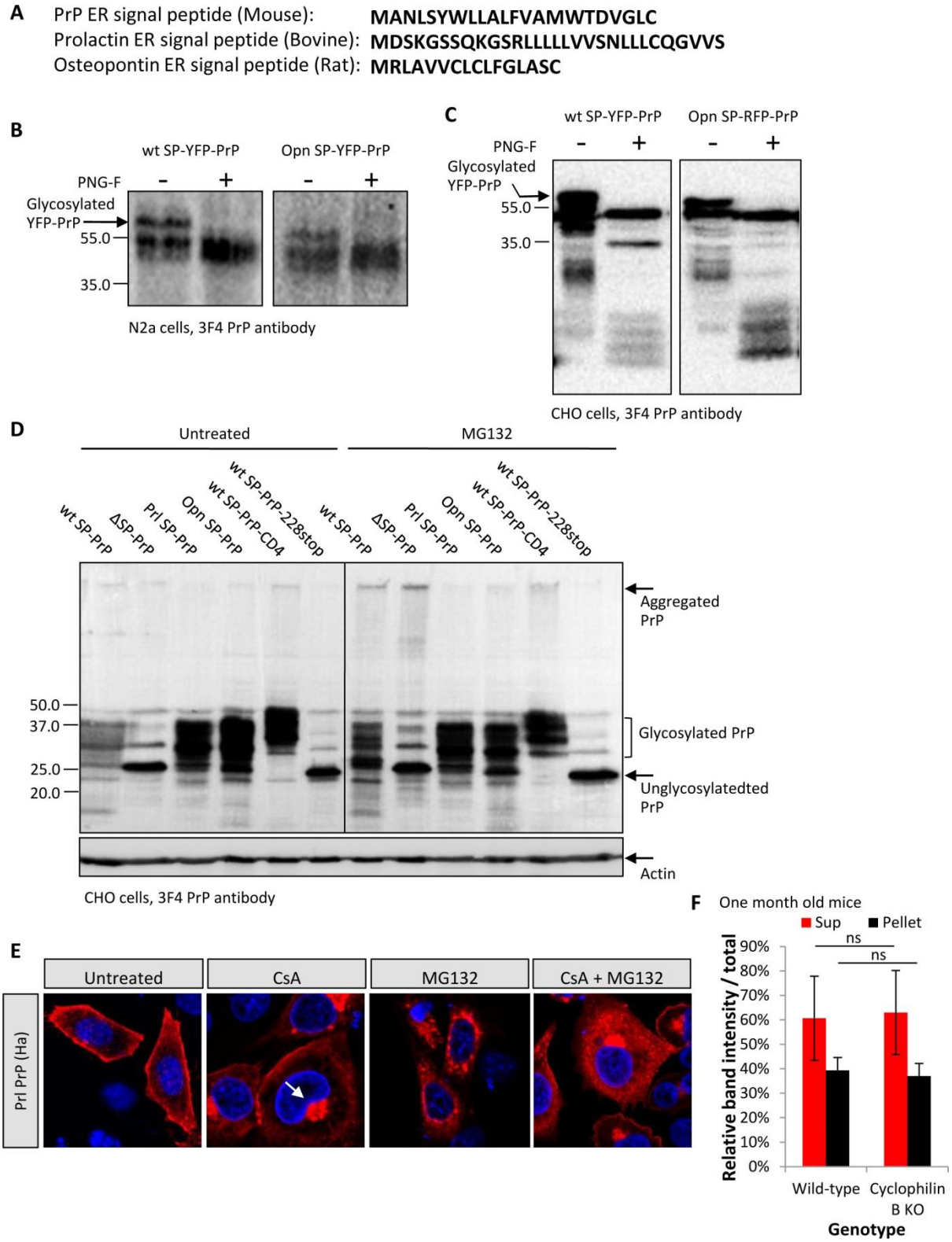
PrP-containing Aggresomes are Cytosolic Components of an Endoplasmic Reticulum Quality Control Mechanism

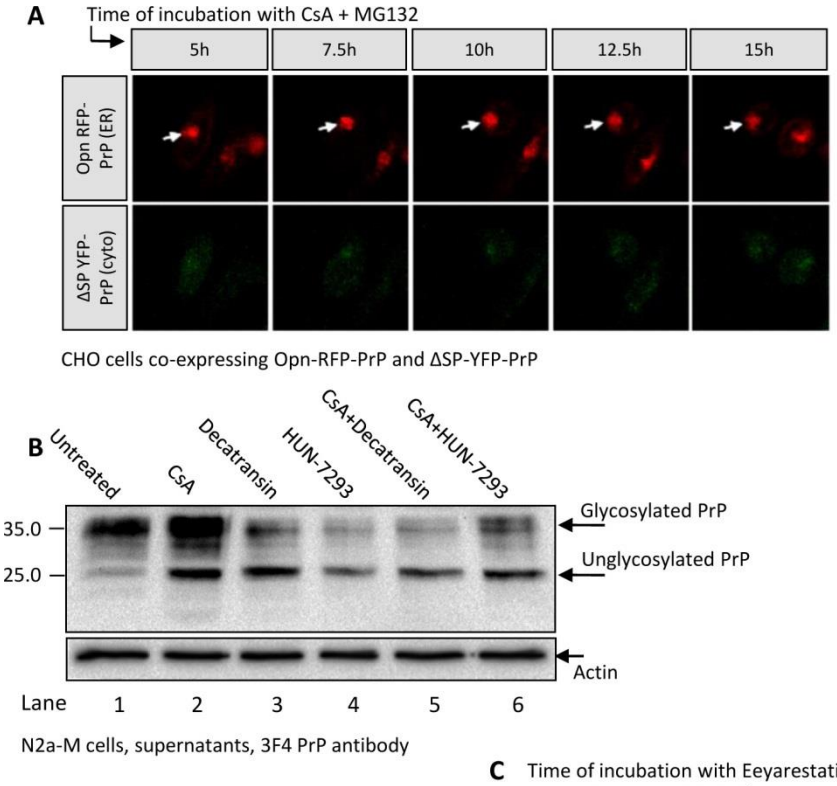
Tatyana Dubnikov et al.,

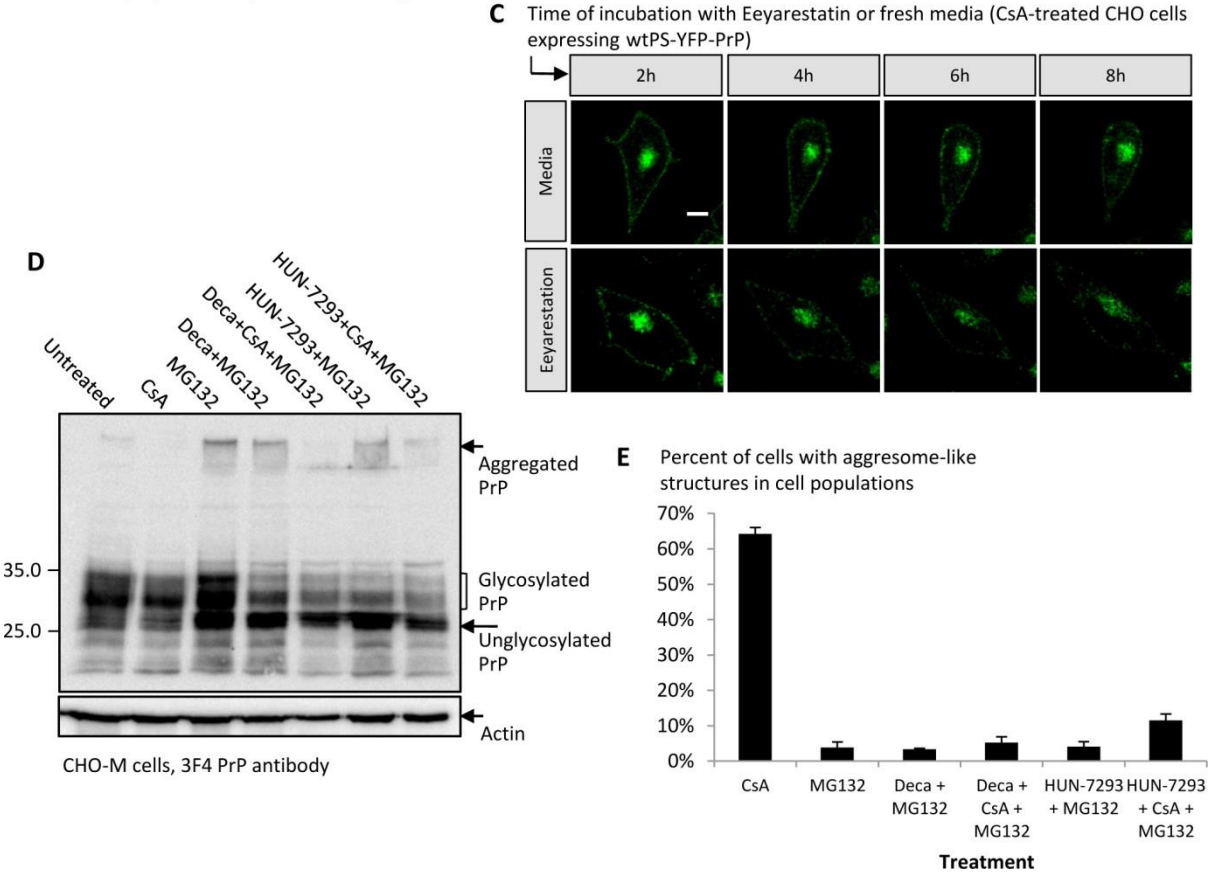


Journal of Cell Science • Supplementary information

Figure S1: A. Amino acid sequences of ER localization signal of PrP (mouse), Prolactin (Bovine) and Osteopontin (Rat). **B.** N2a cells expressing either wt SP-YFP-PrP or Opn SP-YFP-PrP were homogenized. Half of each homogenate was treated with PNGase F while the other half was left untreated. The samples were subjected to WB analysis using the 3F4 PrP antibody. PNGase F removed glycans from both PrP molecules (arrow) indicating that they entered the ER. **C.** CHO cells stably expressing either Opn SP-RFP-PrP or wt SP-YFP-PrP were homogenized, treated with PNGase F or left untreated and blotted as described in B. The removal of glycans by PNGase F indicates that these fluorescently-tagged PrP constructs have also entered the ER. **D.** The indicated PrP constructs were stably expressed in CHO cells (none of the constructs in this experiment was fluorescently tagged). Cells expressing each construct were either treated with MG132 (10μM, 6 hours) or left untreated and PrP was blotted by the 3F4 antibody. High-molecular weight PrP aggregates (arrow) accumulated in MG132-treated cells that express PrP which bears its natural ER localization signal or lack such signal but much less in cells expressing the PrP which carries the Opn or Prl signal peptides. **E.** PrP bearing the Prl ER localizations signal (not fluorescently tagged) accumulates in aggresomes of CsA-treated CHO cells (arrow) but not as a result of proteasome inhibition by MG132. **F.** Four brains of young (one month of age) CypB KO mice and four brains of their wild-type siblings were harvested, subjected to high-speed sedimentation and PrP was blotted using a PrP antibody. Band intensities in supernatants and pellets were measured by the ImageJ software. No difference in PrP sedimentation rates was seen among brains of young CypB KO and control mice.

Journal of Cell Science • Supplementary information





Journal of Cell Science • Supplementary information

Figure S2: A. Live visualization of CsA-treated CHO cells expressing Opn SP-RFP-PrP (transiently) and the ΔSP-YFP-PrP (stably) that were treated with both CsA and the proteasome inhibitor MG132, shows that cytosolic PrP (yellow) molecules do not accumulate in pre-existing aggresomes (red) when proteasome activity was inhibited. **B.** N2a-M cells were either left untreated or exposed to CsA, the Sec61p inhibitors Decatransin or HUN-7293 or to the combination of CsA and either one of the sec61p inhibitors. The cells were homogenized, subjected to high-speed sedimentation by ultra-centrifugation and PrP was blotted using the monoclonal antibody 3F4. While the majority of PrP molecules in untreated and in CsA-treated cells were glycosylated, only small minorities of the molecules that were present in cells which were exposed to Decatransin or HUN-7293 carried glycans, indicating that both Sec61p inhibitors efficiently inhibit the entry of PrP into the ER. **C.** Exposing CsA-treated CHO cells that express wt SP-YFP-PrP to the p97 inhibitor Eeyarestatin leads to the disintegration of pre-existing aggresomes. **D.** CHO-M cells were treated for 16 hours with the indicated combinations of CsA (60µg/ml), MG132 (10µM), Decatransin (1µM) or HUN-7293 (1µM) and subjected to WB analysis. While proteasome inhibition with MG132 leads to the accumulation of glycosylated, unglycosylated and aggregated PrP molecules in the cells, combined treatments with MG132 and either one of the Sec61p inhibitors (Decatransin and HUN-7293) foremost results in the accumulation of unglycosylated PrP. This shows that both Sec61p inhibitors efficiently block the entry of PrP into the ER of CHO-M cells. **E.** CHO cells expressing the wt SP-YFP-PrP were treated with the indicated combinations of compounds, visualized by a confocal microscope and aggresomes were counted. While more than 60% of the CsA-treated cells contained aggresomes, less than10% of the cells that were treated with CsA and Sec61p inhibitor (Decatransin or HUN-7293) contained such structures regardless whether their proteasomes were inhibited by MG132 or not.

Journal of Cell Science • Supplementary information

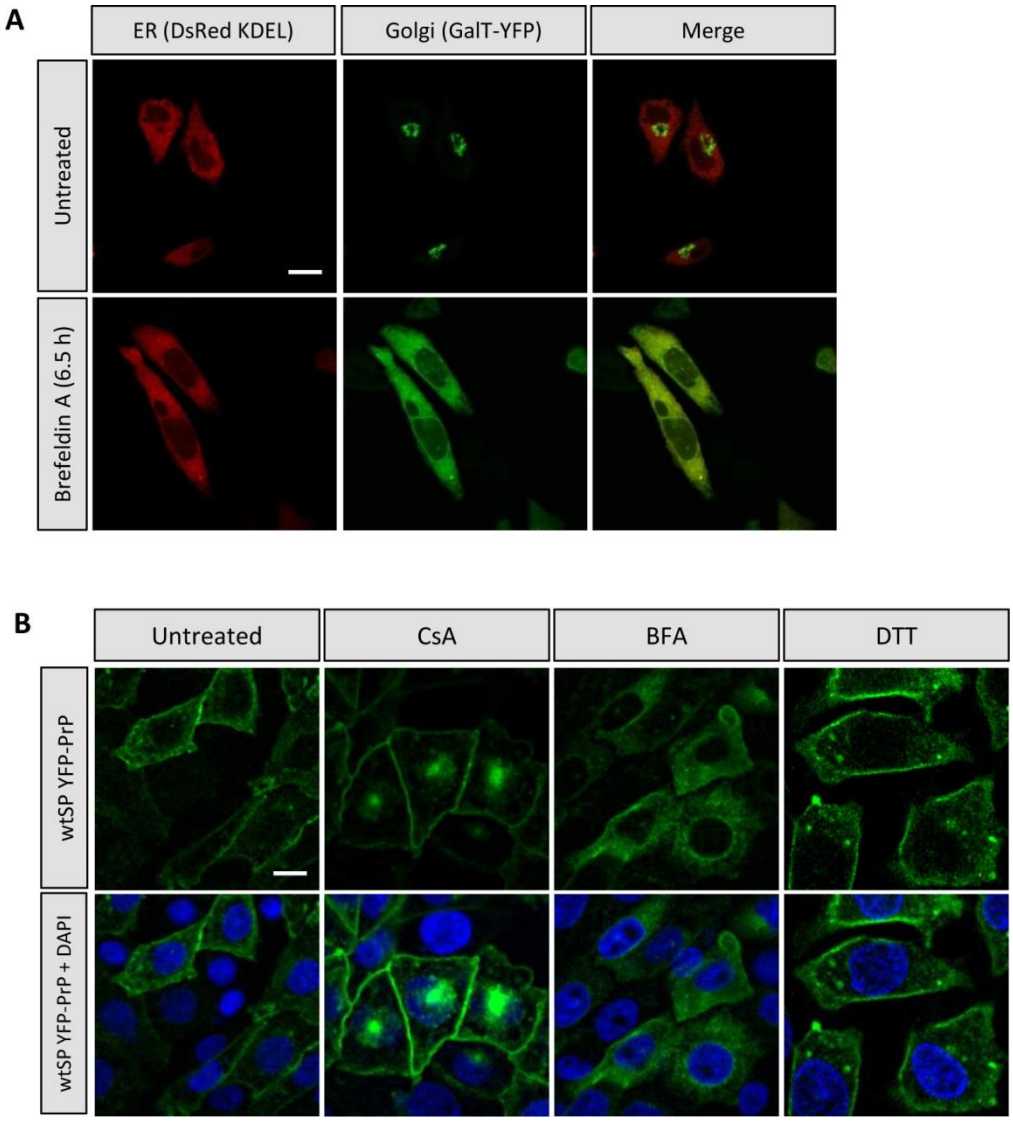
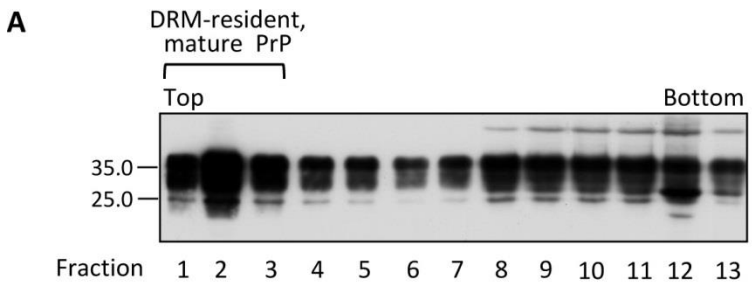


Figure S3: A. CHO cells were transfected with ER-labelling DsRed-KDEL and Golgi-labelling GalT-YFP. The cells were either left untreated (upper panels) or incubated with 1nM Brefeldin A (BFA) for 6.5 hours and visualized by a confocal microscope. Our results show that these conditions are sufficient to confer the collapse of the Golgi apparatus into the ER (scale bar 3µm). **B.** CHO cells expressing the wt SP-YFP-PrP were either treated with CsA, the UPR^{ER} inducers Brefeldin A (BFA) or Dithiothreitol (DTT) or left untreated. The activation of UPR^{ER} by BFA or DTT did not result in the deposition of wt SP-YFP-PrP in aggresomes.

Journal of Cell Science • Supplementary information



B CD4 sequence (mouse):

VFLACVLGGSFGFLGFLGLCXLCCVRCRHQQRQAARMSQIKRLLSEKKTCQCPHRMQKSHNLI

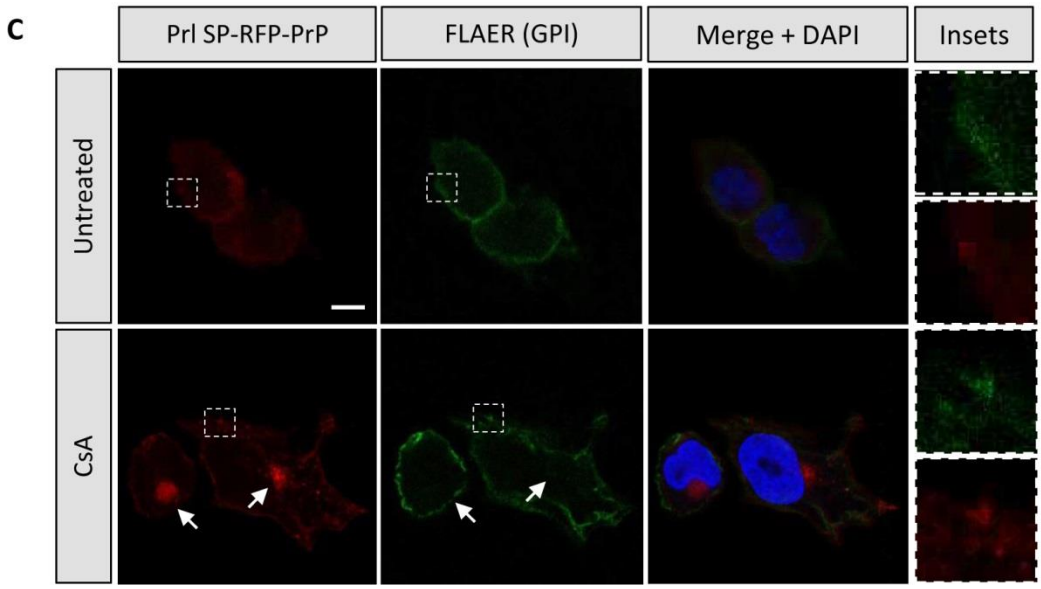
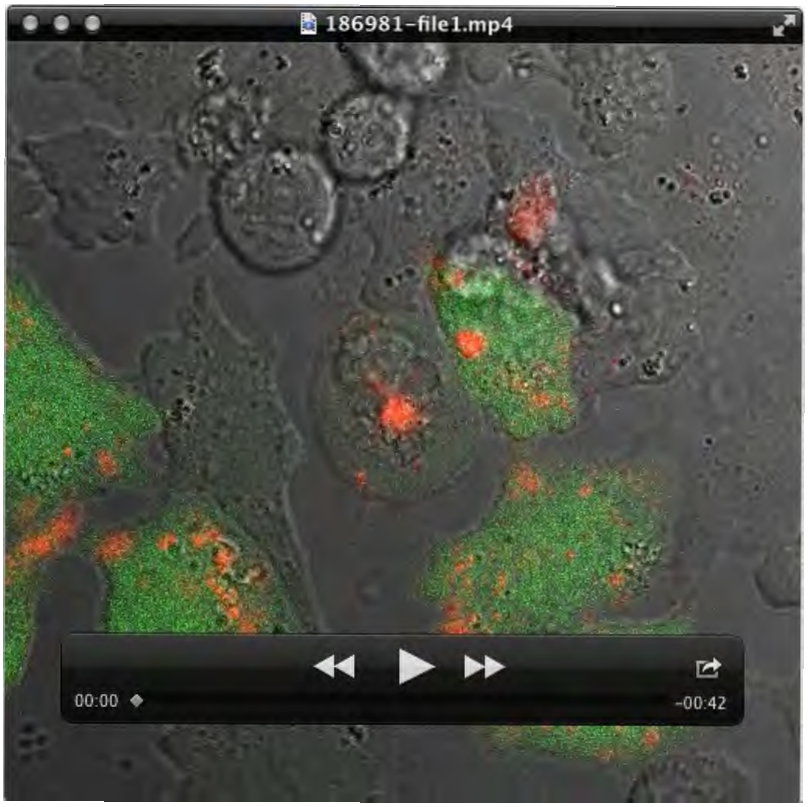


Figure S4: A. N2a-M cells were subjected to preparative floatation, thirteen fractions were isolated and PrP was blotted using a specific antibody (clone 3F4). Mature PrP that was collected from fractions 1 and 2 (top of the gradient) was used as size marker at Fig. 4A. **B.** Amino acid sequence of the mouse CD4 transmembrane domain used to clone the Prl-YFP-PrP-CD4 construct (Fig. 4E). **C**. Prl SP-RFP-PrP expressing CHO cells were treated as in figure 4F but were not permeabilized. FLAER did not label PrP aggresomes under these experimental conditions confirming that the labelling of GPI by this reagent is intracellular (scale bar 2μm).

Journal of Cell Science • Supplementary information



Movie 1 legend: Live visualization of CsA-treated CHO cells co-expressing Opn SP-RFP-PrP (transiently) that efficiently enters the ER and ΔSP-YFP-PrP (stably) stays entirely cytosolic. The movie shows that cytosolic PrP (yellow) molecules do not accumulate in existing aggresomes (red, arrow). Corresponding figure, 2A.

Journal of Cell Science • Supplementary information