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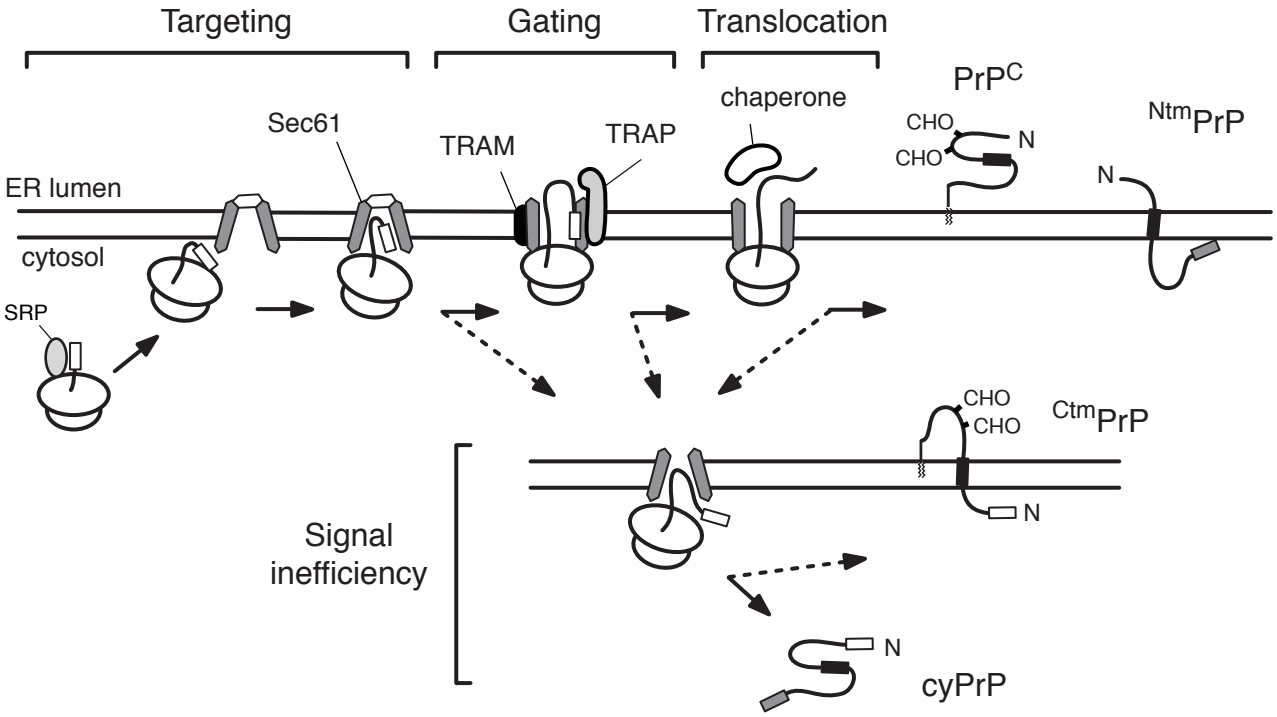
Supplementary Figure S1



Supplementary Figure S1. Schematic diagram of PrP biogenesis at the ER membrane. Steps in PrP translocation. Starting at the left, PrP is targeted to a Sec61-translocon via its N-terminal signal sequence (white rectangle). The signal then interacts with Sec61, and with the aid of accessory components including the TRAM and TRAP proteins, gates open the channel to initiate translocation. Further protein synthesis results in complete translocation into the ER lumen. This is the normal pathway followed by the majority of PrP polypeptides synthesized (i.e., normal PrP^{C}, or cellular PrP). In vitro, successful translocation of the N-terminus, but membrane insertion of the central hydrophobic domain (black rectangle) results in ^{Ntm}PrP. This form presumably still contains the GPI anchoring signal sequence (grey). Whether this form is generated in vivo is not known.

The bottom panel shows that intrinsic inefficiencies in the signal sequence interaction with the translocon can cause a small proportion of PrP polypeptides to fail at the crucial gating/initiation steps. Here, the nascent chain is targeted properly, but is synthesized on the cytosolic face of the translocon. In these cases, the polypeptide is typically expelled into the cytosol to generate cytosolic PrP (cyPrP). However, the central hydrophobic domain (black rectangle), particularly if it carries a mutation that increases hydrophobicity, can engage the nearby translocon to generate a transmembrane species termed ^{Ctm}PrP. Thus, ^{Ctm}PrP is made at the expense of cytosolic PrP, and is dependent on both signal inefficiency and the central hydrophobic domain. Note that depending on exactly when translocation fails, cyPrP and ^{Ctm}PrP may have a processed N-terminus lacking a signal sequence.

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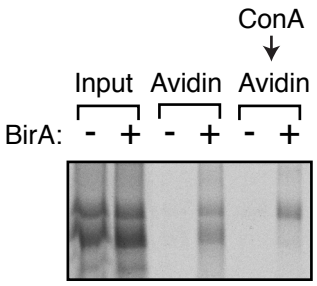
Supplementary Figure S2



Supplementary Figure S2 - Specificity of Avidin and ConA pulldowns. A mixture of glycosylated and non-glycosylated PrP produced by in vitro translation was biotinylated with BirA as indicated. An aliquot of the total products is shown in the first two lanes. These products were denatured in SDS and subjected to either Avidin pulldown, or sequential pulldowns with ConA followed by Avidin. Note that both glycosylated and non-glycosylated PrP are pulled down by Avidin, but only if they are first biotinylated with BirA. ConA pulldown is selective for the glycosylated PrP, and these products can be effectively eluted and pulled down by Avidin.

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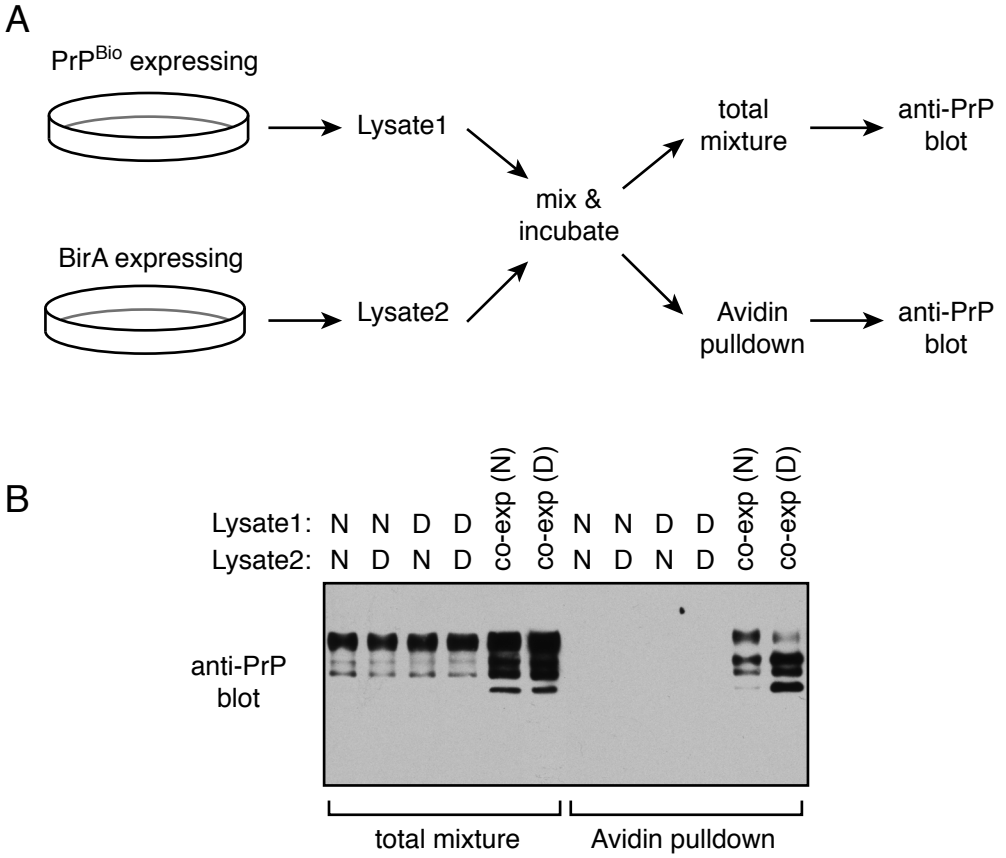
Supplementary Figure S3



Supplementary Figure S3 - No detectable post-lysis biotinylation by BirA. (A) Experimental design. Cells transfected with either a BioTag-containing substrate or BirA are made into lysates that are then mixed, incubated for 1 h on ice, and either reserved for direct analysis (total mixture) or subjected to pulldown with immobilized Avidin. The samples are then analyzed by anti-PrP immunoblot. (B) Results of mixing experiments in which the lysates were prepared under denaturing (D) or non-denaturing (N) conditions. As a positive control, the PrP^{Bio} and BirA were co-expressed in the same cells, and subjected to the same procedure using either denaturing (D) or non-denaturing (N) conditions. The blot is intentionally over-exposed to illustrate no detectable PrP recovered in the avidin pulldowns of mixed lysates.Note that the improved recovery of the control reaction under denaturing conditions is reproducible, and likely reflects either improved solubility of the substrate under these conditions or better access of the biotin to the immobilized Avidin under fully denaturing conditions.

Denaturing = 1% SDS, 0.1 M Tris, pH 8 -> boil -> dilute 10X in non-denaturing buffer lacking SDS.
Non-denaturing = 1% Triton X-100, 1% Deoxycholate, 0.1% SDS, 100 mM NaCl, 50 mM Hepes, pH 7.4.

Thus, both conditions achieve the same final conditions, but the denaturing samples are first boiled in SDS.

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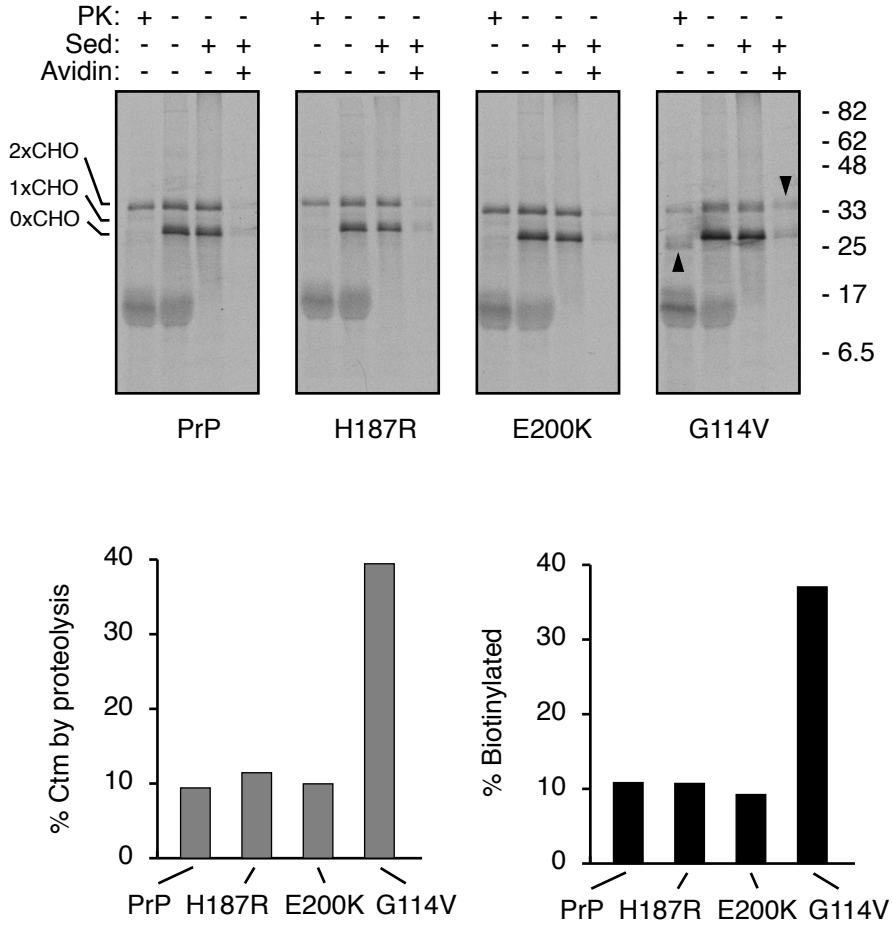
Supplementary Figure S4



Supplementary Figure S4 - Analysis of wild type and mutant PrP for ^{Ctm}PrP in vitro. The indicated BioTagged constructs were translated in vitro in the presence of microsomes and analyzed for topology by either a protease-protection assay or the BirA biotinylation assay exactly as in Fig. 2. The proportion of PrP made in the ^{Ctm}PrP form was quantified from the gels (top panel) and plotted (bottom panel). Note that mutations in the C-terminal domain of PrP such as PrP(H187R) and PrP(E200K) do not increase ^{Ctm}PrP, as described earlier (Stewart, R.S. and Harris, D.A., J. Biol. Chem., 276:2212-20). By contrast, a mutation that increases hydrophobicity of the HD such as PrP(G114V) results in increased ^{Ctm}PrP, as described before (Rane, N. et al., J. Cell Biol., 188(4):515-26).The upward and downward arrowheads indicate the increased ^{Ctm}PrP band for PrP(G114V) in the protease digested and Avidin-pulldown lanes, respectively.

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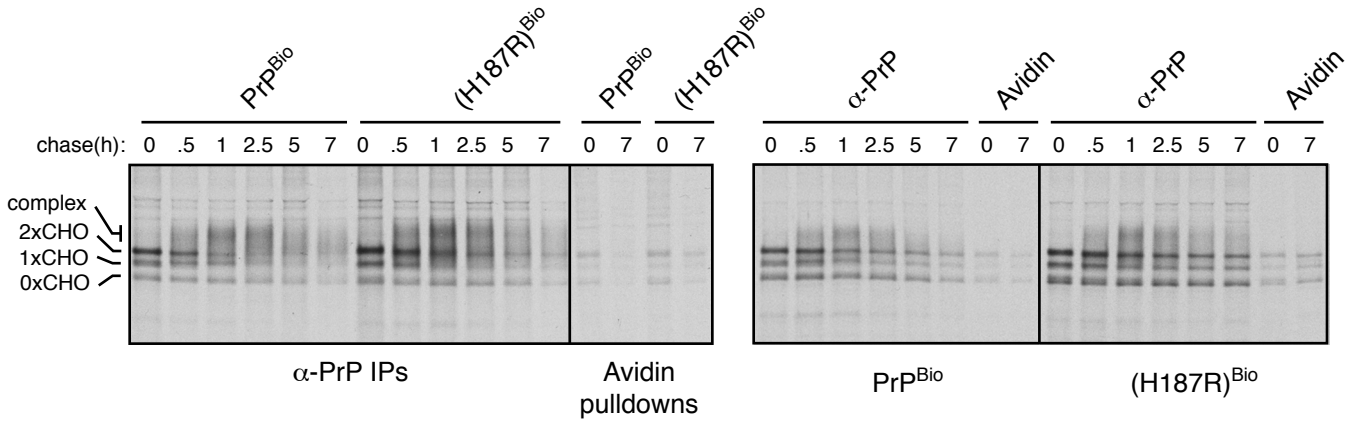
Supplementary Figure S5



Supplementary Figure S5 - The turnover of ^{Ctm}PrP is delayed by the H187R mutation. Pulse-chase analysis of PrP^{Bio} and PrP(H187R)^{Bio} in N2a cells co-expressing BirA. Pulse-labeling was for 30 min, and chase times were as indicated. Samples were either immunoprecipitated with anti-PrP antibodies or pulled down with immobilized Avidin as indicated. The two panels represent two independent experiments. Note that the relative amount of ^{Ctm}PrP in the two experiments is different. However, in both cases, the amount of biotinylated products produced by wild type and mutant PrP is the same, and the reduced degradation of biotinylated products for the mutant is observed in both cases.

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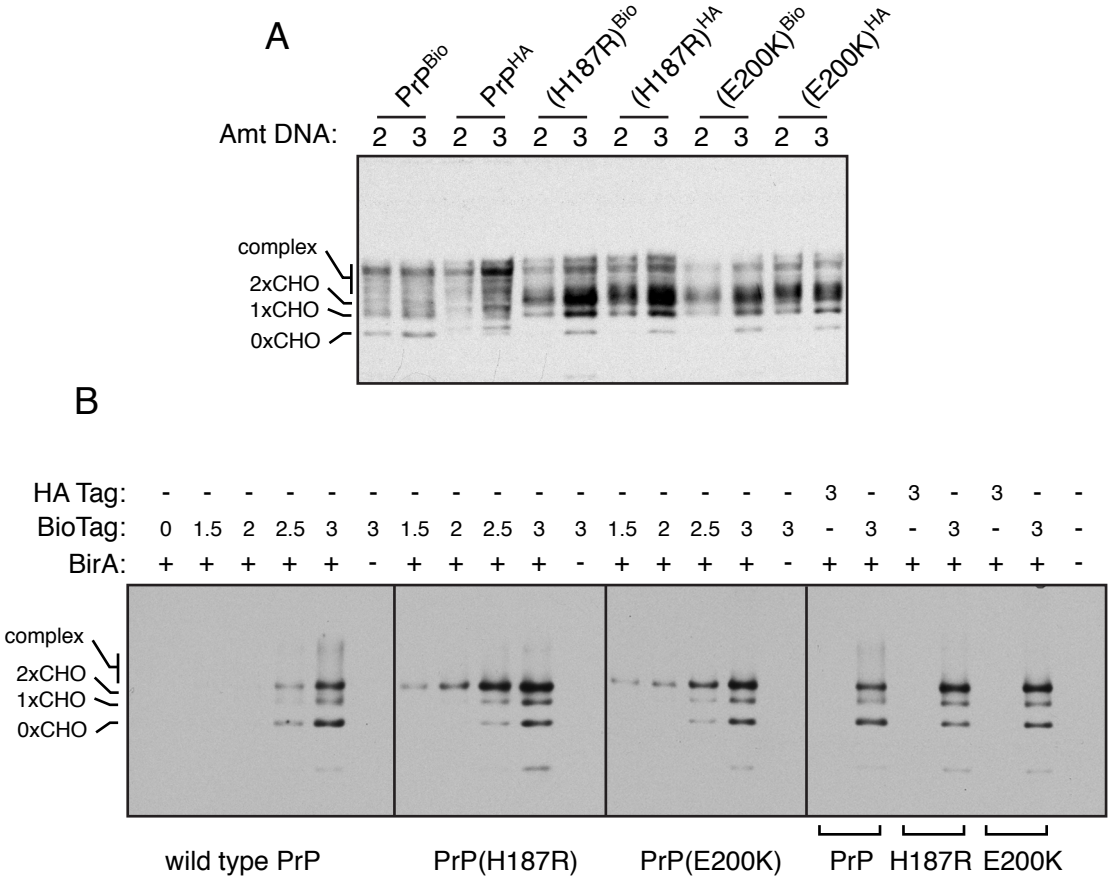
Supplementary Figure S6



Supplementary Figure S6 - Analysis of wild type and mutant PrP for ^{Ctm}PrP in HEK293 cells. (A) The indicated constructs were co-transfected with BirA into HEK293 cells and total cell lysates analyzed for total PrP expression by immunoblotting. Two different amounts of the PrP construct (in ug per 35 mm dish) were transfected. Note that as characterized before (Ashok and Hegde, 2009), the glycosylation pattern of the mutant PrPs is different than wild type. In separate experiments, pulse labeling confirmed that the rates of expression of all construct are equal, meaning that the differences in overall steady state levels is due to differences in turnover rates, as has been characterized (Ashok and Hegde, 2009). (B) The indicated amounts (in ug per 35 mm dish) of the indicated BioTagged or HA-tagged constructs were co-transfected with BirA (or a control GFP-expressing plasmid), and after 24 h, harvested. The biotinylated products in the total lysates were isolated with immobilized Avidin and PrP detected by immunoblotting with anti-PrP. Note that at any given expression level, the amount of biotinylated glycosylated PrP (corresponding to ^{Ctm}PrP) is greater for the mutants than for wild type. Unlike the results in N2a cells, the amount of unglycosylated biotinylated PrP is very similar among the constructs. To ensure that differences were not due to analysis of the samples on different gels (all of which were run, blotted, and exposed in parallel), a subset of the samples was analyzed together on the same gel (right-most panel) and gave identical results.