Developmental Cell *15*

Supplemental Data

Reduced Translocation of Nascent Prion Protein During ER Stress Contributes to Neurodegeneration

Neena S. Rane, Sang-Wook Kang, Oishee Chakrabarti, Lionel Feigenbaum, and Ramanujan S. Hegde

Supplemental Note

During the production of Ifn-PrP and Opn-PrP transgenic mice, we found that the Ifn-PrP transgene seems to be detrimental and can lead to either embryonic or neonatal death. This conclusion emerged as founders arising from these transgene injections (performed in parallel) were being weaned. Both constructs gave ~10% positive founders (as judged by southern analysis), although the Ifn-PrP was slightly lower (8 of 97 animals, or 8.2%, compared to 18 of 165, or 10.9%, for Opn-PrP) and in general had lower copy number of trangenes. Subsequently, 3 of 8 Ifn-PrP founders died within 2 months (in contrast to 1 of 18 for Opn-PrP). Of the remaining five founders, one died at 8.5 months without giving any transgene-positive progeny in five litters encompassing 29 animals. Two others also were bred five times during their life without any positive progeny in 35 animals. One founder was sacrificed for analysis, and the last founder was the only one yielding positive progeny. This contrasted with Opn-PrP in which 4 of the 6 founders we initially attempted to breed gave transgene-positive founders in roughly the expected 50% ratio (35 of 76 animals), from which two independent lines were developed for analysis. Other transgenes injected at the same time gave similar results, indicating that the Ifn-PrP transgene was uniquely detrimental for reasons that remain to be determined.

Furthermore, all positive pups from the successfully breeding Ifn-PrP line over many generations were female. These transgene-positive females were noticably smaller than non-transgenic female (or male) littermates. In addition, males were under-represented by ~50% among the litters while females contained the transgene at the expected ~50% frequency (see Table).

	MALES		FEMALES	
Genotype	Tg-positive	Tg-negative	Tg-positive	Tg-negative
No. Animals	0	30	26	32

We therefore surmise that the transgene is either more toxic to the development of males relative to females, or more likely, that the transgene is carried on the X-chromosome. Hence, expression in females would be lower than in males (due to X-inactivation), allowing their progression to birth. Regardless of the reason, there are several reasons to believe that the developmental toxicity of Ifn-PrP, while interesting in its own right, may not be relevant to the pathogenesis of

prion diseases. First, naturally occuring diseases caused by PrP are, with few exceptions, late onset diseases of the latter half of life. Second, the physiologic significance of decreased translocation of PrP (and therefore increased generation of cytosolic PrP) during development is unclear since pathologic induction of ER stress is not typically observed during development. By contrast, ER stress has been repeatedly observed during the course of prion disease pathogenesis. Thus, Ifn-PrP expression, whose biosynthesis mimics PrP during ER stress, is of most relevance under the setting where ER stress would be pathologically induced. For this reason, we focused our analysis on adult-onset phenotypes when PrP-mediated neurodegeneration occurs concomitant with ER stress.

Supplemental Experimental Procedures

In vitro transcription employed PCR-generated templates containing the SP6 promoter, SP6 polymerase from NEB, and the following reaction conditions: 40 mM Hepes, pH 7.4, 6 mM $MgCl_2$, 2 mM spermidine, 0.1 mM GTP, 0.5 mM each of ATP, CTP, and UTP, and 0.5 mM di-Guanosine Cap-analogue from NEB. Reactions proceeded for 1 h at 40°C. Templates for full length proteins (Fig. 2E and F) were generated by PCR using a 5' primer just preceeding the SP6 promoter and a 3' primer beyond the stop codon. Templates for truncated products used the same 5' primer and a 3' primer lacking a stop codon and annealing to the PrP coding region at codon 150 (Fig. 2C, D).

In vitro translation utilized reticulocyte lysate either prepared by the method of Jackson and Hunt (Methods Enzymol., 1983, 96:50-74) or purchased from Green Hectares. In both cases, the crude lysates were supplemented with Hemin, nuclease treated, and supplemented with salts, calf liver tRNA (from Novagen), an energy regenerating system, 19 amino acids (except Methionine), ^{35}S-Methionine, and in some cases, microsomal membranes from canine pancreas [prepared by the method of Walter and Blobel (Methods Enzymol., 1983, 96:84-93)] or hamster brain. Optimal concentrations of each component were determined in preliminary experiments. Translation reactions were at 32°C for 1 h (Fig. 2E, F), 32°C for 15 min (Fig. 2C, D), or 26°C for 40 min (Fig. 1C and Sup. Fig. 1B) after which they were placed on ice for further manipulations as described below.

For crosslinking, the microsomes were isolated by sedimentation through a sucrose cushion, resuspended in physiologic salt buffer (100 mM KAc, 2 mM $MgAc_2$, 50 mM Hepes, pH 7.4, 250 mM sucrose), and treated with crosslinkers essentially as described before (Kim and Hegde, 2002, Mol. Biol. Cell, 13:3775-86). In Fig. 2C, 200 uM of the cysteine-reactive crosslinker BMH (from Pierce, freshly prepared as a 50X stock in DMSO) was added for 30 min on ice, and quenched with 10 mM 2-mercaptoethanol. In Fig. 2D, 250 uM of the lysine-reactive crosslinker DSS (from Pierce, freshly prepared as a 50X stock in DMSO) was added for 30 min at room temperature, and quenched with 100 mM Tris-HCl, pH 8. Following crosslinking the samples were either analyzed directly (as in the left panel of Fig. 2C), denatured in 1% SDS and subjected to immunoprecipitation (Fig. 2C and 2D), or fractionated into membrane and lumenal components exactly as described previously (Kim and Hegde, 2002, Mol. Biol. Cell, 13:3775-86). Note that parallel samples without crosslinker were also analyzed to confirm the specificity of each of the crosslinking products (data not shown), and that these crosslinking products (i.e., SRP54, Sec61α, and PDI) have been characterized in detail previously by our lab.

For ubiquitination analysis, translation reactions contained 50 uM His-Ubiquitin and 5 uM Ubiquitin-aldehyde (both from Boston Biochem.). Ubiquitinated products were recovered from the samples following denaturation in 1% SDS. The samples were then diluted 10-fold into a

buffer containing 50 mM NaCl, 25 mM Hepes, pH 7.4, and 0.5 % Triton X-100. Talon beads (Invitrogen) comprised of immobilized Co+2 were added, incubated for 1h, washed twice in the above dilution buffer, and the products eluted in SDS-PAGE sample buffer containing 50 mM EDTA.

Total RNA was isolated from brain tissue using the RNeasy Protect mini kit from Qiagen. The optional DNAse treatment step was included to ensure no DNA contamination. RNA was quantified by absorbance at 260 nm. Standards for calibration of the RT-PCR reactions were generated by SP6 polymerase mediated in vitro transcription using PCR-generated templates encoding Ifn-PrP and Opn-PrP. The in vitro synthesized transcripts were DNAse treated, purified, and quantified as above. RT-PCR utilized SuperScript III reagents from Invitrogen. Serial dilutions ensured that the RT-PCR reactions used for estimating quantities of starting mRNA were in the linear range. RNAse digestion controls prior to the RT-PCR reactions confirmed that none of the products resulted from DNA contamination. Ifn-PrP and Opn-PrP amplification used signal sequence selective 5' primers and a common 3' primer in the mature domain of Hamster PrP. Endogenous mouse PrP amplification used primers to the 3'UTR at regions that diverge from the Hamster PrP 3' UTR.

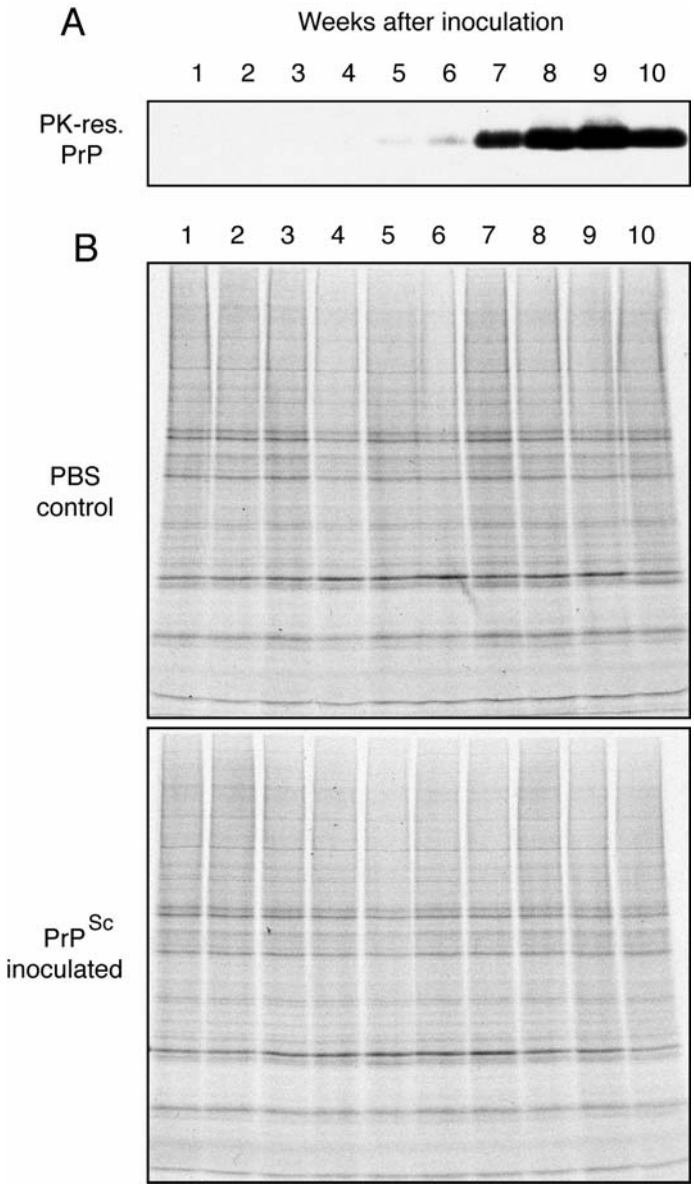

Figure S1. Characterization of Microsomes from PrP^{Sc}-Inoculated Hamsters
(A) Brain homogenate from hamsters inoculated with PrP^{Sc} for the indicated times were analyzed for protease-resistant PrP. Prior to analysis by immunoblotting, samples were deglycosylated with PNGase to compress all of the glycoforms into a single 20 kD band. Equal loading was confirmed by total protein staining of the blot (not shown). No protease-resistant PrP was observed in any of the matched samples from saline-inoculated animals (not shown). (B) ER-derived rough microsomes from hamsters inoculated for the indicated times with PBS or PrP^{Sc} were mixed with an in vitro translation mix containing [35]S-Methionine and in vitro synthesized PrP transcript. Following incubation for 1 h at 32 °C to allow translation of both the endogenous and exogenous mRNAs, an aliquot of the products were analyzed by SDS-PAGE and autoradiography to visualize the newly translated products. Note equal labeling and very similar profiles of synthesized products in all samples, indicating that recovery of the microsomes and the associated mRNAs was uniform.

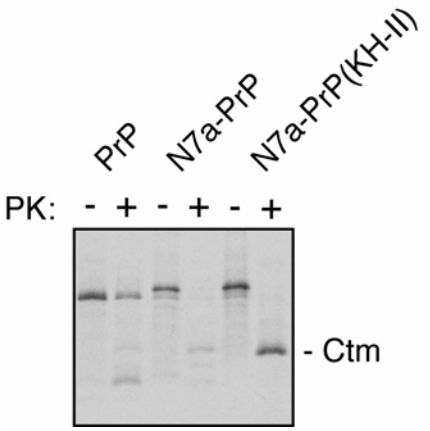

Figure S2. Relative Effect of the Signal Sequence and Transmembrane Domain on ^{Ctm}PrP
The indicated constructs were translated in vitro and analyzed for translocation and topology using a protease-protection assay (see Kim et al., 2001, JBC, 276:26132-40). N7a refers to a signal sequence mutant that allows successful targeting of PrP to the ER translocon, but is not efficient in initiating translocation into the lumen. Note that this 'weak' signal sequence results in little or no increase in the total amount of the ^{Ctm}PrP form. Instead, most of the synthesized protein is cytosolic, as evidenced by its lack of protease protection. However, if the N7a signal sequence is combined with another mutation (KH-II) that increases the hydrophobicity of the potential transmembrane domain, nearly all of the synthesized protein is in the ^{Ctm}PrP form.

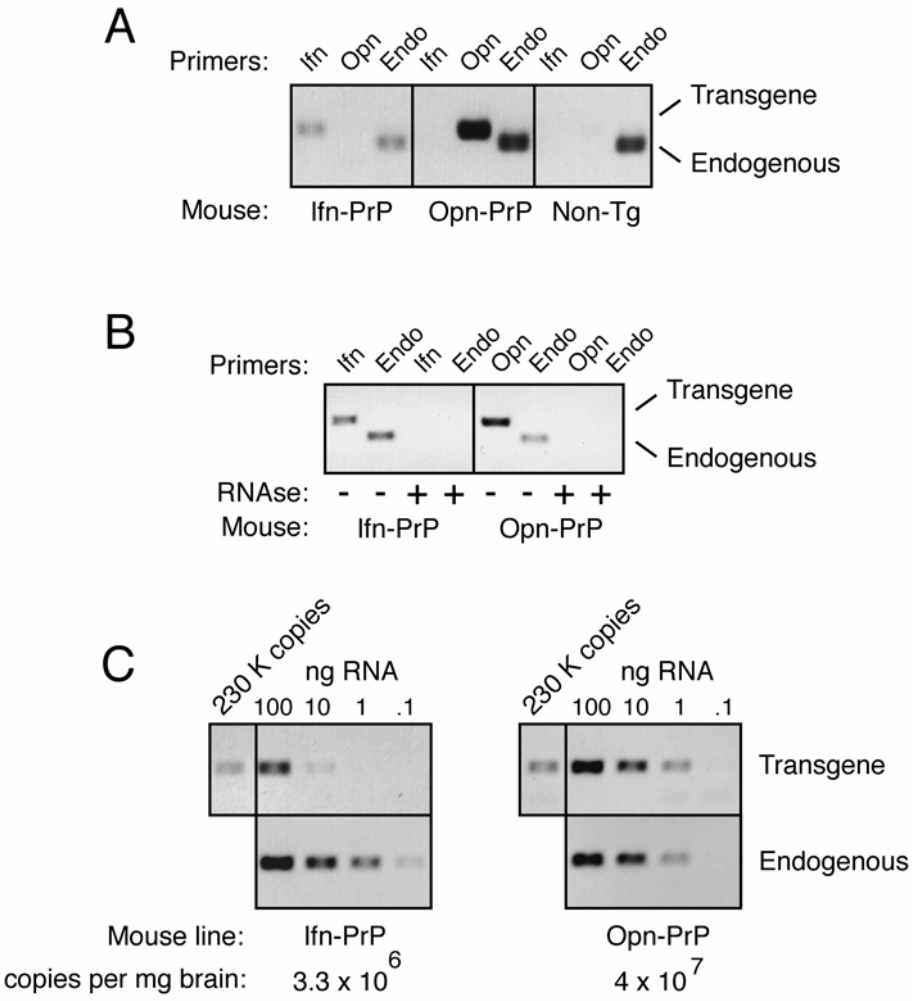

Figure S3. Quantitation of Transgene mRNA Levels

(A) Total RNA isolated from total brain tissue of the indicated mouse lines were used in RT-PCR reactions with primer sets correpsponding to the Ifn-PrP transgene, Opn-PrP transgene, or the 3'UTR of endogenous mouse PrP. Transgene and endogenous primer sets were designed to give products of 283 and 190 bp, respectively. Shown are Ethidium Bromide stained agarose gels of 20% of the products (the image is inverted for clarity). Note that each primer set is selective.
(B) Treatment of the brain RNA template with RNAse before use in RT-PCR reactions abolishes amplification, illustrating that amplification was not from contaminating DNA.
(C) RT-PCR reactions were carried out using either 230,000 copies of synthetic transcript (made by in vitro transcription) or the indicated amounts of total brain RNA. The left panel used RNA from an Ifn-PrP mouse, and the right panel an Opn-PrP mouse. Parallel reactions were also carried out on the same samples using the endogenous PrP primer set to confirm equal efficiencies of amplification when identical amounts of total RNA were used. Based on densitometric quantification of the amplified product and of the amount of total RNA isolated per mg brain tissue (~285 ng), we could calculate the number of copies of each trangene per mg brain tissue. Similar numbers were obtained in additional experiments where different amounts of synthetic standard was used for quantification (data not shown).

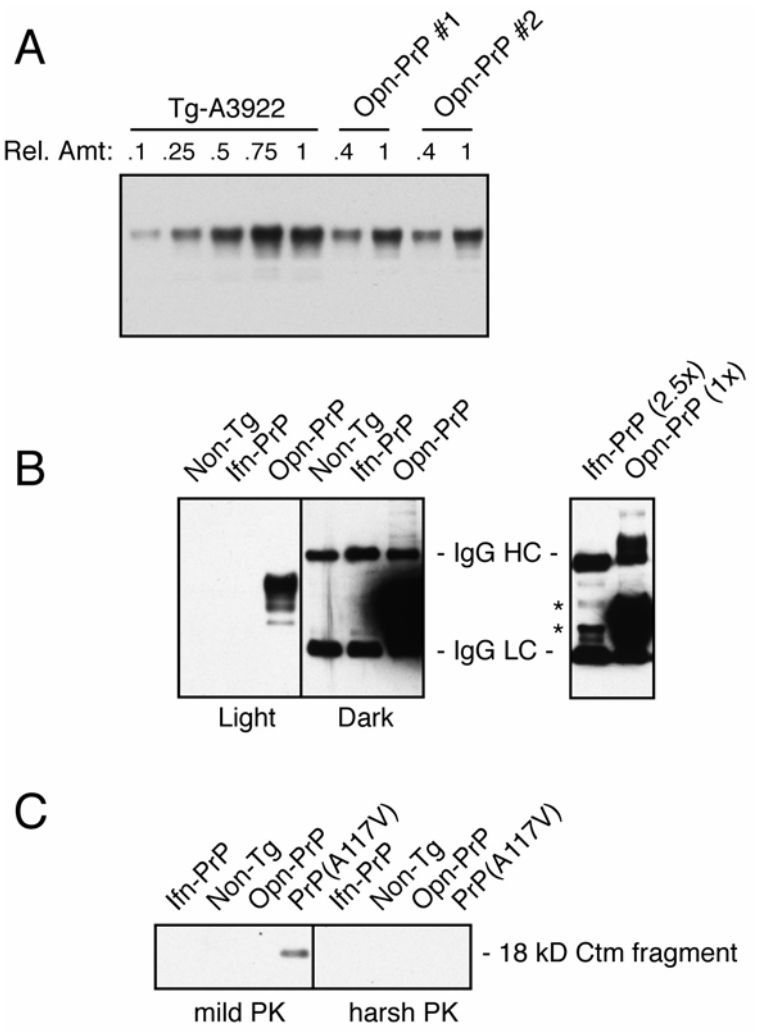

Figure S4. Analysis of PrP Expression in Tg Mice

(A) Total brain homogenate from mice overexpressing wild type Hamster PrP at 4x normal levels (Tg-A3922; see Hegde et al., 1998, Science, 279:827-834) were compared to homogenate from two independent lines of Opn-PrP mice. Detection was with the 3F4 antibody against Hamster PrP. The relative amount of homogenate analyzed is indicated above each lane. From densitometric analysis of this blot, we determined that Opn-PrP expression is roughly half of the A3922 line, and therefore approximately twice the level of endogenous PrP.

(B) Equal amounts of brain homogenate from the indicated transgenic animals was analyzed by immunoblotting using the 3F4 antibody. Note that even though the mRNA level of Ifn-PrP is ~10% of Opn-PrP (see Sup. Fig. 3), little or no protein was reliably detected, even on gross overexpression of the blot (middle panel). Only when 2.5x more homogenate was analyzed could a small amount of mostly immature forms of PrP be detected (right panel, asterisks), as also seen in neuronal cultures from these mice (see Fig. 7A).

(C) Analysis of brain homogenate from the indicated transgenic mice for the presence of ^{Ctm}PrP using the limited PK digestion assay characterized before (Hegde et al., 1998, Science, 279:827-834). Here, an 18 kD fragment derived from ^{Ctm}PrP is observed under the 'mild' but not 'harsh' PK digestion conditions. Note that Ifn-PrP and Opn-PrP did not show detectable ^{Ctm}PrP, in contrast to mice expressing PrP(A117V).