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Baumann, F; Pahnke, J; Radovanovic, I; Rülicke, T; Bremer, J; Tolnay, M; Aguzzi, A (2009). Functionally relevant domains of the prion protein identified in vivo. PLoS ONE, 4(9):e6707.

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Originally published at:
PLoS ONE 2009, 4(9):e6707.
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Abstract

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Functionally Relevant Domains of the Prion Protein Identified In Vivo

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Abstract

The prion consists essentially of PrPSc, a misfolded and aggregated conformer of the cellular protein PrP. Whereas PrPSc-deficient mice are clinically healthy, expression of PrPSc variants lacking its central domain (PrP[CD]), or of the PrP-related protein Dpl, induces lethal neurodegenerative syndromes which are repressed by full-length PrP. Here we tested the structural basis of these syndromes by grafting the amino terminus of PrPSc (residues 1–134), or its central domain (residues 90–134), onto Dpl. Further, we constructed a soluble variant of the neurotoxic PrP[CD] mutant that lacks its glycosyl phosphatidyl inositol (GPI) membrane anchor. Each of these modifications abrogated the pathogenicity of Dpl and PrP[CD] in transgenic mice. The PrP-Dpl chimeric molecules, but not anchorless PrP[CD], ameliorated the disease of mice expressing truncated PrP variants. We conclude that the amino proximal domain of PrP exerts a neurotrophic effect even when grafted onto a distantly related protein, and that GPI-linked membrane anchoring is necessary for both beneficial and deleterious effects of PrP and its variants.

Introduction

PrPSc is the main constituent of prions [1], the infectious agents causing transmissible spongiform encephalopathies (TSE). PrPSc is an aggregated and misfolded isomer of the cellular protein PrP [2] which is expressed in a broad range of tissues of most vertebrates [3]. Nascent PrP is exported to the lumen of the endoplasmic reticulum, deprived of its amino terminal signal sequence, glycosylated at two asparagine residues, and endowed with a GPI moiety which anchors it to the outer cell surface. Ablation of the Prnp gene, which encodes PrP, abrogates prion replication [4] and toxicity [5]. Prnp deficient mice enjoy a normal life expectancy [6], but suffer from subtle neurological effects of PrP and its variants.

Transgenic expression of amino proximally truncated PrPSc mutants [PrP[ACD], PrP[CD] and PrP[AF], henceforth collectively termed ΔPrP] causes early-onset ataxia and white-matter degeneration (Fig. 1A). Toxicity appears to correlate with partial or complete deletions of the conserved PrP central domain (CD, residues 94–134) [9,10,11] which bridges the flexible amino proximal tail and the globular carboxy proximal domain [12].

Another neurotoxic phenotype was detected in compound-heterozygous Prnp[ZHIII] mice and in homozygous Prnp[ZHIII/ZHIII] mice [13] whose Prnp[ZHIII] allele leads to ectopic expression of the PrP[CD]-related protein Dpl [14,15,16,17]. Neuronal expression of Dpl in Tg[Dpl] or Tg[N-Dpl] mice induces ataxia within 40–60 days [18,19]. Despite 80% amino acid sequence dissimilarities [14], the overall 3D structure of Dpl is similar to that of PrP [Fig. 1B] and includes an unstructured amino proximal tail, a globular three-helix domain [20], and a GPI anchor. However, Dpl is physiologically not expressed in the adult nervous system [21] and, importantly, lacks any sequences comparable to the CD. Therefore, Dpl resembles the neurotoxic ΔPrP mutants. What is more, the toxicity of both Dpl and ΔPrP is counteracted by co-expression of full-length PrP [9,10,18,22,23], implying that it exploits common molecular pathways.

We reported previously that the removal of just the CD domain confers dramatic neurotoxicity to PrP. This suggests that the toxicity of Dpl may also result from the absence of a CD-like domain. Here, we tested this hypothesis by transgenic expression of two chimeric proteins, PrP_Dpl (residues 1–65 of Dpl replaced by residues 1–133 of PrP) and CD_Dpl (residues 90–133 of PrP inserted between residues 65 and 66 of Dpl). Transgenic mice expressing these proteins did not develop any clinical phenotypes. Additionally, coexpression of PrP_Dpl or of CD_Dpl ameliorated the clinical syndromes and prolonged the life expectancy of mice
expressing neurotoxic ΔPrP mutants, in agreement with a previous report [24]. Since PrP is thought to be involved in signal transduction, we tested whether the toxicity of CD-deficient PrP mutants (PrP_{ACD}) may require localization to membrane lipid rafts. Indeed, removal of the GPI addition signal from PrP_{ACD} prevents its neurotoxic effects.

**Results**

Transgenic mice expressing chimeric PrP-Dpl proteins and PrP_{ACD}s

All chimeric mutants of Dpl and PrP described here are based on the ‘half-genomic’ pPrPHG backbone [25] whose expression...
pattern has been recently studied in detail [26]. This construct contains a redacted murine Prnp gene which lacks intron #2 and is flanked by 6 and 2.2 kb of 5′ and 3′ genomic regions, respectively. Neuronal expression of Dpl leads to autolysis, neuronal loss and demyelinating neuropathy [17,18,19,22] while most of the toxicity of truncated PrP can be assigned to the lack of the central domain CD (residues 94–134) [10]. If the absence of a CD-like domain were responsible for its toxicity, addition of domains containing 

We constructed CD_Dpl, a chimeric fusion protein consisting of codons 90–133 of mouse Prnp inserted between codons 65 and 66 of Pmnd (Fig. 1A, F). This particular insertional position was chosen because hydrophobicity comparisons suggested that the resulting chimeric protein would resemble wild-type PrP (Fig. 1C, D). In a second construct termed PrP_Dpl, the amino terminus of PrP comprising codons 1–133 was fused to the carboxy terminus of Dpl comprising codons 66–179 (Fig. 1A; E). Pronuclear injection was performed into Pmnd/+ wild-type (wt) C57BL/6N mice giving rise to transgenic founders on a Pmnd/+ background (henceforth termed PrP_Dpl, PrP_Dpl, PrP_Dpl, PrP_Dpl, and PrP_Dpl with superscripts defining the Prp allele status and subscripts denoting the respective hemizygous transgenics).

PrP and Dpl are tethered to the cell membrane by a C-terminal GPI anchor. PrP has been proposed to act as a signal transducer acting on various signaling pathways [9,10,27,28,29,30], and in this context it was speculated that PrP toxicity may require membrane localization. To test this hypothesis, we introduced two point mutations at codons 232 and 233 (original mouse numbering) of the half-genomic construct PrPACD [10], resulting in two in-frame stop codons. This prevents the translation of the carboxy terminal hydrophobic membrane anchoring domain of the precursor protein (see Fig. 1A), resulting in a secreted PrP mutant termed PrPACD. Because of the possible toxicity of the transgene, pronuclear injection was performed into hybrid B6D2F1 Prp+/+ zygotes to generate PrpACD (shorthand as above) transgenic mice. The latter mice were predicted to be viable due to the coexpression of wild-type PrP.

CD_Dpl founder mice #1070, #1071 and #1073, as well as PrP_Dpl founder mice #1023, #1024, #1025 and #1026 and PrPACD, founder mice #36, #37, #38, #39, #40, #41, #42, #43 all exhibited undistorted Mendelian transmission of the respective hemizygous transgenes). PrP and Dpl are tethered to the cell membrane by a C-terminal GPI anchor. PrP has been proposed to act as a signal transducer acting on various signaling pathways [9,10,27,28,29,30], and in this context it was speculated that PrP toxicity may require membrane localization. To test this hypothesis, we introduced two point mutations at codons 232 and 233 (original mouse numbering) of the half-genomic construct PrPACD [10], resulting in two in-frame stop codons. This prevents the translation of the carboxy terminal hydrophobic membrane anchoring domain of the precursor protein (see Fig. 1A), resulting in a secreted PrP mutant termed PrPACD. Because of the possible toxicity of the transgene, pronuclear injection was performed into hybrid B6D2F1 Prp+/+ zygotes to generate PrpACD (shorthand as above) transgenic mice. The latter mice were predicted to be viable due to the coexpression of wild-type PrP.

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**Table 1.** Characterization of transgenic mice.

| Construct | Deletion | Transgenic copy numbers | mRNA | Protein | Mouse line |
|-----------|----------|-------------------------|------|---------|------------|
| PrPwt     |          | 1                       | 1°/0° | 1°      | B6 WT      |
| PrPzf     | Δ32–134  | 70                      | 2°    | 2°      | TgF35      |
| PrPACD    | Δ94–134  | 1                       | n.d.  | 0.2°    | Tg1046     |
| PrPACD    | Δ94–134  Δ231–254 | 6                       | 3.5°  | 0.3°    | Tg40       |
| PrPACD    | Δ94–134  Δ231–254 | 5                       | 3°    | 0.3°    | Tg42       |
| Dpl       |          | 1                       | 0°/1° | 1°      | B6 Nagasaki|
| CD_Dpl    |          | 126                     | 1.6°/2° | 5°/1°  | Tg1071     |
| PrP_Dpl   |          | 180                     | 4°/120 2°/0.2° | Tg1025     |
| PrP_Dpl   |          | 220                     | 7°/180° 3°/0.4° | Tg1026     |

PrP mRNA and protein levels are expressed relatively to wild-type mice (°) or, in the case of PrpNgsk/Ngsk mice, relatively to Dpl expression (°). 

doi:10.1371/journal.pone.0006707.t001
Figure 2. Expression and localization of transgenic proteins. (A) Similar glycosylation patterns of PrP\textsuperscript{CD}, PrP\textsubscript{Dpl}, CD\_Dpl and Dpl. Brain homogenates were subjected to PNGase F treatment as indicated, and analyzed by Western blotting using anti-PrP mouse monoclonal antibody POM3 (upper panel) or anti-Dpl mouse monoclonal antibody E2 (lower panel). The spurious band at 20–25 kDa in the 1\textsuperscript{st} lane of the lower panel may indicate incomplete deglycosylation of Dpl. (B) The glycosylation patterns of full-length PrP, PrP\_ACD and PrP\_ACDS are similar. PNGase-treated brain homogenates were analyzed by Western blotting using anti-PrP mouse monoclonal antibody POM11. (C) Protein levels in brain extract of transgenic mice compared to PrP in Bl6 mice (filled black columns and left ordinate) and compared Dpl in Nagasaki mice (open columns and right y-axis) using either PrP specific antibodies POM11 or POM3 or Dpl specific antibody E2 for western blot. Each column represents the average of 3 mice. (D) Detergent-resistant membrane (DRM) preparations from transgenic mouse brains were separated by density gradient centrifugation and analyzed by Western blotting with monoclonal antibody POM3. Significant amounts of PrP\textsuperscript{CD}, PrP\_Dpl, and CD\_Dpl buoyed similarly to flotillin (48 kDa) confirming localization within DRMs. Non-buoyant fractions may indicate raft disruption or may represent immature protein fractions. (E) Density gradient DRM preparations of wild-type and anchorless PrP (PrP\textsuperscript{CD}), PrP\_ACD and PrP\_ACDS, transgenic brains analyzed after deglycosylation with PNGase F with monoclonal antibody POM1. PrP and PrP\_ACD buoyed similarly to flotillin, whereas PrP\_ACDS (lower band in fraction 13, arrowhead) were never DRM-associated irrespectively of the presence or absence of wild-type PrP (*). (F) Plasma concentration of prion protein variants. Plasma from wild-type PrP\textsuperscript{CD}, PrP\_ACD, PrP\_ACDS, Tg1046, PrP\_ACD\_Tg40, Tg42 and anchorless PrP, (Tg44) mice was studied by ELISA with POM antibodies. PrP plasma levels were vastly elevated in all transgenic mice expressing anchorless versions of PrP. (G–N) Cerebellar sections immunostained with antibodies directed against PrP (POM3) (G–J) and Dpl (K–N). POM3 immunoreactivity was seen in the molecular and granule cell layers of wt (G), PrP\textsuperscript{CD} (H) and PrP\_CD\_Dpl (I) mice but was absent, as expected, from PrP\_Ngsk\_Ngsk cerebella (J). Cerebellar molecular and granule cell layers are immunostained with anti-Dpl antibody in PrP\_Dpl (M) and PrP\_Ngsk\_Ngsk mice (N). No Dpl staining was observed in wt mice (K). Scale bar 100 \mu m.

doi:10.1371/journal.pone.0006707.g002
they all reside in similar membrane microdomains. Therefore, most aspects of PrP_Dpl and CD_Dpl biogenesis appear to be similar to those of PrP^C. In contrast, both PrP_ACD and PrP displayed less buoyancy, suggesting no association with rafts in agreement with their biogenesis as soluble proteins. We then prepared DRMs from Tg42 PrP^ACD mice expressing PrP^C and PrP_ACD. Fractions were deglycosylated with PNGase F prior to western blotting. This experiment revealed that coexpression of wild-type PrP fails to recruit PrP_ACD to DRMs. Upon pretreatment with phosphatidylinositol-specific phospholipase C (PI-PLC) the buoyancy of the GPI-anchored PrP variants became similar to that of their anchorless counterparts (Fig. S2).

Finally, we determined the serum PrP concentration in PrP^ACD, PrP_ACD, and PrP_Dpl mice, as well as in GPI-Tg42 mice expressing anchorless full-length PrP, [32] (Fig. 2F). Despite similar PrP levels in brain homogenates, mice expressing anchorless versions of PrP (PrPs or PrP_Dpl) reached an age of 26 days [10]. Despite higher total PrP levels, mice expressing anchorless versions of PrP (PrPs or PrP_Dpl) survived only 32 and 60 days respectively [18,19] (data not shown).

Phenotypes of mice expressing PrP-Dpl chimeric proteins
All transgenic lines (Tg1025; Tg1026; Tg1071) were maintained in the Prnp^+/o or Prnp^o/o allelotype (PrP^ACD^o/o or PrP^Dpl^o/o, PrP^ACD^o/CD_Dpl and PrP^Dpl^o/CD_Dpl), and monitored using a four-degree clinical score [10]. It has previously been shown that onset and development of disease correlate with expression levels of Dpl. Tg(Dpl)20272/Zrc1l and (TgN-Dpl)32 mice, which express high amounts of Dpl, survived only 32 and 60 days respectively [18,19] whereas mice expressing lower Dpl levels, such as Prnp^Ngsk/Ngsk mice [16], showed progressive symptoms of ataxia and were euthanized according to clinical scoring at ≈70 weeks of age. Instead, none of the PrP^ACD^o/o or PrP^Dpl^o/o, PrP^ACD^o/CD_Dpl and PrP^Dpl^o/CD_Dpl mice showed abnormal behavior even after >100 weeks of age, and most of them died at 26–35 months of age (Fig. 3A). This suggests that the presence of amino terminal domains of PrP reduces the toxicity of Dpl.

Phenotypes of mice expressing anchorless PrP_ACDs proteins
Transgenic lines Tg40 and Tg42, henceforth termed PrP^ACD^o/o and PrP_ACDs were monitored using the same clinical score as with Dpl-PrP chimeric mice. Onset and development of disease caused by PrP_ACD correlated inversely with expression levels of the transgene and was ameliorated by coexpression of PrP^C. Mice expressing high amounts of PrP_ACD survived 35 (Tg1050) or 80 days (Tg1047) in a PrP_ACD genotype whereas Tg1046 mice, which express less PrP_ACD only developed pathology in the absence of PrP^C and reached an age of 26 days [10]. Despite higher total expression levels in PrP_ACDs than in PrP_ACD, even after >60 weeks none of the PrP^ACD^o/o or PrP^ACD^o/CD_Dpl and PrP^Dpl^o/CD_Dpl from both transgenic lines Tg40 and Tg42 showed abnormal behavior, and most of them died at a similarly advanced age as wt mice (Fig. 4A). Therefore, removal of the membrane anchor prevents the toxicity caused by deletion of the central domain (CD) of PrP^C.

Histological phenotype
Wt, PrP^ACD^o/o, PrP^ACD^o/CD_Dpl, and PrnP^Ngsk/Ngsk mice were sacrificed at 100, 200, and 420 days of age, and brains as well as spinal cords were analyzed histologically. By the age of 200 days these mice displayed no pathological alterations with the exception of some Purkinje cells loss in PrnP^Ngsk/Ngsk mice (data not shown). When brains of 60-week-old wt (Fig. 3B, F, J), Tg1026 PrP^Dpl^o (Fig. 3C, G, K), Tg1071 PrP^CD_Dpl^o (Fig. 3D, H, L) and PrnP^Ngsk/Ngsk mice (Fig. 3E, I, M) were compared, GFAP immunostains (Fig. 3B–I) showed moderate activation of astrocytes within the molecular layer of the cerebellum in PrP^CD_Dpl^o mice (Fig. 3D). No such pathological changes were seen in PrP^ACD^o or wt mice (Fig. 3B).

White matter pathology characterized by vacuolation and astrogliosis was seen in the cerebellum (arrows Fig. 3E) and in the corpus callosum of PrnP^Ngsk/Ngsk mice (Fig. 3I). None of these changes were observed in wt, Tg1026 PrP^Dpl^o and Tg1071 PrP^CD_Dpl^o mice (Fig. 3F–H). Transverse semithin sections of spinal cords (mid-thoracic level, Fig. 3J–M) and of sciatic nerves (Fig. 3O–R) revealed coarse vacuolar degeneration (white arrowheads) in myelinated fiber tracts in PrnP^Ngsk/Ngsk mice and axonal loss (white arrows, Fig. 3M, R). No such changes were observed in wt, Tg1026 PrP^ACD^o and Tg1071 PrP^CD_Dpl^o mice (Fig. 3J–M, O–Q).

Wt, Tg1046 PrP^o/o, Tg40 PrP^CD^o o/ACD, and PrnP^Ngsk/Ngsk mice were sacrificed at 23 days and 60 weeks of age, and brains as well as sciatic nerves were analyzed histologically (Fig. 4). Tg40 PrP^o/o/ACD mice of 23 days of age (Fig. 4C, G) displayed no pathological alterations compared to Tg1046 PrP^o/o/ACD mice which showed strong cerebellar white-matter astrogliosis (Fig. 4D). Transverse semithin sections of the sciatic nerve revealed peripheral neuropathy in Tg1046 PrP^ACD^o/CDs with axonal loss white arrows and myelin degeneration white arrowheads (Fig. 4H) but not in wt (Fig. 4F), PrP^o/o (Fig. 4I) or Tg40 PrP^CD^o/ACD mice (Fig. 4G) at 23 days of age. No PrP_ACD toxicity was observed also at later time points (data not shown).

Functional rescue of truncated PrP variants
PrP_Dpl and CD_Dpl did not elicit any clinical or histopathological syndrome in PrnP^o/o mice. This may indicate that PrP_Dpl and CD_Dpl have lost all functional characteristics of PrP-like proteins. We assessed this possibility by intercrossing Tg1026 PrP_Dpl and Tg1071 CD_Dpl transgenic mice with the neurototoxic PrP deletion mutants Tg1046 PrP^o/o/ACD and TgF35 PrP^o/AF mice [9], whose toxicity can be ameliorated by the coexpression of full length PrP. The resulting Tg1046 PrP^o/o/ACD developed first signs of disease at 18-20 days post birth and reached terminal disease at 25±0.7 days (n = 22) of age, as described previously. Double transgenic Tg1046x1071 Tg1046x1071 PrP^ACD^o/CDs mice survived until 43±2.3 days (n = 8), whereas Tg1046x1071 PrP^ACD^o/CDs littermates survived 25±2.0 days (n = 11) (Fig. 5A and Table 2). Double-transgenic Tg1046x1071 Tg1026 PrP^ACD^o/CDs mice survived 36±1.3 days (n = 6), as opposed to 26±1.7 days (n = 6) for Tg1046x1071 Tg1026 PrP^ACD^o/CDs littermates (Fig. 5C and Table 2). A similar trend was also seen in the transgenic line Tg1025 PrP^ACD^o/CDs and in intercrosses of the PrP^ACD^o/CDs lines Tg1047 and Tg1050 (data not shown), with significant prolongation of survival (ANOVA; p<0.001). TgF35 PrP^o/AF mice developed ataxia and were euthanized at 96±5.3 days of age (Fig. 5B, D and Table 2) as described [9]. Double transgenic TgF35x1071 TgF35x1071 PrP^o/AF/CDs mice survived 150±12.7 days (n = 8; 5B; Table 2), whereas double transgenic TgF35x1071 TgF35x1071 PrP^o/AF/CDs mice survived 139±4.1 days (n = 11; 5D; Table2). In both cases survival was significantly longer (ANOVA; p<0.001) than for the single transgenic littermates TgF35x1071 PrP^o/AF and TgF35x1071 PrP^o/AF. In both paradigms one Prnp allele sufficed to fully suppress the phenotype of the toxic mutant (data not shown).

Histological analysis of terminally sick Tg1046 PrP^o/CDs mouse brains revealed astrogliosis both in the corpus callosum (not shown) and in the cerebellar white matter (Fig. 5E) while TgF35 PrP^o/AF mice displayed additional severe cerebellar granule cell...
Figure 3. Survival and histological phenotype of PrP/Dpl chimeric mice. (A) Survival of transgenic mice. The longevity of both PrP_Dpl and CD_Dpl mice was unaffected by their endogenous Prnp status. All mice survived longer than Nagasaki mice and did not develop clinically apparent pathologies. Each line represents data derived from ≥8 individuals. (B–R) Histopathological changes in 60 week old wt (1st column from left), PrP^{PrP_Dpl}_1026, Tg1026 (2nd column), PrP^{PrP_Dpl}_1071 (3rd column), and PrP^{NGsk/NGsk} mice (4th column). Panels B–I represent GFAP immunostains of the cerebellum (1st row) and of the corpus callosum (2nd row), whereas panels J–M depict paraphenylene diamine-stained semithin sections of the mid-thoracic spinal cord (3rd row) and sciatic nerve (4th row). PrP^{PrP_Dpl}_Tg1026 mice showed mild cerebellar astrogliosis (D), whereas PrP^{NGsk/NGsk} mice had additional vacuolar white matter changes (arrows) and Purkinje cell loss (E). No pathological changes were seen in Tg1026 PrP^{PrP_Dpl}_Tg1026 (C), Tg1025 PrP^{CD_Dpl} (not shown) and wt mice (B). Vacuolar white matter pathology and astrogliosis in the corpus callosum of PrP^{NGsk/NGsk} mice (I) but not in wt (F), PrP^{PrP_Dpl}_Tg1026 (G) and PrP^{CD_Dpl} mice (H). Semithin sections revealed coarse vacuolar degeneration of myelinated fiber tracts in PrP^{NGsk/NGsk} mice (M, R), whereas no such changes were observed in wt (J, O), PrP^{PrP_Dpl}_Tg1026 (K, P) and PrP^{CD_Dpl} mice (L, Q). Arrows: areas with axonal loss; arrowheads: axons with degenerated myelin sheaths (M, R). Scale bars: 100 μm in panels B–I; 25 μm in panels J–R.

doi:10.1371/journal.pone.0006707.g003
Milder white-matter changes and much less severe CGC loss (Fig. 5H). Western blot analysis of brain homogenates indicated that expression levels of the various transgenic proteins were unchanged in the compound transgenic mice independently of the respective combination. The steady-state levels of CD_Dpl exceeded those of PrP_DCD PrP_Dpl and PrPwt (Fig. 5K, M), whereas those of PrP_Dpl and PrP_DCD were similar and much lower than those of PrP_Dpl (Fig. 5L, N). Although expression of CD_Dpl was higher than that of PrP_Dpl, compound PrP_DCD PrP_Dpl and PrP_DCD PrP_Dpl mice displayed longer survival than PrP_DCD PrP_Dpl and PrP_DCD PrP_Dpl mice, CD_Dpl seemed to be less effective than PrP_Dpl to suppress cerebellar granule cell loss. This finding may point to a specific function of the amino proximal regions in suppressing neurodegeneration.

In order to address the functionality of PrP_DCDs, we intercrossed Tg42 PrP_DCDs and Tg1046 PrP_DCD mice and monitored the offspring for clinical signs of disease. Tg1046 × Tg42 PrP_DCD_Dpl were found to develop first signs of disease at 18–20 days post birth, and reached terminal disease at 25 ± 0.71 days of age (n = 22; Fig. 4A and Table 2). Double transgenic Tg1046 × Tg42 PrP_DCD_Dpl mice survived for 25 ± 1.9 days. Hence there was no significant difference in survival. All single or double transgenic mice coexpressing PrP_DCDs, Tg1046 × Tg42 PrP_DCDs, and Tg1046 × Tg42 PrP_DCD_Dpl survived to old age without any signs of clinical disease, indicating that PrP_DCDs does not diminish the potential of PrP_DCD induced toxicity. In contrast, PrP_Dpl and PrP_DCD were previously shown to compete for the rescue effect of PrP_Dpl in double transgenic mice Tg1046 × TgF35 PrP_DCD_Dpl [10]. We therefore conclude that removal of the lipid anchor from PrP_DCD completely abolishes its neurotoxic properties.

Discussion

The results presented here confirm and extend a recent report that fusion of the complete amino-terminus of PrP detoxifies Dpl.
Figure 5. Survival of compound transgenic mice. Survival of compound transgenic mice derived from intercrosses between the transgenic lines described above. (A–D) Survival curves of mice lacking PrPC and expressing various transgenes (PrP_D, PrP_ACD, CD_Dpl, PrP_Dpl) as indicated by the subscripts. Each line summarizes the survival animals with the respective genotype (group size: 6–16 as indicated). (E–J) Comparison of histopathological phenotypes in terminally sick PrP_{oD}D mice showed astrogliosis both in cerebellar cortex and white matter (E). Milder changes were present in Tg1046 Tg1071 PrP_{oD}D mice (F). (G) Subtotal granule cell loss associated with severe astrogliosis was seen in the cerebellum of TgF35 Tg1026 PrP_{oD}D mice (H). However, granule cell loss and astrogliosis was less severe in TgF35 Tg1026 PrP_{oD}D mice (I) and almost absent from TgF35 Tg1026 PrP_{oD}D mice (J). Scale bar = 5 μm. (K–N) Brain expression of PrP and transgenic PrP deletion mutant as well as PrP/Dpl fusion proteins. Specific bands are indicated with arrowheads (K). The expression of CD_Dpl was higher than that of PrP_{ACD} and PrP_{D}. Lanes 1–3 represent a serial dilution of a Tg1046 Tg1071 PrP_{ACD}Dpl mouse compared to a PrP_{ACD}Dpl mouse (lanes 4–6). (L) PrP_Dpl expression is similar to that of PrP_{ACD} and significantly lower than that of PrP_{D}. Lanes 1–3 represent serial dilutions of Tg1046 Tg1026 PrP_{Dpl}D mouse. The asterisk indicates a carboxy terminal fragment formed from wild-type PrPC. (M) Indirect comparison indicates similar PrP_{DF} and CD_Dpl levels in TgF35 Tg1071 PrP_{DF}Dpl mice which were higher than those of PrP_{DF}. Lanes 1–4 depict a serial dilution of a TgF35 Tg1026 PrP_{DF}Dpl mouse compared to a TgF35 Tg1026 PrP_{DF}Dpl mouse (lanes 4–6). All brain homogenates were treated with PNGase F, and replica western blots were decorated with antibodies POM1, POM3, and POM11 as indicated below each blot.

doi:10.1371/journal.pone.0006707.g005
Tg(PrPN-Dpl) mice expressing a fusion protein consisting of amino acids 1–124 of PrP and amino acids 58–179 of Dpl failed to show Dpl typical neurological disorder and were able to prolong the onset of ataxia in mice with exogenous Dpl expression [24]. By generating chimeric proteins that contain either the entire amino-terminus of PrP linked to the carboxy-terminus of Dpl (PrP_Dpl) or the central domain of PrP alone (CD_Dpl), we found specific domains within the amino-terminus of PrP that are involved in the detoxification of Dpl in two distinct brain regions and cell types. While PrP_Dpl showed no signs of cerebellar granule cell degeneration for at least 60 weeks, PrP_CD_Dpl mice displayed mild ataxia within the CCG layer. This may point to some residual neurotoxicity of CD_Dpl. In contrast, white matter degeneration was observed in Dpl-expressing Nsgk mice yet was not seen in mice expressing either of the two transgenes, PrP_Dpl and CD_Dpl. Since leukoencephalopathy is the major life-shortening pathology associated with expression of truncated PrP and Dpl [10,33], both addition of the whole amino-terminus, or addition of the central domain alone resulted in a normal life expectancy in transgenic mice.

In addition to detoxifying Dpl, chimeric fusion proteins were able to partially antagonize the toxic effects of the PrP deletion mutants PrP_{AF} and PrP_{ACD}. While PrP_Dpl was able to antagonize cerebellar granule cell loss in PrP_{AF} mice, CD_Dpl was not. Cerebellar white matter gliosis was milder in both PrP_{AF} CD_Dpl and PrP_{AF} PrP_{Dpl} mice. This lends further support to the conclusion that distinct domains within PrP exert neurotrophic functions in a variety of brain regions and cell types.

We have excluded that differences in expression level were responsible for the observed effects: Western blotting with antibody POM3 [31], which recognizes a domain common to both transgenes, showed a higher expression for CD_Dpl than for PrP_Dpl. All transgenic constructs were expressed using the same backbone, thereby reducing the likelihood of differential expression in distinct cell types. Thus the cell-specific effects of the different transgenes appear to be related to their structural features rather than to the levels or tissue-specific patterns of their expression.

Despite sequence homologies of <20%, the carboxy terminal domains of Dpl and PrP have very similar folding patterns of the respective carboxy proximal regions, whereas their amino proximal portions are much less structured [20,34,35]. Hence the selective permutations of the less structured domains of the two proteins performed here are not very likely to alter the overall global fold of the resulting fusion proteins. We found that both PrP_Dpl and CD_Dpl underwent correct intracellular sorting and posttranslational processing (Fig. 2A, C). Furthermore, in none of the transgenic mice (including the lines expressing the highest levels of transgene) did we detect any spontaneous formation of PK-resistant transgenic protein or PrP aggregates by Western blotting and histology (data not shown).

Further evidence for specific differences in the function of PrP_{AF} comes from the previously studies on transgenic mice expressing PrP_{AF} in a cell-type specific manner. While cerebellar granule cell loss in PrP_{AF} mice was reversed by neuronal expression of PrP, white matter degeneration was rescued by myelin-specific expression of PrP [36].

Cell-specific requirements for distinct PrP domains might explain the discrepancies regarding the domains reported to be involved in cytotoxic functions. Several studies suggest that the octapeptide repeat region is crucially linked to the neuroprotective functions of PrP_{AF} [37,38,39]. On the other hand, a feature common to all the toxic PrP deletion mutants is the lack of the central domain (encompassing at least residues 105–125) within PrP_{ACD}. This in turn points to a role of the central domain of PrP_{ACD}.

The results presented here may help clarifying this controversy. The central domain (aa 94–134) appears to be crucial for myelin maintenance, while other domains within the amino terminus (aa 23–94) may be required for neuroprotection. Residues 23–94 consist of the amino-terminal charged cluster (aa 23–89) involved in cytotrophic functions [40]. It will be interesting to study whether chimeric PrP/Dpl proteins exert PrP_{AF} like functional regulation of the NMDA receptor and whether central domain, octapeptide repeat region or amino-terminal charged cluster are involved in this function.

| Table 2. Survival of compound transgenic mice. |
|-----------------------------------------------|
| Crosses | Number of animals | Genotype | Average survival (days) | Standard deviation of mean (days) | Significance |
|---|---|---|---|---|---|
| Tg1046 | 22 | PrP^{+/+}_{ACD} | 25.2 | 0.7 | |
| Tg1046 × Tg1071 | 8 | PrP^{+/+}_{ACD} CD_{Dpl} | 42.9 | 3.3 | *** p < 0.001 |
| Tg1046 × Tg1026 | 6 | PrP^{+/+}_{ACD} PrP_{Dpl} | 36.3 | 1.3 | *** p < 0.001 |
| TgF35 | 15 | PrP^{+/+}_{AF} | 95.6 | 5.3 | |
| TgF35 × Tg1071 | 8 | PrP^{+/+}_{AF} CD_{Dpl} | 150.1 | 12.7 | *** p < 0.001 |
| TgF35 × Tg1026 | 11 | PrP^{+/+}_{AF} PrP_{Dpl} | 139.1 | 4.1 | *** p < 0.001 |
| Tg1046 | 7 | PrP^{+/+}_{ACD} | 27.3 | 0.6 | |
| Tg1046 × Tg42 | 10 | PrP^{+/+}_{ACD} CD_{Dpl} | 24.8 | 0.6 | Ns p > 0.05 |

Mice of various genotypes were housed and monitored according to a 4-degree clinical score system. Terminally sick animals were euthanized. Mean survivals of single-transgenic littermates were compared to double transgenic mice and statistical significance of difference was tested by ANOVA.

doi:10.1371/journal.pone.0006707.t002
It was suggested that homodimerization of PrP<sup>C</sup> mediates the transduction of extracellular signals [44,45,46]. The toxicity of truncated PrP and Dpl is counteracted by overexpression of full-length PrP<sup>C</sup> [9,10,18,19] and exacerbated by removal of the endogenous Prnp gene, suggesting that PrP<sup>C</sup> and its variants compete for a common interacting molecule. The PrP/Dpl fusion proteins appear to partake in this competition as well, as both CD<sub>Dpl</sub> and PrP<sub>Dpl</sub> prolonged survival of PrP<sup:o</sup> and PrP<sup>AF</sup> mice. Perhaps the CD region is responsible for stringent protein-protein interactions, whereas the structured carboxy termini of PrP and Dpl allow for more relaxed interactions and are therefore interchangeable. Such interactions might also include the formation of functionally relevant homodimers or homooligomers [47]. The residues 113-128 of PrP mediate interaction of PrP with stress inducible protein 1 (STI) [48] and heparan sulfate [49]. The incompleteness of the rescue in all tested paradigms of PrP<sup>o</sup>CDs CD<sub>Dpl</sub> PrP<sup>o</sup>CD PrP<sup>AF</sup> CD<sub>Dpl</sub> and PrP<sup>AF</sup> PrP<sub>Dpl</sub> mice may relate to insufficient amounts of the respective fusion proteins, or possibly to reduced affinity for their binding partners.

In addition to the findings described above, we extended our analysis of functional domains within PrP to those determining the localization of the protein. Mice expressing anchoreless PrP accumulate high titers of prions and protease-resistant PrP when challenged with scrapie [32,50], yet develop only subtle pathologies [51]. Here, anchoreless PrP<sub>AMCD</sub> was expressed to high levels in transgenic mice, and was very efficiently sequestered into the extracellular space of brain and in serum as a mature, fully glycosylated soluble form [52]. Although the deletion within PrP<sub>AMCD</sub> was identical to that of the neurotoxic membrane anchored PrP<sub>AMCD</sub>, it did not induce any pathology in transgenic mice, irrespective of the presence or absence of full-length PrP<sup>C</sup>. Since the total concentration of PrP<sub>AMCD</sub> in brain homogenates was as high as that of PrP<sub>AMCD</sub> and even higher than that of PrP<sub>AMCD</sub> in the serum, lack of toxicity was unrelated to its expression level. Also, PrP<sub>AMCD</sub> failed to influence the survival of PrP<sub>AMCD</sub> mice coexpressing PrP<sup>E</sup>, confirming that it exerts neither beneficial nor detrimental effects on the central nervous system.

PrP<sub>AMCD</sub> did not localize to detergent-resistant membrane (DRM) fractions, even when wild-type PrP<sup>E</sup> was coexpressed. This observation suggests that the genetic interaction between PrP<sup>E</sup> and its neurotoxic variants may physically necessitate membrane anchoring of all relevant partners. In contrast, soluble-dimeric prion protein (PrP-F<sub>C</sub>) was found to translocate to the DRM compartment and to associate with PrP<sup>E</sup> upon prion infection of mice coexpressing PrP<sup>E</sup> and PrP-F<sub>C</sub> [51]. In this context, it may be of interest to study the localization of PrP<sub>AMCD</sub> in prion infected mice.

In conclusion, the above findings indicate that (1) the amino proximal domain of PrP contains minimal elements that are necessary and sufficient for PrP function, that (2) distinct domains within the amino-terminus of PrP exert site- and/or cell-specific functions, and that (3) GPI membrane anchoring is mandatory for exerting said function. The understanding of the physiological and pathophysiological functions of the prion protein will benefit from functional analyses of the proteinaceous [48] and non proteinaceous [49] constituents interacting with PrP and its variants. Finally, it will be of particular interest to explore whether the phenomena studied here share functional and molecular aspects with the neurotoxicity observed in prion diseases [53].

Materials and Methods

Ethics Statement

All mice were maintained under specific pathogen-free (SPF) conditions. Housing and experimental protocols were in accordance with the Swiss Animal Protection Law and in compliance with the regulations of the Veterinariaamt, Kanton Zurich.

Construction of the transgenes

The coding region of murine Prnp and Prnd gene were analyzed using DNAMAN software (Lynnon Biosoft, Canada), and hydrophobicity plots were generated using a window of 9 amino acid residues. The regions identified in these plots were used to define the CC, CD and HC domains. The chimeric fusion proteins of PrP and Dpl were designed such that their hydrophobicity characteristics would mimic that of wild-type PrP. Based on pPrPHG [25], a Prnd/Nnil fragment was subcloned in the pMECA [54] backbone. To create the CD<sub>Dpl</sub> cDNA, mouse genomic cDNA was used as template to obtain two PCR fragments with primer sets JP1 (5′-ATA ATG ATG CAT ACC ATG AGC AAG ACC CGG CTG TGG AGC AC/JP2 (5′-TAC TGC CCC AGC TGC TGC GCG CCC TGC CAC ATG CTG GAG GTT GGT TTG TGG TTG GGT GCT GGT CTT GGT CC CCA CGT ATT ATG GGT ACC CCC TCC CCG GGC TTG CCT GTT GAA GG) and JP3 (5′-CCT GAA GGA TGT GGC AGG AGG TGC GGC AGC TGC AGT GGT GGG CCG TGG TGG TGG CTA CAT GCT GGG GAG CGC GTG CAG GCC CAT GAA GCT GTA CGC ATCG/JP4 (5′-ATA ATG CAT TTA CTT CAC AAT GAA CCA AGC)). The two initial products were fused in a third PCR with the flanking primers JP1 and JP4. This product was digested with Nsil and ligated to the Nnil sites of the pMECA vector containing the pPrPHG subcloned into the pMECA vector sequence into which a second Nsil site had been engineered. After confirming insertion with the correct orientation, the insert was cloned back into the pPrPHG backbone using the Prnd/Nnil sites.

PrP<sub>Dpl</sub> was created based on the plasmid pPrPHG [25]. A fragment (480 bp) was amplified using the primers pE2 (5′-CAA CCG AGC TGA AGC ATT CTG CCT)/X2 (5′-CCT GCT CAC GGC GCT CCC CAC GAT G) containing sequence information from Exon3 to codon 132/133 of the murine PrP. In a second PCR using genomic DNA as template and primers X3 (5′-GGG AGC GCC GAC ATC GAC)/X4 (5′-AAA GAA TTC CAT ACC ATG ATT GTA TCT TAT ATC CAC ATG) a fragment (360 bp) containing codon 68 until polyadenylation site of Dpl was amplified. After purification both fragments were cut with NotI and directly ligated into the pCR-Blunt II-Topo vector. The transgene was then excised with AgeI/EcoRI and, after blunting the 3′ EcoRI sites, ligated into the original AgeI/BbrI site of pPrPHG. The presence of the new insert was confirmed by restriction analysis using Nsil.

PrP<sub>AMCD</sub>, was generated using the pMECA Prnd/Nnil subclone pPrPHG previously described [10]. The oligonucleotide primers dCDSol5′ (5′-CCT ATT ATG ACG AGC GGA GAA GAT CCT GAT GAA CCG TGC TTT TCT CCT CCC-3′) dCDSol3′ (5′- GGA GGA GAA AAC CAC GGC GTT GTA TCA GGA GCT TCT CCT CCC GTC GTA ATG GG-3′) each complementary to opposite strands of the vector, were extended during temperature cycling by PfuTurbo DNA polymerase. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks was generated. After temperature cycling and treatment with DpnI to digest the parental DNA template and select for the desired DNA construct, the nicked vector DNA incorporating the mutations was transformed into E. coli. Clones were picked and sequenced. Finally the Prnd/Nnil fragment containing the desired point mutation was religated into the pPrPHG vector as described before [10].

Generation, Identification, and Maintenance of Transgenic Mice

The pPrPHG plasmids containing the PrP or Dpl coding sequences were propagated in E. coli XL1 blue, the minigene
excised with NdeI and SfiI, and processed as described [25]. Pronuclear injections into fertilized oocytes were carried out as described [55]. Transgenes on a Prnp<sup>o/o</sup> background were identified by PCR using the exon 2 primer pE2 (5′-CCA CCG ACC TGA AGC ATG CCT CCT C) and the exon 3 primer Ubl floxed Dpl (5′-CTC GCT GGT GGA GCT TGC TAT C) resulting in a PCR product of 618 bp for CD Dpl and 670 bp for PrP Dpl or pE2<sup>α</sup> and exon 3 primer Mut217 (5′-GCC AGG CCT TCT GTT ACC GGG TGA CCG) resulting in a PCR product of 619 bp. PCR analysis in order to verify the outbreeding of the Prnp<sup>α</sup> allele was carried out using primers P10 (Prnp exon 3, 5′-GTA CCC ATA ATC AGT GGA ACA AGC GCC GCA GC) and 18S rc (5′-CAA TT) giving a product of 352 bp for the Prnp<sup>α</sup> allele, and P3 and 3′ NC gave an 560 bp signal for the Prnp<sup>α</sup> allele, and P3 and 3′ NC gave a 562 bp product for the Prnp<sup>α</sup> allele. Alternatively, to test for the presence or absence of the Prnp<sup>α</sup> allele an additional PCR was performed using primers P2 (Prnp int 2, 5′-ATA CTT CGG ACC ACT GAT ACC TGG TTG CTC AT) and P10rev (reverse complementary of P10 5′-AAA TGT CTC GCT GGT GGA GAA AAC CAC GGT GGT CAC GGT) to give a product of 352 bp for the Prnp<sup>α</sup> allele. In order to distinguish between transgenic mice expressing Prp<sub>MC</sub> and Prp<sub>ACD+C</sub>, two separate PCR reactions were performed using primers pE2<sup>α</sup> and pCdRev (5′-GGA GGA GAA AAC CAC GGT GGT CCT) yielding a diagnostic amplicon of 666 bp, or using pE2<sup>α</sup> and pCdRev (5′-GGA GGA GAA AAC CAC GGT GGT CCA TCA) yielding a diagnostic amplicon of 666 bp.

Q-PCR to determine genomic copy numbers

Total genomic DNA was prepared from mouse tails after PK digestion and purified according to standard procedures. Copy numbers were assessed by Taqman PCR using 2 ng of total genomic DNA and primer pairs CD Sonde3′ (5′-GGA CCC AGC CAT AAT) and CD Sonde3′ (5′-GCC CTC CCC AGC ATG TAG) on C57B16, Tgα29, Prnp<sup>α/α</sup>, Tg1025, Tg9726 and Tg9727 mice. For determination of copy numbers of Tg40, Tg42 primer pairs p60 (5′-GCC TAC CCT AAC CAA GTG T) and p61 (5′-GAT CCT CTT CCG TCG TAA T) were used. To standardize Taqman PCR on GAPDH using primers GAPDH up (5′-CCA CCC CAG CAA GGA GAC T) and GAPDH down (5′-GAA ATT GTG AGG GAG ATG GT) was done in parallel.

mRNA analysis

Total brain RNA was isolated in Trizol (Life Technologies), purified and DNase treated according to the manufacturer’s manual (Roche). After reverse transcription (GeneAmp; Roche) cDNA was used for Taqman PCR using primer pairs Dpl Tag3′ (5′-CTG CGG GGC TAA CTA TTG/Tag3′ (5′-CTG CGG TGT GTC CAC) and Prp Tag3′ (5′-CAG TGG AAC CAG CCA CAC ACC/Prp Tag3′ (5′-CCC CAG CAT GTA GCC ACC). To standardize expression levels GAPDH using primers GAPDH up (5′-CCA CCC CAG CAA GGA GAC T) and GAPDH down (5′-GAA ATT GTG AGG GAG ATG CT) and 18S rRNA using primers 18S fw (5′-GTA ACC CCG TGA ACC CCG T) and 18S rev (5′-CCA CTT ACC AAT CGG TAG TAC) were used. Taqman PCR using SYBR-green (Roche) and determination of ΔACT-values were done on a Applied Biosystems 7900 device. As control for possible DNA contamination, DNase-treated RNA from wt and tg mice that had not been reverse transcribed was used.

Western blot analysis

Brain hemispheres were homogenized in 7 vol PBS, 0.5% Nonidet P-40, and 0.5% deoxycholate and the solution was centrifuged 5 min in an Eppendorf centrifuge. For deglycosylation, up to 50 μg denatured total protein were incubated at 37 °C for 4 h with 500 U PNGase F (New England Biolabs) according to the manufacturer’s instructions. The protease inhibitors Pefabloc (1 mg/ml), Leupeptin (10 μg/ml), Pepstatin (10 μg/ml), Aprotinin (1 μg/ml) (all from Boehringer, Mannheim), and 0.5 mg/ml EDTA were added. After electrophoresis of protein samples through 12% SDS-polyacrylamide gels, samples were transferred to nitrocellulose membranes (Schleicher & Schuell) and incubated with mouse monoclonal anti-PrP antibodies POM1, POM5 and POM11 [31], followed by incubation with peroxidase-labeled anti-mouse antiserum (1:2500; Amersham) and developed with the ECL detection system (Pierce). Antibody incubations were performed in 1% Top Block [Juro] in Tris-buffered saline-Tween (TBS-T) for 1 h at room temperature or overnight at 4 °C.

Flotation assays

Flotation of detergent insoluble complexes was performed as described [56]. Appropriate brain homogenates were extracted for 2 h on ice in cold lysis buffer (150 mM NaCl, 25 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1% Triton X-100; total protein: 1 mg in 1.6 ml. Extracts were mixed with two volumes (3.2 ml) of 60% Optiprep® (Nycomed) to reach a final concentration of 40%. All lysates were loaded at the bottom of Beckman ultracentrifuge tubes. A 5–30% Optiprep® step gradient in TNE (150 mM NaCl, 25 mM Tris-Cl, pH 7.5, 5 mM EDTA) was then overlaid onto the lysate (8.4 ml of 30% Optiprep® and 3.6 ml of 5% Optiprep®). Tubes were centrifuged for 24 h at 4 °C in a TLS55 Beckman rotor at 100,000 g. Fractions (1 ml) were collected from the top of the tube and processed for immunoblotting and visualization with anti-PrP antibody POM3 [31], anti-flotillin 1, and anti-GAPDH antibody (both BD Transduction Laboratories).

In order to release GPI anchored proteins from membranes, brain homogenates were treated for 2 h at 37 °C with 10 U/ml Phospholipase C (PI-PLC from Sigma) as described [37].

ELISA

PrP ELISA was performed as described in [58] 96-well plates (Nunc-Immuno Maxisorb; prod. no. 439454) were coated with 50 μL per well of POM1 (2 mg/ml, 1:5000 in 0.1 M sodium carbonate buffer pH 9.6 (1.56 g Na2CO3,2H2O, 0.1 g NaHCO3 in 500 ml H2O)) over night at 4 °C. All following incubation steps were made at room temperature. The plates were washed by immersing them 4–5 times in PBS with 0.1% Tween-20 (PBST). Plates were then incubated with 100 μL per well of blocking buffer (5% Top-Block in PBST) for two hours. A 1:3 dilution of recombinant murine PrP (rmPrP) (starting from 50 ng/ml) was used for a standard curve. Blood plasma from respective mice was diluted appropriately in sample buffer (1% Top-Block in PBST) and incubated for 1 h. Then, plates were washed 4–5 times in PBST and incubated with biotin-labeled POM2 (1 ng/ml, 1:5000 in sample buffer, 100 μL per well) for 1 h. Plates were washed 4–5 times and incubated with avidin-HRP (1 mg/ml, 1:1000 in sample buffer, 100 μL per well) for 1 h followed by another round of washing, 4–5 times in PBST and 2–3 times with PBS alone. Chromogenic substrate (Bio source, prod. no. SB02, 50 μL per well) was applied for up to 10 min. The reaction was stopped with 0.5 M H2SO4 and absorbance was read at 450 nm.

Clinical scoring and observation

Mice were examined once weekly for clinical signs as described previously [10]. Mice were euthanized when they reached a score of 3.5 or higher. Statistical significance was assessed as indicated.
Morphological analyses

Brains, spinal cords and sciatic nerves were removed and fixed in 4% formaldehyde in PBS, pH 7.5, paraffin embedded, and cut into 2-4 μm sections. Sections were stained with hematoxylin-eosin (H&E), Luxol-Nissl (myelin and neurons), and commercial antibodies to GFAP (glial fibrillary acidic protein; activated astrocytes), MBP (myelin basic protein), NF200 (neurofilament 200), IBA1 (microglia) and SAF94 (PrPSc- and PrPSc-negative aggregates). For semithin sections and electron microscopy mice were perfused with ice-cold 4% PFA/3.9% glutaraldehyde. Spinal cord tissues were removed, immersed in the same solutions, and kept in Phosphate buffer at 4°C until processing. Tissues were embedded in Epon, and semithin sections were stained with toluidine blue and para-phenylene diamine. Frozen sections for POM3 and Dpl staining were blocked with M.O.M Mouse IgG Blocking Reagent (Vector Laboratories) stained with anti Dpl GX-2D10-B1 (Dpl) or POM3 antibodies to GFAP (glial fibrillary acidic protein; activated astrocytes), MBP (myelin basic protein), NF200 (neurofilament) and specific cerebellar lesions. Cell 93: 203–214.

Supporting Information

Figure S1 Characterization of transgenic mice (A) Gene copy numbers per haploid genome in transgenic lines as determined by genomic Q-PCR. (B) relative mRNA level in brain extracts of transgenics compared to PrP mRNA in C57BL/6 mice (filled black columns and left y-axis) and compared Dpl mRNA in Prnp<sup>back/Nkg</sup> mice (open columns and right y-axis) using either PrP or Dpl specific primer sets for Q-PCR. Each column represents the average of 3 mice.

Found at: doi:10.1371/journal.pone.0006707.s001 (0.55 MB TIF)

Figure S2 Characterization of membrane anchored and PI-PLC treated transgenic proteins. Density gradient DRM preparations of wild-type, PrP GPI anchorless (PrP<sup>P<sub>C</sub></sup>), PrP<sup>ACD<sub>C</sub></sup> and PrP<sup>ACD<sub>R</sub></sup> transgenic brains analyzed after PI-PLC treatment and deglycosylation with PNGase F with monoclonal antibody POM1. After PI-PLC treatment PrP and PrP<sup>ACD<sub>C</sub></sup> had similarly buoyancy like PrPs and PrP<sup>ACD<sub>R</sub></sup>, whereas flotiltin a non GPI-anchored DRM associated protein still was found in fractions with higher buoyancy indicating the intactness of the DRMs.

Found at: doi:10.1371/journal.pone.0006707.s002 (0.84 MB TIF)

Acknowledgments

We thank Petra Schwarz, Rita Moos, Marianne König, Andrea Schifferli, Cinzia Tiberi, Li-Chun Infanger, and Dimitri Gourionov for technical assistance. We also thank Drs. Bruce Chesebro and Michael Oldstone for kindly providing PrP, mice.

Author Contributions

Conceived and designed the experiments: FB JP IR AA. Performed the experiments: FB TR JB. Analyzed the data: FB JP MT AA. Contributed reagents/materials/analysis tools: IR TR AA. Wrote the paper: FB JP JB MT AA.

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