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Posttranslational modifications (PTMs) are common among proteins that aggregate in neurodegenerative disease, yet how PTMs impact the aggregate conformation and disease progression remains unclear. By engineering knockin mice expressing prion protein (PrP) lacking 2 N-linked glycans (Prnp^{180Q/196Q}), we provide evidence that glycans reduce spongiform degeneration and hinder plaque formation in prion disease. Prnp^{180Q/196Q} mice challenged with 2 subfibrillar, non-plaque-forming prion strains instead developed plaques highly enriched in ADAM10-cleaved PrP and heparan sulfate (HS). Intriguingly, a third strain composed of intact, glycophosphatidylinositol-anchored (GPI-anchored) PrP was relatively unchanged, forming diffuse, HS-deficient deposits in both the Prnp^{180Q/196Q} and WT mice, underscoring the pivotal role of the GPI-anchor in driving the aggregate conformation and disease phenotype. Finally, knockin mice expressing triglycosylated PrP (Prnp^{187N}) challenged with a plaque-forming prion strain showed a phenotype reversal, with a striking disease acceleration and switch from plaques to predominantly diffuse, subfibrillar deposits. Our findings suggest that the dominance of subfibrillar aggregates in prion disease is due to the replication of GPI-anchored prions, with fibrillar plaques forming from poorly glycosylated, GPI-anchorless prions that interact with extracellular HS. These studies provide insight into how PTMs impact PrP interactions with polyanionic cofactors, and highlight PTMs as a major force driving the prion disease phenotype.

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Introduction
Amyloid plaques accumulate in the brain of patients with Alzheimer’s disease and certain familial prion diseases, and are often associated with a clinical course that progresses for more than 3 years (1–3). In contrast, in sporadic Creutzfeldt-Jakob disease (sCJD), prion aggregates more commonly form diffuse, synaptic, or plaque-like deposits in the brain and symptoms advance with extraordinary rapidity, with a median of 6 months from clinical onset to terminal disease (4–6). Similar to sCJD, in animal prion diseases, such as chronic wasting disease of cervids, prion aggregates frequently form diffuse or punctate deposits and the clinical phase is brief (7–11). Although the histopathology has been extensively characterized, the molecular mechanisms that drive the prion fold is brief (7–11). Although the histopathology has been extensively characterized, the molecular mechanisms that drive the prion fold

Posttranslational modifications (PTMs) are common among proteins that aggregate in neurodegenerative disease, yet how PTMs impact the aggregate conformation and disease progression remains unclear. By engineering knockin mice expressing prion protein (PrP) lacking 2 N-linked glycans (Prnp<sup>180Q/196Q</sup>), we provide evidence that glycans reduce spongiform degeneration and hinder plaque formation in prion disease. Prnp<sup>180Q/196Q</sup> mice challenged with 2 subfibrillar, non–plaque-forming prion strains instead developed plaques highly enriched in ADAM10-cleaved PrP and heparan sulfate (HS). Intriguingly, a third strain composed of intact, glycosphatidylinositol-anchored (GPI-anchored) PrP was relatively unchanged, forming diffuse, HS-deficient deposits in both the Prnp<sup>180Q/196Q</sup> and WT mice, underscoring the pivotal role of the GPI-anchor in driving the aggregate conformation and disease phenotype. Finally, knockin mice expressing triglycosylated PrP (Prnp<sup>309</sup>) challenged with a plaque-forming prion strain showed a phenotype reversal, with a striking disease acceleration and switch from plaques to predominantly diffuse, subfibrillar deposits. Our findings suggest that the dominance of subfibrillar aggregates in prion disease is due to the replication of GPI-anchored prions, with fibrillar plaques forming from poorly glycosylated, GPI-anchorless prions that interact with extracellular HS. These studies provide insight into how PTMs impact PrP interactions with polyanionic cofactors, and highlight PTMs as a major force driving the prion disease phenotype.

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PrP has the highest affinity to heparin and that each additional glycan decreases the binding affinity. Finally, using knockin mice that express PrP having a third N-linked glycan, we show a complete reversal of a typical plaque-forming disease phenotype, as mice instead developed primarily subfibrillar aggregates that lack HS binding and showed a rapid disease progression. Collectively, these studies support the conclusion that PrP glycans can impede fibril formation, potentially through reduced binding of shed, glycosylated PrP to extracellular HS, and suggest that the PTMs on PrP contribute to the profoundly rapid clinical progression observed in the majority of prion diseases.

Results

Prnp<sup>180Q/196Q</sup> mice generated using the CRISPR-Cas system show normal PrPC expression and trafficking. Mice expressing unglycosylated PrP<sup>C</sup> were generated using a single guide RNA to create 2 point mutations in the endogenous Prnp locus, corresponding to asparagine to glutamine substitutions at positions 180 and 196 (mouse PrP numbering) thereby altering the N-glycosylation sequons. Six founder lines were generated, 2 were sequenced, and 1 (line 191) was selected and bred to homozygosity on a C57BL/6 background. Prnp<sup>180Q/196Q</sup> mice developed normally with no clinical signs or histologic lesions observed in the brain (n = 12 mice, 150–639 days of age) (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI131564DS1). PrP<sup>C</sup> was expressed in the brain at levels similar to WT mice, and the unglycosylated forms showed an equivalent electrophoretic mobility (Figure 1A). To assess the PrP<sup>C</sup> distribution in neurons, cortical neurons were isolated from Prnp<sup>180Q/196Q</sup> and WT mice, immunolabelled for PrP, and evaluated by confocal microscopy, revealing an indistinguishable PrP distribution throughout the neuronal cell body and neurites (Figure 1B).

PrP<sup>C</sup> is GPI-anchored in the outer leaflet of the cell membrane, yet whether unglycosylated PrP traffics to the cell membrane has been controversial and may depend on the specific amino acids substituted into the N-linked glycan consensus sequence, Asn-X-Ser/Thr (39). Asparagine-to-glutamine substitutions were chosen here due to their structural similarity, differing only by a single methylene. To quantify surface expression in primary cortical neurons isolated from WT and Prnp<sup>180Q/196Q</sup> mice, phosphatidylinositol-specific phospholipase C (PIPLC) hydrolysis was performed on live primary neurons to cleave the GPI-anchor. Similar levels of cleaved PrP were detected in the media, indicating that unglycosylated PrP<sup>180Q/196Q</sup> localized to the cell surface (Figure 1C). To further confirm that PrP<sup>180Q/196Q</sup> traffics to the cell surface, PrP-deficient RK13 cells were transfected with Prnp<sup>WT</sup>, Prnp<sup>180Q/196Q</sup>, or Prnp<sup>180A/196A</sup>, a mutant reported to show impaired PrP trafficking (40). Again PrP<sup>180Q/196Q</sup> was released into the media by PIPLC similar to WT PrP<sup>C</sup>, whereas only approximately 40% of PrP<sup>180A/196A</sup> was released, supporting that PrP with asparagine-to-glutamine substitutions traffics to the cell surface (Supplemental Figure 1B).

To assess whether unglycosylated PrP localizes to lipid rafts in

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**Figure 1. PrP<sup>180Q/196Q</sup> traffics similarly to WT PrP<sup>C</sup> in primary neurons and in mice.** (A) Representative Western blot of PNGase-F–treated brain extracts from age-matched Prnp<sup>180Q/196Q</sup> and WT mice reveal similar PrP<sup>C</sup> expression levels (quantified in right panel) (100–250 day old mice); n = 4/group. (B) PrP immunocytochemistry shows that unglycosylated PrP<sup>180Q/196Q</sup> traffics to neuronal processes in primary cortical neurons, as does PrP in WT neurons; n = 3 experiments. Scale bars: 10 μm. (C) Representative Western blots of phospholipase C–cleaved (PIPLC-cleaved) PrP<sup>180Q/196Q</sup> and WT PrP from the surface of cortical neurons show that surface PrP<sup>C</sup> levels are similar (media); n = 3 experiments. The additional band in the media (~23 kDa) may be a cleaved form of PrP. (D) PrP<sup>180Q/196Q</sup> and WT PrP<sup>C</sup>, together with flotillin, localize to detergent-resistant membranes in the brain; n = 3/group. Unpaired, 2-tailed Student’s t test, no significant differences (A and C).
WT and PrP<sub>180Q/196Q</sub> brain extracts were treated with low PK concentrations, which revealed no differences in PrPC digestion (Supplemental Figure 1C). Last, to test for insoluble PrP, brain homogenates were ultracentrifuged, which revealed that PrP<sub>180Q/196Q</sub> was highly soluble, similar to WT PrP<sub>C</sub> (approximately 92% and 95% soluble, respectively), and differed from a prion-infected brain (approximately 22% soluble) (Supplemental Figure 1D). Thus, Prnp<sup>180Q/196Q</sup> mice show no evidence of aggregated PrP, consistent with their normal lifespan and lack of neurologic disease.

Prnp<sup>180Q/196Q</sup> mice infected with 4 prion strains develop plaque and plaque-like deposits. We next assessed the susceptibility of the Prnp<sup>180Q/196Q</sup> mice to prion infection.

Aged Prnp<sup>180Q/196Q</sup> mice show no evidence of spontaneous prion disease. To determine whether unglycosylated PrP<sup>C</sup> spontaneously aggregates, we histologically and biochemically investigated 12 aged Prnp<sup>180Q/196Q</sup> mice. Brain was immunolabelled for PrP and showed no evidence of prion aggregation (Supplemental Figure 1A). To test for the presence of proteinase K-resistant (PK-resistant) PrP, WT and PrP<sub>180Q/196Q</sub> brain extracts were treated with low PK concentrations, which revealed no differences in PrP<sup>C</sup> digestion (Supplemental Figure 1C). Last, to test for insoluble PrP<sup>C</sup>, brain homogenates were ultracentrifuged, which revealed that PrP<sup>C</sup> was highly soluble, similar to WT PrP<sup>C</sup> (approximately 92% and 95% soluble, respectively), and differed from a prion-infected brain (approximately 22% soluble) (Supplemental Figure 1D). Thus, Prnp<sup>180Q/196Q</sup> mice show no evidence of aggregated PrP, consistent with their normal lifespan and lack of neurologic disease.

Prnp<sup>180Q/196Q</sup> mice infected with 4 prion strains develop plaque and plaque-like deposits. We next assessed the susceptibility of the Prnp<sup>180Q/196Q</sup> mice to prion infection. Prnp<sup>180Q/196Q</sup> and WT mice...
were inoculated intracerebrally with 4 mouse-adapted prion strains known as RML, 22L, ME7, and mCWD. RML, 22L, and ME7 are subfibrillar strains derived from sheep scrapie and show no fibrils ultrastructurally in situ (41–43), whereas mCWD is a mouse-adapted fibrillar strain derived from deer chronic wasting disease that is characterized by dense plaques composed of bundles of long, extracellular fibrils (44). All RML- and 22L-inoculated Prnp180Q/196Q mice developed prion disease. Following inoculation with RML prions, WT and Prnp180Q/196Q mice showed no significant difference in survival times on first passage (WT: 164 ± 1 days after inoculation [dpi]; Prnp180Q/196Q: 160 ± 3 dpi [mean ± SEM]), and a modest decrease in survival on second passage in Prnp180Q/196Q mice (148 ± 5 dpi) (Figure 2A). Following inoculation with 22L prions, Prnp180Q/196Q mice showed a prolonged survival (WT: 127 ± 0 dpi; Prnp180Q/196Q: 155 ± 5 dpi), which was unchanged on second passage in Prnp180Q/196Q mice (155 ± 6 dpi) (Figure 2A). In contrast, there was a significant transmission barrier following inoculation with ME7 prions, as there was an incomplete attack rate (6/7 mice, 86%) and a highly variable and significantly prolonged incubation period (WT: 169 ± 1 dpi; Prnp180Q/196Q: 389 ± 27 dpi). Yet by the second passage, the attack rate was 100% and the incubation period decreased by 60% (142 ± 5 dpi), and by third passage the incubation period was similar to ME7 in WT mice (177 ± 5 dpi), suggestive of strain adaptation. Similarly, the fibrillar mCWD prions led to an incomplete attack rate on first passage (5/9, 56%), suggestive of a transmission barrier, although the incubation period in WT mice was longer (WT: 569 ± 7 dpi; Prnp180Q/196Q: 508 ± 24 dpi) (Figure 2A). Notably, most Prnp180Q/196Q mice positive for mCWD prions (4 of 5 positive mice) had low PrPSc levels in brain and were euthanized due to age (n = 3) or concurrent disease (n = 1), thus were not likely at terminal disease stages.

A comparison of the histopathologic lesions in RML-, 22L-, ME7-, and mCWD-infected WT and Prnp180Q/196Q mice revealed intriguing differences in spongiform degeneration, gliosis, and PrPSc distribution (Figure 2, B and C). First, the spongiform degeneration was consistently scored as more severe for all 3 strains in the Prnp180Q/196Q mice as compared with the WT mice (Figure 2B). Additionally, in the Prnp180Q/196Q mice infected with ME7 prions, the hippocampus was consistently marked by atrophy due to severe neuronal loss, with complete loss of the CA1 pyramidal neurons and numerous gemistocytic astrocytes, which persisted upon second passage, whereas WT mice showed a moderate loss of hippocampal neurons (Supplemental Figure 2A). Finally, the cerebellum lacked PrPSc in all infected Prnp180Q/196Q mice, a striking difference from WT mice in which all 3 strains were present in the cerebellum (Figure 2, C and D, region 2). This was not due to a lack...
ME7 showing the highest stability. the 3 unglycosylated prions correlated with plaque formation, with μm) (Figure 2B). In the mCWD prions form large parenchymal plaques. In WT mice, RML and 22L prions typically form diffuse, ME7 prions form diffuse and small plaques (>100 μm) (Figure 2B). In the Prnp180Q/196Q mouse brain, the RML prion deposits remained mostly diffuse and occasionally formed plaque-like clusters (Figure 2B). In contrast, 22L and ME7 prions formed diffuse and significantly larger plaques (up to 100 μm for ME7) that were congophilic (Figure 2, B and E; Supplemental Figure 2, C and D). There were also numerous florid ME7 plaques that were surrounded by small vacuoles, similar to those described in variant CJD-infected human brains (45) (Supplemental Figure 2, C and D). There were also numerous florid ME7 plaques that were surrounded by small vacuoles, similar to those described in variant CJD-infected human brains (45) (Supplemental Figure 2E). Notably, large mCWD plaques were morphologically identical in the WT and Prnp180Q/196Q mice (Figure 2B). Thus the Prnp180Q/196Q mice challenged with the 3 subfibrillar prion strains shared certain newly acquired disease features, including an increase in plaques and plaque-like structures, more severe cortical spongiosis, and a notable lack of prions in the cerebellum.

PrP biochemistry was altered in Prnp180Q/196Q mice. To assess the differences in the biochemical properties of the unglycosylated prions, we first assessed the presence of PK-resistant PrP in all Prnp180Q/196Q mice. All RML- and 22L-challenged and some ME7- and mCWD-challenged mice showed PK-resistant PrP (Figure 3A). The solubility and stability of RML, 22L, and ME7 PrP were assessed (mCWD prion levels were too low to assess). Notably, the insoluble PrP levels were similar for RML- and 22L-infected WT and Prnp180Q/196Q mice, yet for ME7, we found that further passage in the Prnp180Q/196Q mice was associated with significantly increased levels of insoluble PrP in the WT and Prnp180Q/196Q mice (Figure 3B, second passage).

To next measure the aggregate stability, aliquots of brain homogenate were first denatured with guandine hydrochloride (GdnHCl) ranging from 0 to 6 M, and then diluted and digested with PK. The PrP was measured by ELISA to determine the [GdnHCl]1/2 concentration at which half the PrP remained. While there were no stability differences for RML and 22L prions in the WT and Prnp180Q/196Q brains, ME7 prions were more stable in the Prnp180Q/196Q brain (Figure 3C). Therefore, the aggregate stability of the 3 unglycosylated prions correlated with plaque formation, with ME7 showing the highest stability.

To further probe the conformation of unglycosylated PrP, we next applied a conformationally sensitive amyloid probe, heparin-binding affinity. Why does ADAM10-cleaved, unglycosylated PrP have a higher tendency to form fibrils than glycosylated PrP? We previously found high levels of HS bound to ADAM10-cleaved PrP having 0 to 1 glycan (51). We reasoned that unglycosylated PrP may bind with higher affinity to extracellular HS due to a lack in electrostatic repulsion between the anionic glycans and HS. To test this hypothesis, we assessed how PrP glycans impact the binding affinity to heparin, a highly sulfated surrogate of HS, using affinity chromatography to test this hypothesis, we assessed how PrP glycans impact the binding affinity to heparin, a highly sulfated surrogate of HS, using affinity chromatography and PrP with 0, 1, 2, or 3 glycans from transfected RK13 cells. PrPc was cleaved from the cell surface by PPLC and applied to the heparin sepharose column. While PrPc with 2 to 3 glycans showed a low affinity to heparin, unglycosylated PrPc showed a high affinity to heparin; the affinity progressively decreased with each additional glycan (Figure 6A). Notably, even within the glycoform mixtures of WT PrPc (0–2 glycans), the unglycosylated PrPc showed the highest affinity to heparin (Figure 6B).

To determine whether brain-derived unglycosylated PrP also shows a higher affinity to heparin, we next tested the heparin-binding affinity of PrP from age-matched WT and Prnp180Q/196Q...
mouse brain homogenates (Figure 6C). Again we found that unglycosylated PrPSc had a higher heparin-binding affinity than glycosylated WT PrPC (Figure 6, C and D), which also held true for their ADAM10-cleaved counterparts. Interestingly, for both genotypes, ADAM10-cleaved PrP showed a higher heparin-binding affinity than full-length PrP (Figure 6E), suggesting that the GPI anchor with the 3 C-terminal amino acids markedly reduces heparin binding. Collectively, these data suggest that N-linked glycans and the GPI-anchor hinder PrPC binding to HS.

Unglycosylated, ADAM10-cleaved PrP binds HS in vivo. Sulfated glycosaminoglycans (GAGs) promote fibril assembly in vitro (57, 58) and the sulfated GAG, HS, codeposits with amyloid beta and prion plaques in vivo (59, 60). However, whether HS preferentially bind to unglycosylated prions is unknown. To determine whether the 22L, ME7, and mCWD plaques harbor HS in the Prnp180Q/196Q mice, we immunolabelled prion-infected brain sections for HS using the 10E4 antibody, which recognizes an N-sulfated glucosamine residue and does not react with hyaluronan, chondroitin sulfate, or dermatan sulfate. We found that HS clearly localized to prion plaques in Prnp180Q/196Q mice, as compared with only a low level of diffuse HS stain in RML-, 22L-, and ME7-infected WT mice (Figure 6F, Supplemental Figure 3B) and RML-infected Prnp180Q/196Q mice (Supplemental Figure 3B). Pretreating brain sections with heparin lyases abolished the HS immunolabelling (Supplemental Figure 3C), supporting specificity of the antibody binding.

Mass spectrometry was next used to quantify HS bound to ME7 prions from Prnp180Q/196Q and WT brain. ME7 was selected due to the abundance of plaques in the Prnp130Q/136Q mice. PrPSc was purified by solubilization and multiple rounds of ultracentrifugation (61), levels were quantified against a recombinant PrP standard, and the sample was denatured with sodium hydroxide. HS chains were then depolymerized with a cocktail of heparin lyases, the resulting disaccharides were [12C6] aniline-tagged, and disaccharides were identified by liquid chromatography-mass spectrometry (LC-MS) using [13C6] aniline-tagged disaccharide standards (62, 63). The HS relative to PrP level was calculated for each sample (HS/PrP ratio [μg/μg]). Consistent with the HS immunostaining results, we found significantly higher levels of HS bound to unglycosylated PrPSc compared with WT PrPSc (Figure 6G). In addition, the composition of HS bound to ME7 in the WT versus Prnp180Q/196Q brain differed, as HS was generally less sulfated in the Prnp180Q/196Q brain, with lower levels of N-sulfated and 6-O sulfated HS (Figure 6H). Interestingly, the HS composition in the whole brain lysates from prion-infected WT and Prnp180Q/196Q mice was similar. This finding suggests that the observed composition differences in PrPSc-bound HS were not due to overall differences in HS biosynthesis, but were instead due to PrPSc selectively binding to specific HS molecules (Figure 6I).

A third glycan on PrP reverses the prion disease phenotype. We reasoned that since unglycosylated PrPSc had a higher heparin-binding affinity than glycosylated WT PrPSc (Figure 6, C and D), which also held true for their ADAM10-cleaved counterparts. Interestingly, for both genotypes, ADAM10-cleaved PrP showed a higher heparin-binding affinity than full-length PrP (Figure 6E), suggesting that the GPI anchor with the 3 C-terminal amino acids markedly reduces heparin binding. Collectively, these data suggest that N-linked glycans and the GPI-anchor hinder PrP binding to HS.

Figure 4. Fluorescence lifetime (FLIM) decay of h-FTAA bound to prion aggregates. (A) Fluorescence lifetime images and (B and C) intensity-weighted mean lifetime (ti) distributions of h-FTAA–stained RML, 22L, and ME7 prion plaques in Prnp130Q/136Q (red) or WT (blue) brain show that the FLIM decay curves in Prnp130Q/136Q brain differ from those in WT brain and reveal 3 different aggregate conformers in prion-infected Prnp130Q/136Q mice. The decay data were collected with the excitation wavelength set at 490 nm. The color bar represents lifetimes from 200 ps (orange) to 1000 ps (blue) and the images are color coded according to the representative lifetime. Decay curves were collected from 5–10 prion deposits from a minimum of n = 3 mice/strain. Scale bar: 20 μm.
ease development. We next challenged mice with a strain known to form long fibrils, mCWD (44). Strikingly, despite an amino acid substitution expected to lead to a transmission barrier and prolong survival, the Prnp 187N mice developed a profoundly accelerated disease (326 ± 9 dpi), which was approximately 60% of the incubation period of mCWD in WT mice (Figure 7A). Remarkably, on second passage, the incubation period decreased even further to 194 ± 7 dpi, approximately 35% of the WT incubation period (Figure 7A). This disease acceleration was likely due to the additional glycans and not to a difference in the amino acid side chain, as PrPC-PrPSc sequence differences typically prolong survival.

To investigate whether the Prnp 187N mice harbored the large plaques characteristic of mCWD in WT mice, we assessed the histopathological phenotype. We found a notable lack of large plaques in the Prnp 187N mice, and instead only diffuse aggregates and rare small plaques in the corpus callosum (WT-infected mCWD) were shown. Scale bar: 50 μm.

Figure 5. Increased ADAM10-cleaved PrPSc in prion-infected Prnp 180Q/196Q mice. (A) Western blots reveal 22L- and ME7-infected Prnp 180Q/196Q mice harbor higher levels of ADAM10-cleaved PrPSc in the brain compared with WT mice; n = 3/group. Note that the PK-digested ADAM10-cleaved PrP runs at a lower molecular weight due to lack of the GPI-anchor and terminal 3 amino acids. *P ≤ 0.05, unpaired, 2-tailed Student’s t test. (B) Western blots show that uninfected Prnp 180Q/196Q and WT mice have similar levels of ADAM10-cleaved/total PrP in the brain; n = 5 WT and 11 Prnp 180Q/196Q mice. (C) Brain sections immunolabeled for PrP with SAF84 (labelled PrP) or with sPb228 antibody (labelled ADAM10-cleaved PrP) reveal ADAM10-cleaved PrP localizes to plaque-like and plaque deposits in ME7-infected Prnp 180Q/196Q, mCWD-infected Prnp 180Q/196Q, and WT brain sections. Note that the diffuse aggregates are not labelled by the sPb228 antibody. Cortex (ME7-infected WT), thalamus (ME7-infected Prnp 180Q/196Q), hippocampus (mCWD-infected Prnp 180Q/196Q), and corpus callosum (WT-infected mCWD) are shown. Scale bar: 50 μm.
Figure 6. PrP glycans hinder binding to HS. (A) Immunoblots of heparin affinity chromatography experiments assessing variably glycosylated PrP. Relative levels of PrP from each elution are shown in the graphs. Differences in the binding affinity are most notable in the 0.5 M NaCl elution (red arrow). Asterisk color indicates the mutants with significant differences. The triglycosylated PrP isoform level was low in RK13 cells; n = 3–4 experiments. (B) Among the WT PrP glycoforms, diglycosylated PrP has a lower heparin affinity than mono- or unglycosylated PrP (unbound is PrP in the flow-through); n = 5 experiments, 4 also included in A. (C) Affinity chromatography of the soluble brain fraction reveals that total and ADAM10-cleaved PrP180Q/196Q have significantly higher heparin affinity than the corresponding WT PrP. ADAM10-cleaved PrP shows a second band (blue arrow) that corresponds to ADAM10-cleaved PrPC. (D) Quantification of PrP shown in C; n = 3/strain. (E) ADAM10-cleaved PrP has a higher heparin-binding affinity than total PrP for both WT PrP and PrP180Q/196Q. (F) Immunolabelling reveals HS colocalizes to ME7 plaques in the Prnp180Q/196Q brain only and to mCWD plaques in both the WT and Prnp180Q/196Q brain; n = 4/strain. Scale bar: 50 μm. (G) LC-MS reveals approximately 6-fold more HS bound to unglycosylated ME7 PrPSc than to highly glycosylated ME7 PrPSc (WT); n = 3/strain. (H) Composition analysis of HS bound to purified PrPSc (ME7) reveals less N-sulfated (NS) and 6-O sulfated (6-O) HS bound to unglycosylated (Prnp180Q/196Q) as compared with glycosylated (WT) PrPSc; n = 3/group. (I) The overall HS composition in ME7-infected Prnp180Q/196 and WT whole-brain lysates are similar; n = 3/group. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001; 2-way ANOVA with Bonferroni’s post hoc test (A, D, E, and H), *P < 0.05; 1-way ANOVA with Tukey’s test (B), **P ≤ 0.01, unpaired, 2-tailed Student’s t test (G).
narily rapid disease progression, and the molecular determinants that underlie these disease differences are unclear. Our work here indicates that the PrP glycans and GPI-anchor, together with HS, orchestrate major components of the disease phenotype in prion disease. The absence of glycans was linked to the formation of parenchymal plaques, particularly for prions lacking a GPI-anchor. Therefore, our results support a model in which the PrP posttranslational state markedly influences the fate of a prion aggregate, with cleaved, unglycosylated prions efficiently binding to extracellular HS to form parenchymal plaques.

The molecular mechanisms that underlie extracellular fibril formation in prion disease have not been previously identified and tested. Our approach to determining these mechanisms was

**Discussion**

In contrast to Alzheimer’s disease, amyloid plaques in the brain are rare in prion disease and are often associated with longer disease durations, as observed in Gerstmann–Sträussler–Scheinker disease, for example (3). Prions more commonly form diffuse, synaptic, or punctate aggregates associated with an extraordinarily rapid disease progression, and the molecular determinants that underlie these disease differences are unclear. Our work here indicates that the PrP glycans and GPI-anchor, together with HS, orchestrate major components of the disease phenotype in prion disease. The absence of glycans was linked to the formation of parenchymal plaques, particularly for prions lacking a GPI-anchor. Therefore, our results support a model in which the PrP posttranslational state markedly influences the fate of a prion aggregate, with cleaved, unglycosylated prions efficiently binding to extracellular HS to form parenchymal plaques.

The molecular mechanisms that underlie extracellular fibril formation in prion disease have not been previously identified and tested. Our approach to determining these mechanisms was
promoted by studies of knockin mice expressing unglycosylated PrP, known as G3 mice, which revealed that glycosylation is not essential for prion conversion or transmission of infectivity (21, 24). G3 mice also showed a tendency to form thioflavin-positive plaques, suggestive of fibrillar prions (24). Similar to the G3 mice, transgenic mice expressing GPI-anchorless PrP\(^c\) also develop fibrillar, thioflavin-positive prion aggregates, but fibrils accumulate perivascularly (19). Our use of prion-infected knockin mice expressing unglycosylated and triglycosylated PrP\(^c\), together with WT mice, also reveal a correlation between a lack of PTMs and fibrillar prion formation. Using recently developed antibodies that recognize ADAM10-cleaved PrP\(^c\) (38), we now identify ADAM10-cleaved PrP\(^c\) as a major component of the unglycosylated PrP\(^sc\) in plaques. Thus, prions with the capacity to replicate ADAM10-cleaved PrP\(^sc\), such as ME7, preferentially recruit unglycosylated, cleaved PrP\(^c\) and form large plaques. In contrast, largely GPI-anchored prions, such as RML, recruit unglycosylated, GPI-anchored PrP\(^c\) and show a minimally altered disease phenotype. Collectively, these findings suggest that the presence or absence of glycans is relevant for prion conformers that can convert ADAM10-cleaved PrP\(^sc\), in which case the unglycosylated PrP\(^c\) is recruited by cleaved, GPI-anchorless PrP\(^c\).

Glycans stabilize the prion protein structure (65, 66) and impede prion conversion and fibril formation in vitro (66–68), potentially through modifying or blocking PrP\(^c\)/PrP\(^sc\) recognition domains (69). The diverse glycans on PrP are bi-, tri-, and tetra-antennary and are highly sialated (14, 17, 70, 71), with high sialation levels negatively impacting the prion replication rate (27). In the GPI-anchored prions RML and 22L, we found that the glycosylation state did not seem to markedly affect prion conversion, as the incubation period was only modestly affected. Additionally, for RML prions, the aggregate morphology, aggregate stability, and solubility were remarkably similar in the unglycosylated state. For 22L, however, the prion aggregate morphology was subtly altered, as PrP\(^sc\) recruited ADAM10-cleaved PrP\(^c\) and formed some plaques. Consistent with a morphological change, alterations in the h-FTAA binding environment also suggested differences in the structure of unglycosylated PrP\(^sc\), in agreement with reports that suggest the glycans may affect the tertiary and quaternary structure, but do not significantly affect the secondary structure (72). Thus, in contrast with in vitro reports (67, 68), our data suggest that the glycans do not impede conversion in vivo, as switching from glycosylated to unglycosylated PrP\(^c\) did not accelerate prion conversion. Moreover, triglycosylated PrP\(^c\) was efficiently converted and led to a rapidly progressive prion disease.

In contrast to RML, ME7 prions were markedly altered by the glycosylation state, as unglycosylated ME7 PrP\(^sc\) formed large extracellular plaques, recruited ADAM10-cleaved PrP\(^c\), was more stable in chaotrope adaptation, had more insoluble PrP\(^sc\), and showed an altered h-FTAA decay profile, suggestive of an altered structure due to either adaptation or a switch in the dominant PrP\(^sc\) structure. Why did unglycosylated ME7 prion aggregates assemble as highly stable, large parenchymal plaques? HS serves as an extracellular scaffold (73–75) and is widely recognized to bind prion aggregates, both in vitro and in vivo (76–79). In accordance with these observations, we found that HS localizes to parenchymal plaques, but not to diffuse aggregates within the same brain. Additionally, mass spectrometry quantification revealed 4 times more HS bound to unglycosylated versus glycosylated ME7 prions. This inverse correlation of PrP glycosylation state with HS binding and plaque formation may be explained by the effect of PrP glycans on HS affinity, potentially decreased by electrostatic repulsion between the anionic glycans of PrP and HS. Our experiments indicating that unglycosylated PrP has the highest affinity for heparin support this idea. Another possible explanation is that PrP glycans sterically block heparin and HS binding sites. Further research will be required to distinguish among these possibilities.

Is highly glycosylated PrP compatible with parenchymal plaque formation? We tested this possibility by challenging mice that express triglycosylated PrP with mCWD prions, which are ADAM10-cleaved, monoglycosylated, and form large plaques. Notably, the first mCWD passage in Prnp\(^{–/–}\) mice resulted in a near abrogation of plaques. Instead, prions formed mostly diffuse aggregates concurrent with a sharp reduction in the survival period. Although it remains a possibility that the change in the amino acid side chain and not the third glycans caused a switch in the strain phenotype, this would more likely have led to a lengthening of the incubation period. Instead, these findings suggest that highly glycosylated PrP may not form large, fibrillar parenchymal plaques, at least for certain strains, further supporting a link between PTM-deficient PrP and fibrillar parenchymal plaque formation.

We propose that a triad of factors act in concert to determine parenchymal prion plaque formation: (a) lack of a GPI-anchor, (b) one or no glycans, and (c) extracellular HS. Therefore, prions that replicate ADAM10-cleaved, unglycosylated PrP can bind HS and assemble into fibrils, forming extracellular parenchymal plaques. A key role for HS in parenchymal plaque formation is further supported by the finding that shortening HS chains in mice haploinsufficient in HS polymerase exostosin 1 decreases parenchymal plaques and increases vascular deposition of ADAM10-cleaved PrP\(^sc\) (51). Similarly, in an AD mouse model, depleting neuronal HS chains leads to a reduction in parenchymal plaques and an increase in vascular plaques (80), also indicating a role for HS in amyloid-\(\beta\) parenchymal plaque formation. Future studies will be necessary to understand the exact role of HS in prion parenchymal plaque formation.

Our findings support and provide a possible explanation for the parenchymal plaques observed histologically in certain familial prion diseases, for example those caused by the F198S mutation in which the plaque core consists of an unglycosylated PrP fragment (81). Additional familial mutations associated with parenchymal plaque formation are also composed of mono- or unglycosylated PrP, including V180I (fCJD) (82) and T183A (1, 83, 84). Whether these prions are composed of GPI-anchor or ADAM10-cleaved PrP remains unclear, and antibodies that can distinguish the posttranslationally modified forms of PrP will be useful for future studies. Since most PrP\(^c\) in mammals is posttranslationally modified with a GPI-anchor and N-linked glycans, these results may help explain the rarity of fibrillar plaques in prion disease and the predominance of small aggregates associated with the highest levels of infectivity.

**Methods**

Additional Methods are included in the Supplemental Material.
**Prnp**<sup>180Q/196Q</sup> and **Prnp**<sup>187N</sup> mouse generation using the CRISPR/CAS9 system. The **Prnp**<sup>180Q/196Q</sup> and **Prnp**<sup>187N</sup> knockin mice were generated by the UC Irvine Mouse Genetics core by microinjection of Cas9 ribonucleoprotein (PNA Bio) into B6SJLF1 × C57BL/6NJ zygotes. Briefly, Cas9 (20 ng/μL), gRNA-1 (20 ng/μL), and ssDNA HDR template (10 ng/μL) were mixed in injection buffer (10 mM Tris, 0.1 mM EDTA) and incubated on ice for 10 minutes, as per the manufacturer’s instructions. The Cas9 mixture was microinjected into the pronucleus of single-cell zygotes isolated from super-ovulated females. All founders and select progeny were genotyped by Sanger sequencing genomic DNA from zygotes isolated from super-ovulated females. All founders and select line progeny were backcrossed and bred to homozygosity. Aged mice investigated for spontaneous aggregation were 100–600 days old.

**Statistics.** A Student’s t test (2-tailed, unpaired) was used to determine the statistical significance between the **Prnp**<sup>180Q/196Q</sup> and **Prnp**<sup>187N</sup> versus WT mouse brain samples for the PrP<sup>C</sup> level of expression, PK digestion of PrP<sup>C</sup>, PrP<sup>Sc</sup> conformational stability, PrP<sup>C</sup> solubility, levels of ADAM10-cleaved PrP<sup>C</sup> and PrP<sup>S</sup>, and levels of HS bound to PrP<sup>Sc</sup> in ME7-infected WT and **Prnp**<sup>180Q/196Q</sup> brains. Student’s t test (2-tailed, unpaired) was also used to compare numbers of PrP<sup>Sc</sup> plaques found in ME7-infected WT and **Prnp**<sup>180Q/196Q</sup> mice, PrP<sup>C</sup> cell surface levels from WT and **Prnp**<sup>180Q/196Q</sup> cortical neurons, and cerebellar PrP<sup>C</sup> levels from WT and **Prnp**<sup>180Q/196Q</sup> mice.

One-way ANOVA with Tukey’s post test was performed to assess survival differences between mouse groups, PrP<sup>Sc</sup> solubility differences in the prion-infected mouse brain over multiple passages (**Prnp**<sup>180Q/196Q</sup> and **Prnp**<sup>187N</sup> as compared with WT mice), and to compare the binding affinity of unglycosylated, monoglycosylated, and diglycosylated isoforms of WT PrP<sup>C</sup> to heparin.

Two-way ANOVA with Bonferroni’s post hoc test was performed to assess differences in the lesion profiles, PrP<sup>Sc</sup> levels in each brain region, plaque size of the prion-infected WT and **Prnp**<sup>180Q/196Q</sup> brain, the heparin-binding affinity of RK13-derived PrP<sup>180Q/196Q</sup>, PrP<sup>180Q/196Q</sup>, and WT PrP<sup>C</sup>, the heparin-binding affinity of full-length and ADAM10-cleaved PrP<sup>C</sup> from **Prnp**<sup>180Q/196Q</sup> and WT mouse brains, and the composition of HS bound to PrP<sup>Sc</sup> in ME7-infected **Prnp**<sup>180Q/196Q</sup> and WT mouse brain.

GraphPad Prism 5 software was used for statistical analyses. No measurement was excluded for statistical analysis. For all analyses, P less than or equal to 0.05 was considered significant. Data displayed in graphs represent mean ± SEM.

**Study approval.** All animal studies were performed following procedures to minimize suffering and were approved by the Institutional Animal Care and Use Committee at UC San Diego. Protocols were performed in strict accordance with good animal practices, as described in the Guide for the Use and Care of Laboratory Animals published by the National Institutes of Health.

**Author contributions**

AMS, PAC, TDK, JAL, KPRN, CJS designed experiments, HA and MG provided reagents, AMS, PAC, TDK, JAL, KS, THN, BC, TS, DPP, SN performed the experiments, AMS, PAC, TDK, JAL, THN, TS, DPP, HA, JDE, MG, KPRN, and CJS analyzed the experiments, AMS, PAC and CJS wrote the manuscript. The order of the co–first authors was determined by the study design, data, and experimental interpretation contributed by each co-author.

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