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Additional Declarations: **Yes** there is potential Competing Interest. CS is a Founder, Chief Scientific Officer, consultant and shareholder of Amprion Inc., a biotechnology company that focuses on the commercial use of the protein misfolding cyclic amplification (PMCA) and seed amplification assays (SAA) for high-sensitivity detection of misfolded protein aggregates involved in various neurodegenerative diseases. The University of Texas Health Science Center has licensed patents to Amprion.

In vitro generation of highly infectious recombinant prions adopting structural architectures of *bona fide* prions

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Abstract

Prions are the protein-only infectious agents responsible for prion diseases. Recent cryo-EM studies have revealed that brain-derived prions adopt Parallel In-Register Intermolecular β -Sheet (PIRIBS) structures and share a remarkably similar global folding architecture, characterized by an overall V shape composed of distinct N-terminal and C-terminal lobes. Despite this advance, the molecular basis of prion conversion and propagation remains largely unclear, in part due to the lack of reproducible in vitro platforms capable of generating infectious prions for detailed mechanistic studies. Our previous studies demonstrated the generation of highly infectious prions in vitro using the Protein Misfolding Cyclic Amplification (PMCA) technology; however, it is unknown whether PMCA faithfully replicates the structure of PrP^{Sc}. Here, we report an RML prion-seeded, PMCA-based in vitro propagation assay that enables the robust and reproducible production of recombinant infectious PrP^{Sc} fibrils, which are biologically as pathogenic as the RML prion. Moreover, these PMCA-amplified recombinant PrP^{Sc} fibrils adopt a V-shaped architecture with two distinct lobes, reminiscent of the RML PrP^{Sc} structure. Despite highly conserved biological activity and overall structural similarity, recombinant PrP^{Sc} differs structurally from brain-derived prions, particularly in the C-terminal lobe, suggesting that complete structural identity is not needed for high infectivity. Overall, this recombinant PMCA system provides a powerful and versatile platform for investigating the structure-activity relationship of prion infectivity.

Introduction

Prion diseases are transmissible neurodegenerative disorders that affect a wide variety of mammals, including Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in cervids¹⁻³. Neuropathologically, prion diseases are characterized by the accumulation of misfolded prion protein (PrP), spongiosis, gliosis, and neuronal loss^{1,2,4}. All prion diseases, including CJD, are highly debilitating and incurable, and no disease-modifying therapies are available for these devastating conditions⁵.

Transmission studies in animal models have led to the discovery of the infectious agent termed prion⁶. According to the “protein-only hypothesis”, a prion does not contain nucleic acids and is composed chiefly, if not solely, of aggregated and infectious prion protein (PrP), termed PrP^{Sc} (ref^{1,7,8}). It is believed that PrP^{Sc} propagates through a self-replication mechanism that involves the autocatalytic conversion of the normal cellular PrP^C to the pathogenic PrP^{Sc} (ref^{9,10}). Despite significant advances in prion research, the molecular mechanisms governing prion conversion, the species barrier, prion strain diversity, and prion-induced neurotoxicity/neurodegeneration remain largely unclear¹¹. The high-resolution structure of the pathogenic PrP^{Sc} is believed to hold the key to these fundamental questions¹¹⁻¹⁴. Two structural models of PrP^{Sc} initially emerged from earlier studies: the parallel in-register intermolecular β -sheet (PIRIBS) architecture¹⁵ and the 4-rung β -solenoid model¹⁶. It is likely that prions may adopt multiple distinct structural architectures, determined by the primary structure of PrP^C, the prion strain, and the host species^{12,17}. Recent advances in cryogenic electron microscopy (cryo-EM) and helical reconstruction methodology have enabled the high-resolution structural determination of fibrillar protein aggregates¹⁸⁻²⁰. These methodological breakthroughs have transformed the field by enabling direct structural

interrogation of aggregated proteins extracted from patients' brains. Indeed, recent high-resolution cryo-EM studies demonstrated that at least some experimentally cloned scrapie-derived prion strains in rodents and one CWD prion isolated from a naturally infected deer all have the PIRIBS cores²¹⁻²⁶. Interestingly, these prions, including RML, 22L, and ME7 from mice, 263K from hamsters, and CWD from a white-tailed deer, share a similar overall core architecture, consisting of unique N-terminal and C-terminal lobes. These PrP^{Sc} cores span from the N-terminal residues 92-95 to the C-terminal residues 226-230, corresponding to the sizes of the protease-resistant PrP^{Sc} bands observed on immunoblots. These findings established a conserved structural framework underlying prion infectivity across strains and species.

Although cryo-EM has revealed the architectures of PrP^{Sc} fibrils, the detailed molecular basis of prion conversion and propagation remains poorly understood, in part owing to the absence of *in vitro* generated recombinant prions that faithfully recapitulate both biological activity and structural features. Bacterially expressed recombinant PrP (recPrP) has been widely used to investigate the molecular mechanisms underlying the pathogenic PrP^C-to-PrP^{Sc} conversion. While endogenous PrP^C undergoes post-translational modifications (PTMs), such as the addition of glycans at two Asparagine (Asn or N) residues and a glycosylphosphatidylinositol (GPI) anchor at the very C-terminus, bacterially expressed recPrP does not have these PTMs. It is known that PrP^C molecules with various PTMs, i.e., GPI-anchored or anchorless, di-, mono-, or unglycosylated, can all undergo misfolding into aggregated PrP^{Sc}, suggesting that infectious PrP^{Sc} architectures are structurally compatible with a wide range of PrP PTMs. Indeed, all available high-resolution structures of prions, including the anchored RML and the anchorless RML, prove this is the case²¹⁻²⁶. *In vivo* studies have shown that both GPI-anchored and anchorless PrP^C can be converted to pathogenic anchored and anchorless PrP^{Sc}, respectively. This suggests that the GPI

anchor may not be essential for the PrP^C-to-PrP^{Sc} conversion, although it is critical for the clinical manifestation of prion diseases²⁷. A spectrum of recPrP fibrils has been generated in both seeded and non-seeded in vitro assays, spanning from non-infectious to partially infectious and highly infectious, as characterized in bioassays with wild-type (WT) animals²⁸⁻⁴³. recPrP, full-length or truncated, can spontaneously form amyloid-like fibrils under various conditions⁴⁴, including the use of denaturants such as urea and guanidinium chloride^{29,45-47}. Cryo-EM studies revealed that the cores of these recPrP fibrils consist of residues around 170 to 230^{45,46}, completely lacking the N-terminal lobes found in infectious prions²¹⁻²⁶. Notably, none of these spontaneously formed recPrP fibrils has been reported to be infectious in bioassays⁴⁴⁻⁴⁶. Studies showing the successful generation of highly infectious recombinant PrP^{Sc} have mostly relied on the conversion of the recombinant protein templated by brain-derived prions^{35,36,39,40}. However, these studies have not elucidated the structure of PrP^{Sc} from recombinant origin and thus, high-resolution structural information for infectious recPrP fibrils remains unavailable. This persistent gap has represented a major bottleneck in mechanistic studies of prion replication.

Here, we report the robust production of highly infectious recPrP fibrils using the Protein Misfolding Cyclic Amplification (PMCA) technology^{48,49} invented in our lab. These recPrP fibrils were generated in a seeded PMCA assay, in which a brain-derived murine prion served as the seed and mouse recPrP, supplemented with anionic lipids and RNA cofactors, served as the substrate. These PMCA-generated recPrP fibrils not only possessed large protease-resistant cores comparable to those of *bona fide* prions, therefore termed recPrP^{res}, but also demonstrated a great seeding capacity for converting the glycosylated PrP^C to PrP^{Sc}. Biologically, recPrP^{res} induced typical prion disease in WT mice, with highly synchronized incubation times comparable to that of the RML prion. Structurally, the highly infectious recPrP^{res}, adopted the unique V-shaped

PIRIBS structure, resembling those of infectious prions. Together, these findings establish a recombinant, biologically infectious, and structurally similar prion propagation system that overcomes a longstanding barrier in prion research, providing a powerful platform for dissecting the molecular basis of prion propagation and pathogenesis.

Results

PMCA robustly generates recPrP fibrils with seeding capacity comparable to brain-derived prions.

The development of the PMCA technology represented a major breakthrough in prion research^{48,49}, as it enabled the in vitro reconstitution of the prion replication process and the rapid generation of *bona fide* prions in test tubes within days^{50,51}. Here, we carried out seeded PMCA reactions using the mouse-adapted RML prion as the seed and recPrP as the substrate, rather than mouse brain homogenate (BH), which is therefore referred to as recPMCA. We used conventional proteinase K (PK) digestion and immunoblotting assays to monitor the formation of recPrP fibrils after each round of recPMCA. Our seeded recPMCA reactions were efficient in producing PK-resistant recPrP^{res}, often after only one or two rounds of PMCA (**Figure 1A**). To assess reproducibility, we performed 6 independent experiments, all of which yielded recPrP^{res} in rounds 2 or 3 of PMCA (**Figure S1A**). The newly generated recPrP^{res} could be propagated indefinitely by recPMCA (**Figure S1B**). RecPrP^{res} has a PK-resistant core of approximately 16 kDa (green arrow in **Figure 1A**), similar in size to the PK-resistant cores of unglycosylated and GPI-free brain-derived PrP^{Sc}.

To evaluate the seeding capacity of recPrP^{res}, recPMCA products from round 7 to round 12 of the first experiment (**Figure S1B**) were pooled, centrifuged, and resuspended in sterile PBS

(**Figure 1B**). The pooled recPrP^{res} was then tested in a dilution panel for seeding activity in the standard PMCA assay, using normal mouse BH as the substrate. As a positive control, we used RML PrP^{Sc} (**Figure 1C**, right panel). The pooled recPrP^{res} could be serially diluted up to 10 million-fold and still efficiently propagate PrP^{Sc} (**Figure 1C**, left panel), similar to brain-derived RML prions (**Figure 1C**, right panel). These PMCA results not only demonstrate that the recPMCA propagated-recPrP^{res} is seeding-competent, but more importantly, also suggest that recPrP^{res}, similar to brain-derived prions, may have adopted a conformation that can template the conversion of endogenous PrP^C by incorporating its bulky glycans.

recPrP^{res} fibrils induced classical prion disease in WT mice.

Next, we tested whether recPrP^{res} fibrils are infectious in wild-type animals. Groups of six- to eight-week-old female C57BL mice were intracerebrally injected with the combined recPrP^{res} produced in the first experiment (**Figure 1B**) (n = 8). As a positive control, animals were injected with 10% RML BH (n = 5), and as a negative control, mice were exposed to a 10⁻⁷ dilution of RML (n = 7). The latter control was performed because, in our estimation, the amount of RML PrP^{Sc} seeds used to initiate recPrP conversion that may still remain in the tube should be lower than a 10⁻⁷ dilution of RML BH. After injection, mice were monitored daily for neurological signs. Around 140-150 days post-injection (dpi), all 10% RML- and recPrP^{res}-inoculated mice began displaying classical prion disease clinical signs, such as hindlimb clasping, tail rigidity, and kyphosis, and rapidly progressed to cachexia and lethargy, within 2-3 weeks. Subsequently, the afflicted mice reached the terminal stage in a highly synchronized manner, with an average incubation time of 164.4 dpi in the RML group and 163.4 dpi in the recPrP^{res} group (**Figure 2A** and **Table 1**). Control animals injected with 10⁻⁷ RML BH never developed any clinical signs for

more than 500 days before they were sacrificed at the end of the experiment. Brains of all mice were collected for biochemical and histological analyses. The canonical PK-digestion assay revealed that a significant amount of PrP^{Sc} accumulated in the brains of recPrP^{res}-injected animals, similar to that present in RML-injected mouse brains (**Figure 2B**). In addition, the glycosylation and migration patterns of the PK-resistant PrP^{Sc} were similar among all eight recPrP^{res}-injected animals and were indistinguishable from those of the RML PrP^{Sc} group. As expected, no PrP^{Sc} was detected in the brains of any negative control mice (animals injected with a 10⁻⁷ dilution of RML) when they were sacrificed after 500 dpi (**Figure 2B, bottom panel**). Histopathological analyses further confirmed that prion disease-defining neuropathological features were present in recPrP^{res}-inoculated mouse brains, including spongiosis, astrogliosis, microgliosis, and accumulation of PrP^{Sc} (**Figures 3 and S2**). Similar prion pathologies were observed in all 10% RML BH-infected mice but not in any control animals. These classic biological characterizations strongly support the conclusion that the PMCA-amplified recPrP^{res} is a *bona fide* infectious prion, and it is therefore termed recPrion.

To evaluate the reproducibility of generating recPrion by our recPMCA assay, we studied two additional recPMCA preparations (Experiments #2 and #6 in **Figure S1A**) for bioassay in WT mice. Both recPrions, named recPrion-#2 and recPrion-#6, were propagated and pooled after round 7 to eliminate potential infectivity associated with residual RML seed. Unsurprisingly, inoculation of recPrion-#2 and recPrion-#6 led to the development of typical prion disease in WT mice, including similar incubation periods of 164.6 and 167.5 dpi, respectively (**Figure 2A and Table 1**), as well as the presence of PK-resistant PrP^{Sc} in western blots (**Figure S3A**) and histopathological profile of brain damage (**Figure S3B**).

In spite of the fact that animals injected with RML- and recPrion showed similar incubation period, clinical, biochemical, and histopathological features in our bioassay experiments, it remains possible that the lack of PTMs in recPrions (glycans and the GPI anchor) may change the prion behavior. In particular, recPrions might not be able to efficiently template the conversion of the endogenous PrP^C in vivo, resulting in a transmission barrier in the first passage of recPrion. To assess this possibility, 10% BH from the mouse 7 (P1-A7 in **Figure 2B**) was inoculated into WT mice in a second passage transmission study. All second-passage (P2) animals developed the same neurological disease, exhibiting similar clinical signs and a remarkably synchronized disease course, with an incubation time of 165.4 dpi on average. The essentially overlapping survival curves of recPrion-#1 (P1) and recPrion-#1 (P2) (**Figure 2A** and **Table 1**) suggest that the lack of PTMs in recPrion did not result in a transmission barrier during the first passage. Histological and biochemical studies further confirmed that these P2 mice died of classic prion disease (**Figures 3 and S2**) with substantial brain accumulation of PrP^{Sc} (**Figure 2B**).

Collectively, our in vitro and in vivo data demonstrate that we have established a robust recPMCA assay that reproducibly propagates highly infectious recombinant prion fibrils, offering a valuable tool for elucidating the molecular basis of pathogenic prion conversion.

RML-seeded recPrP^{Sc} adopt the typical V-shaped PIRIBS structure of infectious prions

It is noteworthy that the survival curves for RML, recPrion-#1 (P1 and P2), recPrion-#2, and recPrion-#6 groups of mice are almost identical (**Figure 2A** and **Table 1**), indicating that recPrion fibrils are similarly infectious as the RML PrP^{Sc}. Consistently, the glycosylation and migration patterns of PrP^{Sc} in recPrion-challenged animals are indistinguishable from those of RML PrP^{Sc}. These findings suggest that recPrion generated by RML-seeded recPMCA might have adopted the

RML strain-specific conformation, prompting us to determine the high-resolution structure of these RML-seeded recPrion fibrils by cryo-EM. Specifically, recPrion-#2 and recPrion-#6 fibrils were treated with Benzonase and PK, centrifuged, suspended in cryo-EM sample buffer, and subjected to cryo-EM structural studies (**Figures 4**).

We obtained the cryo-EM structure of the RML-seeded recPrion fibrils at 3.8 Å resolution (**Figures 4C-E, S4 and Table S1**) and built an atomic model directly from the density map with residues T94 to D226 of the mouse PrP sequence, which make up the ordered amyloid core (**Figures 4C-E**). The fibrils follow a PIRIBS-type fibrillar architecture, where each PrP molecule forms one rung in the helical stack, creating the characteristic cross- β arrangement along the fibril axis with a spacing of 4.95 Å between two successive subunits and helical twist of -0.64 degrees (**Figure 4E**). The fibril cross-section is approximately 11.5 × 7 nm (**Figure 4F**), forming a V-shaped fold composed of interconnected N- and C-terminal lobes arranged around a central β -sheet scaffold. Only the core region (T94-D226) is ordered; the N- and C-terminal flanking segments are likely flexible and not resolved in the map. The C178-C213 intramolecular disulfide bond appears to stabilize the C-terminal region, consistent with other PrP^{Sc} structures. The recPrion fibril conformation is maintained through alternating polar and hydrophobic inter-chain contacts, with each subunit interacting vertically along the fibril axis (**Figure 4F**). Hydrogen-bonding networks extend through the stacked β -strands, supporting the PIRIBS architecture. Hydrophilic residues are mainly located on the fibril surface, whereas hydrophobic residues cluster within the interior of the fold (**Figure 4G**). In contrast to the staggered N- and C-terminal lobes reported for ex vivo RML PrP^{Sc} (ref ²³), the recPrion adopts a more planar structure, except for the segment from G130 to P157, which sits slightly lower along the fibril axis and aligns with the corresponding region of the next subunit, producing a modest axial offset (**Figures 4E, G**).

RML-seeded recPrion structure is similar to ex vivo RML PrP^{Sc} but differs at the C-terminal lobe.

Although both RML-seeded recPrion and the ex vivo RML PrP^{Sc} adopt a similar and characteristic V-shaped architecture, the main difference localizes to the C-terminal lobe (**Figure 5A**). In recPrion fibrils, the hairpin tip of the C-terminal lobe bends away from the V-shaped core (**Figure 5A**, left panel), creating a clear distinction from the ex vivo RML fold (**Figure 5A**, middle panel). When the recombinant and ex vivo PrP fibrillar conformations are aligned, their N-terminal arms follow essentially the same trajectory, and the overall curvature of the solenoidal fold is preserved. Superposition of the two models shows a high degree of similarity at the N-terminus, with an RMSD of 1.2 Å calculated over 60 pruned atom pairs, indicating that the N-terminal RML fold is largely maintained in the RML-seeded recPrion (**Figure 5A**, right panel).

The head of the N-terminal β -arch, spanning residues 112-137, is also highly similar between the recPrion and ex vivo RML structures (**Figure 5B**, left panel). In both cases, key hydrophobic residues are oriented toward the fibril interior, and the side chain of Y127 adopts a comparable inward-facing conformation. Hydrophobic residues that form the intra-chain spine of the fold, including V120, V121, L124, M128, A132, I138, and F174, occupy equivalent positions in the two structures. Likewise, the vertical ladders of lysine and arginine residues lining the fibril surface are largely conserved. In addition, residues such as R135, H139, D143, E145, and R150 of the recPrion are oriented toward the fibril core, preserving the overall electrostatic pattern observed in the ex vivo RML structure (**Figure 5B**, middle panel). Furthermore, residues spanning S131-H176 exhibit close structural correspondence between the two conformations, with an RMSD of 1.5 Å, which further supports the strong structural conservation across this region (**Figure 5B**, right panel). Together, these observations indicate that recPMCA preserves a large portion of the RML

prion core fold with high fidelity, with conformational differences primarily confined to a distal C-terminal segment.

Discussion

A major goal in prion research over the past 20 years has been to understand the molecular mechanisms by which a protein can act as an infectious agent, propagate biological information, and transmit a neurodegenerative disease^{1,3,9,12}. An important advance toward this goal was the development of an amplification procedure (PMCA) to mimic prion replication in the test tube⁴⁸, which enabled the production of highly infectious prions in the lab⁵⁰. Initially, infectious prions were generated using whole-brain homogenate as a substrate for the amplification reaction^{50,52,53}. Subsequently, it was possible to generate infectious material from the protein produced in bacteria with the addition of a few synthetic cofactors³⁷⁻⁴³. However, to date, it is unknown whether the infectious material produced from recombinant PrP faithfully retains the biological and structural properties of *bona fide* prions. In this study, we demonstrated that RML-seeded recPMCA in the presence of synthetic POPG lipid and mouse liver RNA cofactors produced recPrP fibrils with a prominent ~16 kDa PK-resistant core (**Figure 1**), and that these fibrils exhibit high prion infectivity in wild-type mice with characteristics (clinical signs, incubation period, brain pathology) indistinguishable from brain-derived prions (**Figure 2**).

Another significant breakthrough towards understanding the molecular basis of prions came with the elucidation of the near-atomic-resolution structure of PrP^{Sc} purified from disease brains using cryo-EM²¹⁻²⁶. High-resolution cryo-EM analyses of pathogenic prions revealed a set of conserved structural features shared across strains and species (**Figures S5A-C**). These include: **1**) fibrillar aggregates composed of a single protofilament containing a large aggregated core divided into two distinct N-terminal and C-terminal lobes; **2**) a wide groove separating the N- and C-

terminal lobes that is large enough to accommodate bulky glycans in PrP^{Sc}; and **3**) the two Asn residues where glycosylation occurs, project outward from the core of the C-terminal lobes. The structural studies reported in this article demonstrate that our PMCA-generated recPrP fibrils exhibit all three defining structural features (**Figure S5D**), suggesting that preserving these features is essential for high prion infectivity. Interestingly, the structure of our recPrP fibrils shows high similarity in the N-terminal lobe, whereas a substantial difference is observed in the C-terminal lobe (**Figure 5A**). These findings suggest that, whereas the proper folding of the N-terminal lobe appears to be critical to retain high infectivity, the C-terminal lobe can adopt a more flexible structure. Interestingly, the main PTMs in PrP (glycosylation, disulfide bridge, and GPI anchor) are present in this lobe.

The putative role of PTMs in prion replication has been studied using recombinant PrP expressed in bacteria, which lacks glycosylation and a GPI anchor. Bacterially expressed recPrP has been extensively utilized to investigate the PrP^C-to-PrP^{Sc} conversion in various in vitro systems⁵⁴⁻⁵⁶. While amyloid fibrils can be generated with recPrP in the presence of denaturants, such as urea and guanidinium chloride, the protease-resistant cores of these recPrP fibrils are typically smaller compared to those of the pathogenic PrP^{Sc} (ref^{29,45-47}). More importantly, none of the recPrP fibrils with high-resolution structural information has been reported to be infectious in animal models (ref⁴⁵⁻⁴⁷). It is plausible that bacterially expressed recPrP, due to the lack of the constraints imposed by PTMs, such as glycosylation, has the freedom to adopt a large spectrum of misfolded conformations, some of which might not be able to convert the endogenous PrP^C in vivo because they cannot accommodate the bulky and charged glycans attached to the mammalian protein.

It is well documented that recPrP fibrils generated in the Real-Time Quaking-Induced Conversion (RT-QuIC) assay seeded by brain-derived prions are not infectious when inoculated into WT animals³⁴. Interestingly, some of these RT-QuIC-propagated recPrP fibrils possess a large PK-resistant core of approximately 17 kDa, despite some containing two smaller cores of around 11 and 12 kDa¹⁵. PIRIBS models for these fibrils were constructed based on solid-state nuclear magnetic resonance (ssNMR) studies¹⁵. A structural model encompassing residues 90-231 contains two main hairpins: the N-terminal and C-terminal hairpins (**Figure S6**). In this model, both Asn 181 and Asn 197 protrude away from the C-terminal hairpin held together by the native disulfide bond between Cys179 and Cys 214, and there is a so-called “glycan cleft” that separates the N- and C-terminal hairpins and may accommodate glycans attached to Asn 181 (**Figure S6**). These models, composed of N- and C-terminal hairpins separated by the “glycan cleft”, resemble in principle the structural features of *bona fide* infectious prions (**Figures S5A-C**). However, a critical difference between RT-QuIC-generated material and the infectious prion structures is that the groove between the N- and C-terminal lobes in infectious prions is much wider than the “glycan cleft” in the RT-QuIC material. This difference might explain the lack of prion infectivity in the RT-QuIC-propagated recPrP fibrils, suggesting that the “glycan cleft” might be too narrow to accommodate the bulky glycans in PrP^C. One of the main differences between the RT-QuIC and PMCA protocols, both of which are prion-seeded assays, is the involvement of cofactors in PMCA and the use of sonication instead of shaking as the fragmentation force. Considering the conformation-changing potential of interactions between recPrP and cofactor molecules⁵⁷⁻⁶⁵, it is plausible that a structural alteration induced by cofactor molecules in PMCA might facilitate the

seeded propagation of recPrP fibrils while maintaining the unique V-shaped architecture of *bona fide* prions.

The significant structural differences in the C-terminal lobe of recPrion compared to brain-derived PrP^{Sc} (**Figure 5**) may be explained by the emergence of a new prion strain. However, our bioassay results do not support this explanation. Indeed, **1)** the average incubation times for RML, recPrion-#1 (both P1 and P2), recPrion-#2, and recPrion-#6 groups of mice are almost identical, and **2)** the glycosylation and gel migration patterns of PrP^{res} in all recPrion-challenged animals are indistinguishable from PrP^{Sc} in RML-injected mice (**Figure 2**). However, detailed strain characterization studies, such as the lesion profile, resistance to proteolytic degradation, and glycosylation pattern quantification, are required to further evaluate the prion structure-strain correlation. An alternative explanation for the differences is that either the lack of glycosylation in recPrP, and/or the intrinsic properties of the cofactor molecules used in our recPMCA, enabled such pronounced conformational evolution during in vitro recPrion propagation without affecting its infectivity. However, when recPrion is passaged in WT animals, in vivo cofactors that are likely distinct from the in vitro cofactors might correct the C-terminal shift, thereby propagating the true RML structure in vivo. In summary, our findings indicate that the C-terminal region appears more adaptable and sensitive to biochemical context, reinforcing the idea that PMCA can faithfully reproduce the infectious fold while also revealing structural elements that depend on in vivo modifications and cofactors. Nonetheless, more work needs to be done to investigate this subject, including high-resolution cryo-EM characterization of PrP^{Sc} extracted from recPrion-injected mice.

The main conclusion of our study is that highly infectious prions can be produced in vitro from recPrP, which retain the biological and pathogenic properties of brain PrP^{Sc} despite folding into a structure similar but not identical to that of brain-derived prions. A similar conclusion was reached

in a recent study using a seed amplification assay (SAA), in which patient-derived alpha-synuclein (α Syn) seeds were amplified at the expense of monomeric recombinant α Syn. Indeed, while the amplified α Syn aggregates generated in vitro were biologically indistinguishable from the α Syn aggregates present in patients' brains, their structures were similar but not identical⁶⁶. The emerging conclusion of these studies is that absolute structural identity among protein aggregates is not required for disease pathogenesis. The structural flexibility of pathogenic protein aggregates may allow subtle biological differences across distinct cells or brain areas, which might help explain the heterogeneity observed in neurodegenerative diseases. Further comprehensive biological-structural characterizations of these in vitro-propagated protein aggregates are warranted to elucidate the molecular mechanisms underlying protein misfolding and pathogenicity in prion diseases as well as other neurodegenerative disorders.

Methods

Expression and purification of recPrP

Full-length recombinant mouse PrP(23-230) was purified as previously reported⁶⁷. Briefly, IPTG-induced *E. coli*, expressing recPrP as inclusion bodies, were harvested at $10,000 \times g$ and washed several times in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl). Pellets were resuspended in lysis buffer and supplemented with 0.5 mg/mL of lysozyme, followed by incubation at room temperature (RT) for 30 min. Additional lysis was performed by subsequent sonication. Inclusion bodies were collected by centrifugation at $15,000 \times g$, followed by washing in lysis buffer supplemented with 0.05% (v/v) Triton X-100 several times. Solubilization of recPrP was performed by incubating inclusion bodies in buffer A (10 mM Tris-HCl, 100 mM Na_2HPO_4 , pH 8.0, 100 mM NaCl, 10 mM β -mercaptoethanol, and 6 M Guanidinium chloride (GdmCl) for 2

hours at RT with gentle agitation. The resulting sample was then purified by conventional immobilized metal ion affinity chromatography (IMAC). Upon sample loading, recPrP was on-column refolded overnight at RT through linear gradient buffer exchange from buffer A to buffer B (10 mM Tris-HCl, 100 mM Na₂HPO₄, pH 8.0, and 100 mM NaCl). recPrP was eluted using buffer B supplemented with 500 mM imidazole and then desalted using Zeba desalting columns (Thermo Scientific). Concentration of recPrP was determined using OD₂₈₀ reading and the molar extinction coefficient (ϵ_{280}) of 63,370 M⁻¹cm⁻¹ for mouse PrP(23–230). For storage, aliquoted recPrP was flash-frozen in liquid nitrogen and stored at -80 °C.

Isolation of total RNA from mouse liver

Total RNA was extracted from liver tissues collected from wild-type C57BL/6 mice using TRIzol reagent (Invitrogen). One hundred milligrams of liver tissue was finely minced and homogenized on ice in 1 mL of TRIzol using a KIMBLE Dounce tissue grinder. 200 μ L of Chloroform was added, vigorously shaken for 30 seconds, and incubated at room temperature for 5 minutes. Following centrifugation at 12,000 $\times$ g for 15 minutes at 4 °C, the aqueous phase (~600 μ L) was transferred to a fresh tube containing 500 μ L of isopropanol. After vortexing and incubation at room temperature for 5 min, RNA was pelleted by centrifugation at 12,000 $\times$ g for 10 minutes at 4 °C. The pellet was washed with 1 mL of 70% ethanol, centrifuged at 7,000 $\times$ g for 5 minutes at 4 °C, air-dried for ~20 min, and resuspended in 100 μ L of nuclease-free water (Invitrogen). RNA purity and concentration were determined by measuring the OD₂₆₀/OD₂₈₀ ratio and OD₂₆₀, respectively, using a NanoDrop spectrophotometer (Thermo Scientific). Purified RNA was flash-frozen in liquid nitrogen and stored at -80 °C.

PMCA with recPrP substrate

RecPrP substrate was prepared following a previously reported procedure with some modifications⁶⁸. Briefly, a 10 mL batch of recPrP substrate was prepared by mixing 1000 μ L of 0.25 mg/mL recPrP, 89 μ L of 2.5 mg/mL POPG (1-palmitoyl-2-oleoylphosphatidylglycerol) prepared in 5% Triton X-100, 1000 μ L of 10X TN buffer (100 mM Tris-HCl pH 7.5, 1.5 M NaCl), 466.5 μ L of 5% Triton X-100, 500 μ L of 6 mg/mL mouse liver RNA, and 6944.5 μ L of nuclease-free ultrapure water (Invitrogen). The resulting PMCA substrate was either immediately used for PMCA amplification or flash-frozen in liquid nitrogen and stored at -80 °C. 2.5 mg/mL POPG solution was prepared by resuspending 25 mg of POPG powder (Avanti Research) in 10 mL of 5% Triton X-100. For recPMCA, the recPrP substrate was thawed at RT in a water bath, then a 90 μ L aliquot was added to a 200 μ L PCR tube (Eppendorf). Three Teflon beads (3/32 inch in diameter, Engineering Laboratories) were added to each tube to increase efficiency and reproducibility. 10 μ L of 10% (w/v) BH from a terminally diseased mouse infected with RML prion was added to the substrate to seed the reaction. Tubes were placed in a water-containing microplate horn (QSonica, Q700) holder inside an incubator set at 32 °C. PMCA was performed by running cycles of 20 sec sonication and 29.6 min incubation for 24 hours. After 1 round (24 h) of amplification, 10 μ L of PMCA product was removed from the current tube and transferred to a new tube containing 90 μ L of fresh recPrP substrate for the next round of PMCA. Products of recPMCA from round 7 and subsequent rounds were pooled, centrifuged at 100,000 $\times$ g, and resuspended in the same volume of 1 $\times$ PBS for downstream applications.

PMCA with normal mouse brain homogenate substrate

Brain tissues were collected and processed as previously described (Morales et al., 2012). A 10% (w/v) brain homogenate (BH) was prepared from whole brains of perfused wild-type C57BL/6 mice (perfusion buffer: 1 $\times$ PBS supplemented with 5 mM EDTA) in conversion buffer (1 $\times$ PBS

containing 1% Triton X-100, 150 mM NaCl, and EDTA-free Complete protease inhibitor). Large debris was removed by brief centrifugation at $810 \times g$ for 1 minute at 4 °C. The resulting supernatant was aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C until use. For BH PMCA, 90 µL of normal mouse BH substrate was added to a 200 µL PCR tube containing three Teflon beads. 10 µL of serially diluted recPMCA product or 10 µL of serially diluted BH from a terminally diseased mouse infected with RML prion was added to the substrate to seed the reaction. Tubes were placed in a water-containing microplate horn (QSonica, Q700) holder inside an incubator set at 32 °C. PMCA was performed by running cycles of 20 sec sonication and 29.6 min incubation for 72 hours.

Proteinase K (PK) digestion

For PMCA products, 10 µL of samples were treated with proteinase K (PK) (Roche) at a final concentration of 50 µg/mL (for recPMCA products) or 100 µg/mL (for BH PMCA products) for 1 hour at 37 °C, with constant agitation at 450 rpm. For infected animals, 10 µL of 10% (w/v) BHs from terminally diseased mice were incubated with 100 µg/mL PK for 1 hour at 37 °C, with constant agitation at 450 rpm. PK-digested samples were then boiled at 95 °C for 10 min in denaturing loading buffer, electrophoresed through SDS-PAGE, and immunoblotted using 6D11 monoclonal antibody (BioLegend). PK-resistant PrP fragments were visualized by developing immunoblots with Amersham ECL Prime Western Blotting Detection Reagent (Cytiva) using a Bio-Rad ChemiDoc imager.

Animals and Housing

Six- to eight-week-old female C57BL/6 mice (JAX) were housed in standard cages under controlled conditions: temperature maintained at 22 ± 2 °C, a 12-hour light/dark cycle, and unrestricted access to fresh water and standard chow. Animal experiments conducted in this study

followed an animal protocol (Protocol #: AWC-23-0020) approved by the Animal Welfare Committee of the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at Houston.

Bioassay

Surgical procedures were performed as previously described⁶⁶, with the following modifications. Two-month-old mice were anesthetized with isoflurane delivered via a precision vaporizer, with concentrations adjusted to maintain a stable surgical plane (1.5–2%), as confirmed by the absence of response to toe pinch. Sterile ophthalmic ointment was applied to both eyes to prevent corneal drying during anesthesia. Preoperative analgesia was provided with a single dose of carprofen (5 mg/kg, subcutaneous), and local anesthesia was achieved by administering 0.25% bupivacaine (2.5 mg/kg) at the incision site. Mice were positioned in a stereotaxic apparatus equipped with a warming platform to maintain body temperature throughout the procedure. The scalp was shaved free of hair, and the surgical site was prepared with alternating scrubs of iodine and 70% isopropyl alcohol, repeated three times to ensure asepsis. Before incision, a subcutaneous injection of bupivacaine or lidocaine was administered along the planned incision site for local analgesia. A midline incision (~1–1.5 cm) was made over the skull, and the underlying connective tissue was bluntly dissected to expose the skull and visualize bony landmarks. The skull surface was cleaned of debris. Using a sterile drill bit, a small hole was drilled at the appropriate coordinates (AP -2 mm; DV +1.8 mm; ML +2 mm from bregma), and a stereotaxic injection of 10 µl of 10% (w/v) BH from terminally diseased mice or 10 µl of recPrP^{res} fibrils was performed into the right hippocampus. Then, the incision was closed with 6-0 non-absorbable monofilament sterile suture. Mice were transferred to a clean recovery cage and provided with supplemental heat until fully ambulatory. Supplemental fluids were administered as needed. Sutures were removed 7 to 14 days

after the procedure. Following surgery, mice were monitored daily for clinical signs. Once any of the predefined clinical signs developed, animals were observed closely until the terminal stage, defined by severe neurological signs and difficulty accessing food and water. At this point, mice were humanely euthanized, and brains were collected for further analysis.

Neuropathological analyses.

Brain samples were fixed in Carnoy solution (60% ethanol, 30% chloroform, and 10% acetic acid) or formalin, dehydrated, and embedded in paraffin. 10 μ m tissue slices were stained with haematoxylin (Sigma-Aldrich)-eosin (Thermo Scientific) (H&E) or immuno-stained with anti-PrP monoclonal antibody (SAF84, 1:500; Cayman Chemical), anti-Iba1 antibody (1:40; Wako), or anti-GFAP antibody (1:500; Abcam). For immunostaining, the color was developed using the DAB Peroxidase Substrate Kit (Vector Laboratories, SK-4100), and hematoxylin was applied as a counterstain. All images were captured using a 10 $\times$ objective on a Leica Stellaris 5 microscope.

Cryo Grid preparation and data acquisition

Cryo-EM grids were prepared in our BSL-2 prion laboratory using a manual plunge-freezing device. Quantifoil C-flat R 2/1 holey carbon grids were used for all experiments. Products of recPMCA from round 7 and subsequent rounds were pooled, treated with Benzonase (MilliporeSigma, E1014), digested with PK, then centrifuged at 100,000 $\times$ g, and resuspended in cryo-EM sample buffer (20 mM Tris, pH 7.4, 100 mM NaCl). A 5 μ L aliquot of the resuspended recPrP fibril sample was applied to the carbon side of the glow-discharged grid and incubated for 10 seconds. Excess liquid was blotted manually using Whatman No. 1 filter paper, after which the grids were immediately plunge-frozen into liquid ethane cooled by liquid nitrogen. Frozen grids were stored in liquid nitrogen until data collection. Cryo-EM data were collected on a Titan Krios microscope operated at 300 kV. Images were recorded at a nominal magnification of 130,000 $\times$,

corresponding to a calibrated physical pixel size of 1.1 Å. All the parameters are summarized in **Table S1**.

Helical reconstruction

Fibrils were reconstructed in RELION-5^{69,70}. Movie frames were corrected for beam-induced motions and dose-weighted in RELION using its own motion-correction system⁶⁹. Non-dose-weighted micrographs were used for CTF estimation with CTFFIND-4.1⁷¹. Out of 23,242 micrographs, 16,892 fibrils were picked manually. Particle segments were extracted using a box size of 1200 pixels and an interbox distance of 29.7 Å, and then downsampled to 300 pixels for 2D classification. This gave an idea about the cross-over distance of the filaments. The initial estimate of the cross-over distance was around 1300 Å. Next, particles were extracted at 600 pixels, and an interbox distance of 14.85 Å, and 2D classification was performed using the extracted particles. Classes with clear cross-over were used for building a de novo 3D initial model using the Relion_helix_inimodel2d program⁶⁹. Suboptimal classes were removed, and the remaining good classes were selected and re-extracted in boxes of 320x320 pixels for initial refinement, followed by multiple rounds of 3D classification and refinement. The final reconstruction of the map was performed after CTF refinement and standard RELION post-processing, using a soft solvent mask that extended to 30% of the box height to estimate the resolution. The Refined map was sharpened using EMReady⁷² and used for model building.

Model building

Atomic models for mouse PrP^{Sc} structures, including PDB entries 7QIG (mouse-adapted RML prion), 8EFU (anchorless 22L prion strain), and 7TD6 (anchorless RML prion strain), were obtained from the Protein Data Bank. Each model was rigid-body fitted into the corresponding cryo-EM density map using the "Fit in Map" tool in UCSF ChimeraX (version 1.9.5). A single

protofilament chain from each fitted structure was extracted and imported into Coot (version 0.9.8.85) together with the corresponding map for manual adjustment. All three models were manually edited to improve local fit to the density. The optimized single-chain models were subsequently refined against the cryo-EM map using phenix.real_space_refine (version 1.21.2) with default parameters. Refined chains were re-imported into UCSF ChimeraX, and a fibril subunit comprising five stacked protofilament repeats was constructed based on the optimized 7QIG model. This multimeric assembly was saved and reloaded into Coot for further inspection. Iterative rounds of manual model adjustment in Coot and automated real-space refinement in Phenix were then performed, enforcing non-crystallographic symmetry (NCS), to further improve the agreement with the density while minimizing steric clashes, rotamer outliers, and Ramachandran outliers. Final model validation statistics were calculated using Phenix. UCSF ChimeraX was used for structural analysis, comparisons, and the generation of the figures.

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Competing interest

CS is a Founder, Chief Scientific Officer, consultant and shareholder of Amprion Inc., a biotechnology company that focuses on the commercial use of the protein misfolding cyclic amplification (PMCA) and seed amplification assays (SAA) for high-sensitivity detection of misfolded protein aggregates involved in various neurodegenerative diseases. The University of Texas Health Science Center has licensed patents to Amprion.

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Inoculum	Attack rate	Survival time (dpi) (mean $\pm$ SEM)	Survival time for individual mouse (dpi)
RML (10^{-7})*	0% (0/7)	>500	>500
RML (10^{-1})	100% (5/5)	164.4 $\pm$ 1.6	161, 161, 164, 168, 168
recPrion-1 (Passage 1)	100% (8/8)	163.4 $\pm$ 1.6	160, 161, 161, 160, 160, 170, 165**, 170
recPrion-1** (Passage 2)	100% (5/5)	165.4 $\pm$ 1.3	161, 164, 166, 168, 168
recPrion-2	100% (5/5)	164.6 $\pm$ 2.6	156, 162, 166, 169, 170
recPrion-3***	100% (4/4)	167.5 $\pm$ 1.9	162, 168, 170, 170

Table 1. Incubation times for intracerebrally inoculated mice.

* Control group: the inoculum was diluted in recombinant PMCA substrate.

** 10% brain homogenate of this animal (P1-#7) was used as inoculum for a passage 2 (P2) transmission study.

*** one of the five mice inoculated with recPrion-3 died of non-prion related, concurrent disease at 100 dpi.

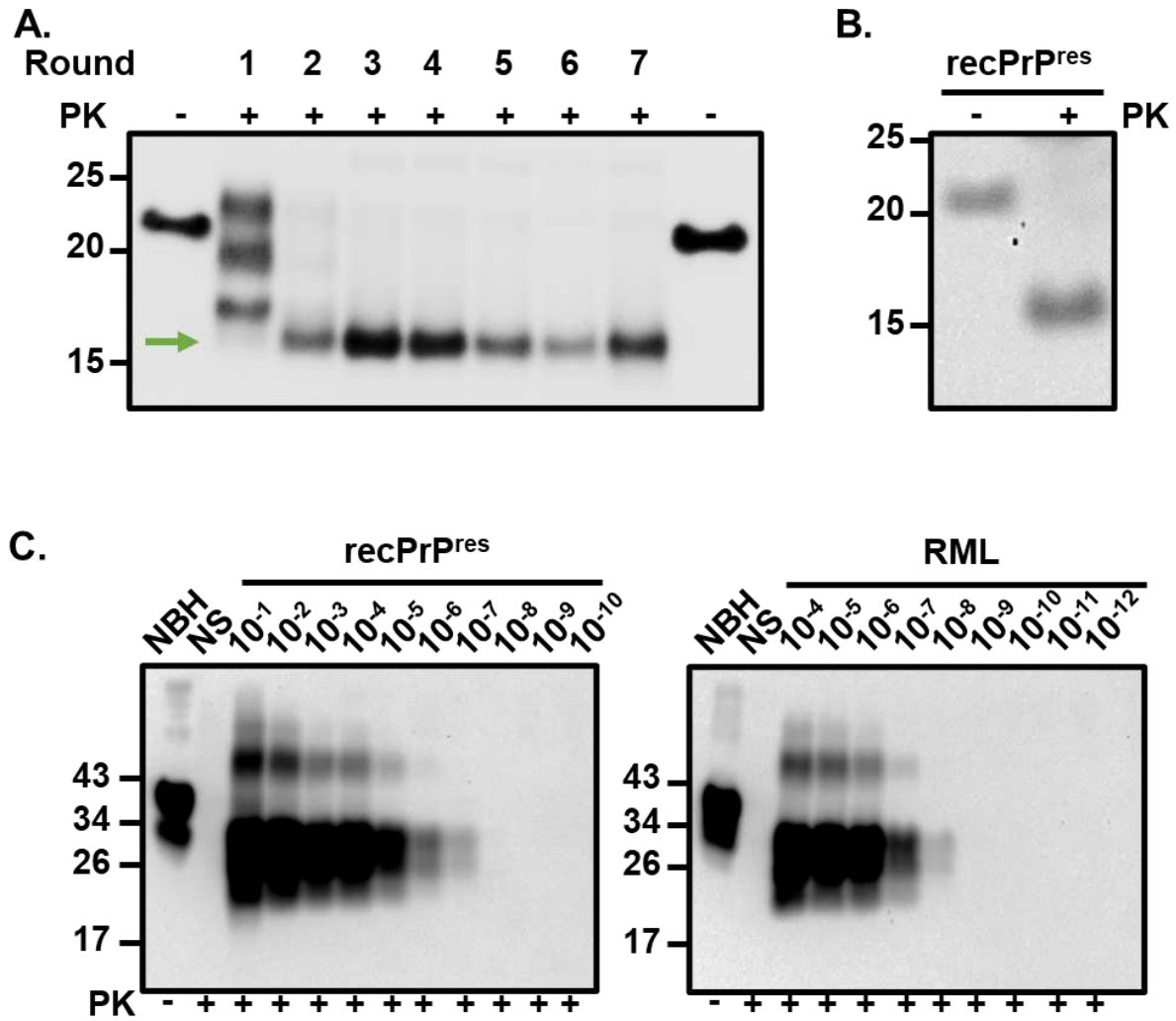


Figure 1. Generation of recPrP^{res} by RML-seeded PMCA. (A) Serial PMCA reactions seeded with RML prion. 10 μ L of 10% RML BH and 90 μ L of the substrate containing mouse recPrP and cofactors were subjected to the first round of PMCA. 10 μ L of round 1 PMCA product was mixed with 90 μ L of fresh substrate to carry out the second round of PMCA. Subsequent PMCA rounds were done in the same manner. After each PMCA round, the generation of recPrP^{res} was monitored by treating 10 μ L of the product with 50 μ g/mL PK. The green arrow indicates the size of the PK-resistant recPrP^{res}. (B) PMCA products from rounds 7 to 12 were pooled together, centrifuged, and resuspended in the same volume of 1 $\times$ PBS. Resuspended recPrP fibrils were subjected to

treatment with 50 µg/mL PK. (C) Left panel: resuspended recPrP fibrils generated in experiment #1 with final dilutions of 10^{-1} to 10^{-10} were used to seed PMCA reactions containing normal mouse BH as substrate. Right panel: RML BH with final dilutions of 10^{-4} to 10^{-12} were used to seed PMCA reactions containing normal mouse BH as substrate. NBH: 5 µL of normal mouse BH as migration control. NS: non-seeded PMCA reactions. The anti-PrP monoclonal antibody 6D11 was used to detect PrP.

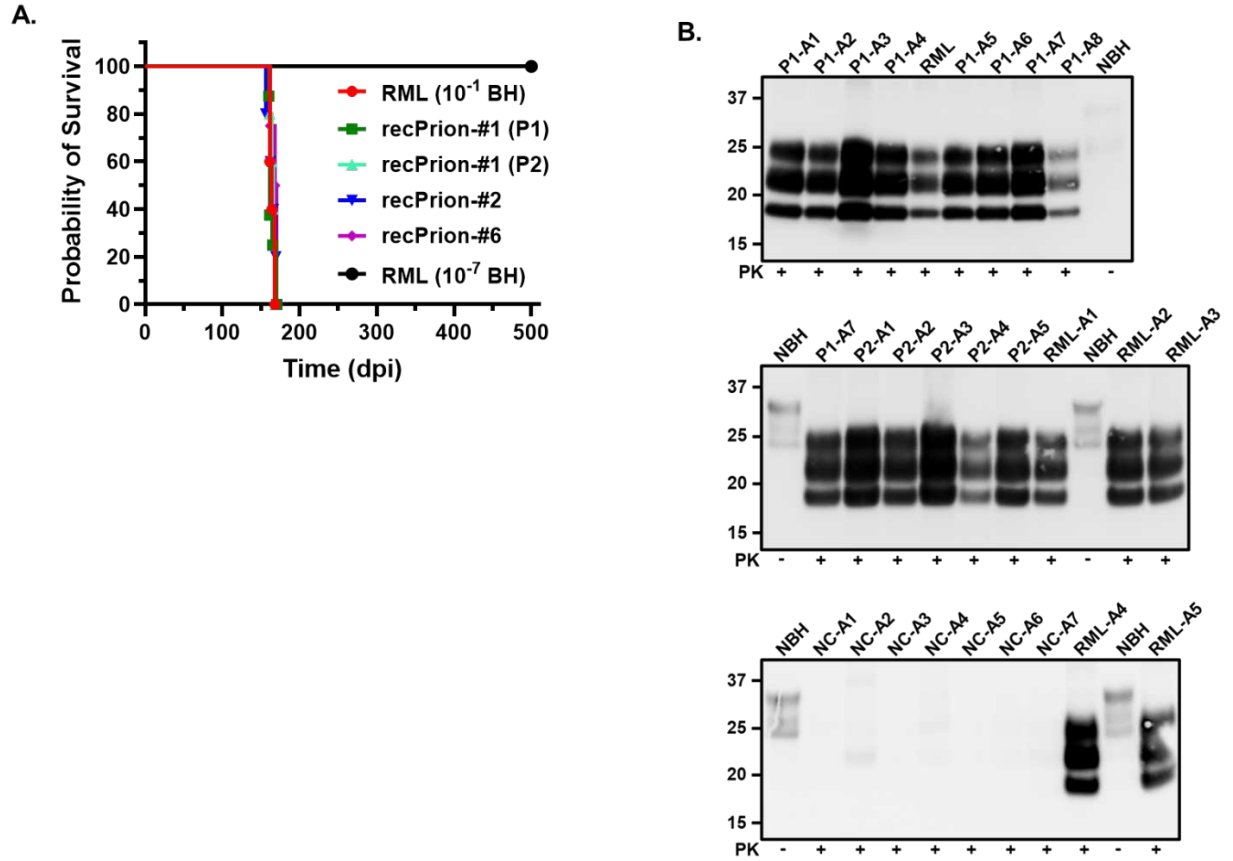


Figure 2. PMCA-propagated recPrP fibrils induced prion disease in wild-type mice. (A) Kaplan-Meier survival curves of wild-type C57BL/6 mice injected with different inocula: 10% (10^{-1}) RML BH (positive control; n=5, average survival time of 164.4 dpi), recPrP^{res} (from Experiment #1 in **Figure 1B**; n=8, average survival time of 163.4 dpi for passage 1), 10% BH from a diseased mouse infected with the first-batch recPrP^{res} (second passage; n=5, average survival time of 165.4 dpi) and 10^{-7} RML BH (Negative control; n=7). For the latter group, none of the mice developed any clinical signs and were sacrificed after 500 dpi. The figure also shows the in vivo results obtained with two other preparations of recPrP^{res} (Experiments #2 and #6) (n=5, average survival time of 164.6 dpi). For the latter group, one of the five mice inoculated with recPrP^{res} (Experiment #6) died of non-prion disease at 100 dpi, and was excluded from the survival curve analysis. Multiple statistical tests found no significant difference between survival curves of

any of the groups (Log-rank (Mantel-Cox) test, Chi square: 2.731, df: 4, p value: 0.6039; Gehan-Breslow-Wilcoxon test, Chi square: 3.304, df: 4, p value: 0.5084), except for the negative control group. **(B)** Immunoblotting analysis of the PK-resistant PrP in the brains of 8 mice from the first passage (P1) of recPrion generated in experiment #1, 5 mice injected with 10% RML (positive control), 5 mice from the second passage (P2) of recPrion, and 7 mice injected with 10^{-7} RML BH (NC, negative control). A1, A2, A3, etc., refer to each animal in the group. NBH: 5 μ L of normal mouse BH used as the migration control. The anti-PrP monoclonal antibody 6D11 was used to detect PrP.

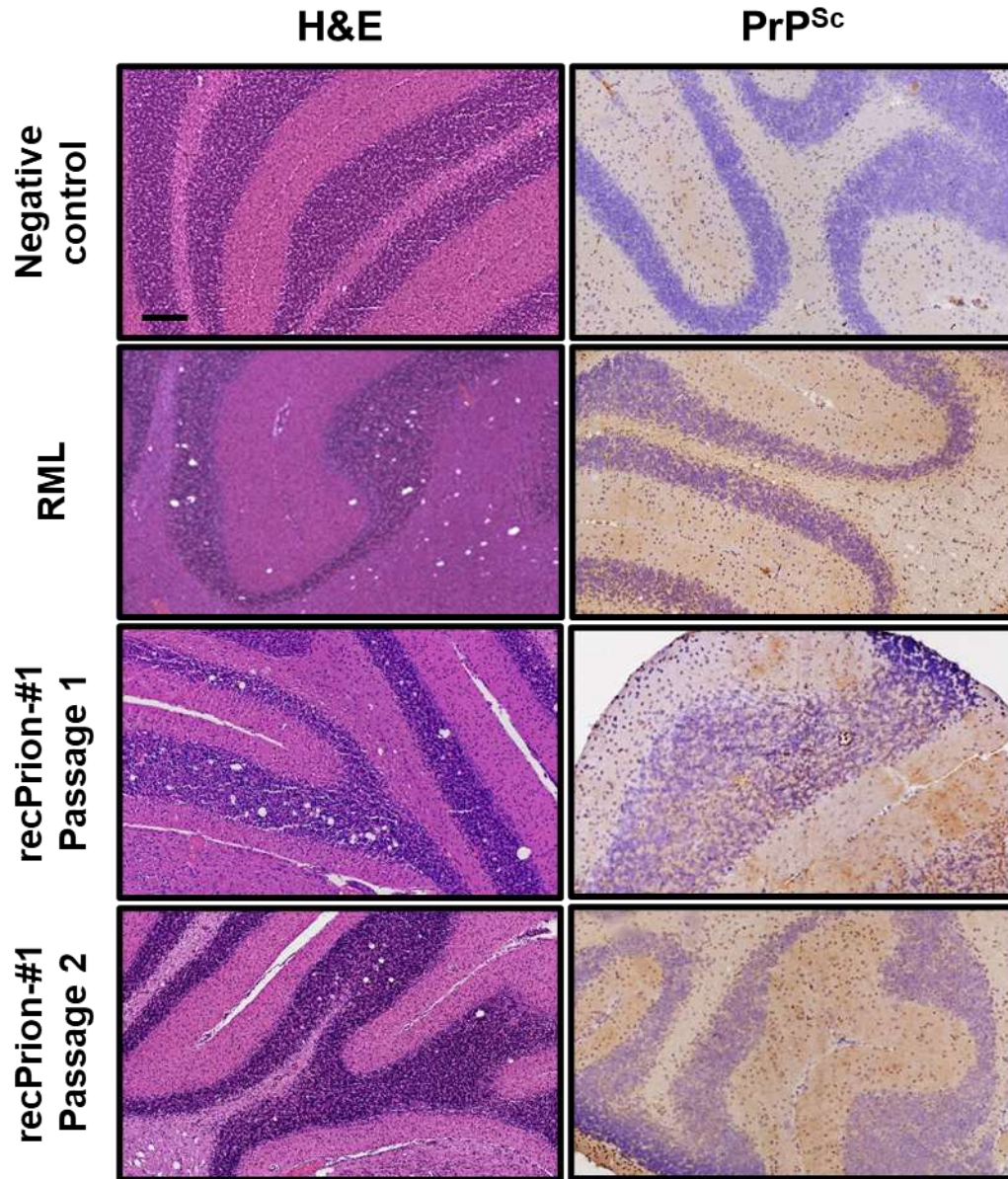


Figure 3. Histopathological analyses of neurodegeneration in recPrP^{res}- and RML-infected mice. Representative images of cerebellar regions from each group show vacuolation, as indicated by hematoxylin-eosin staining and PrP^{Sc} deposition, as indicated by anti-PrP antibody (SAF84) staining. Scale bar: 200 μ m.

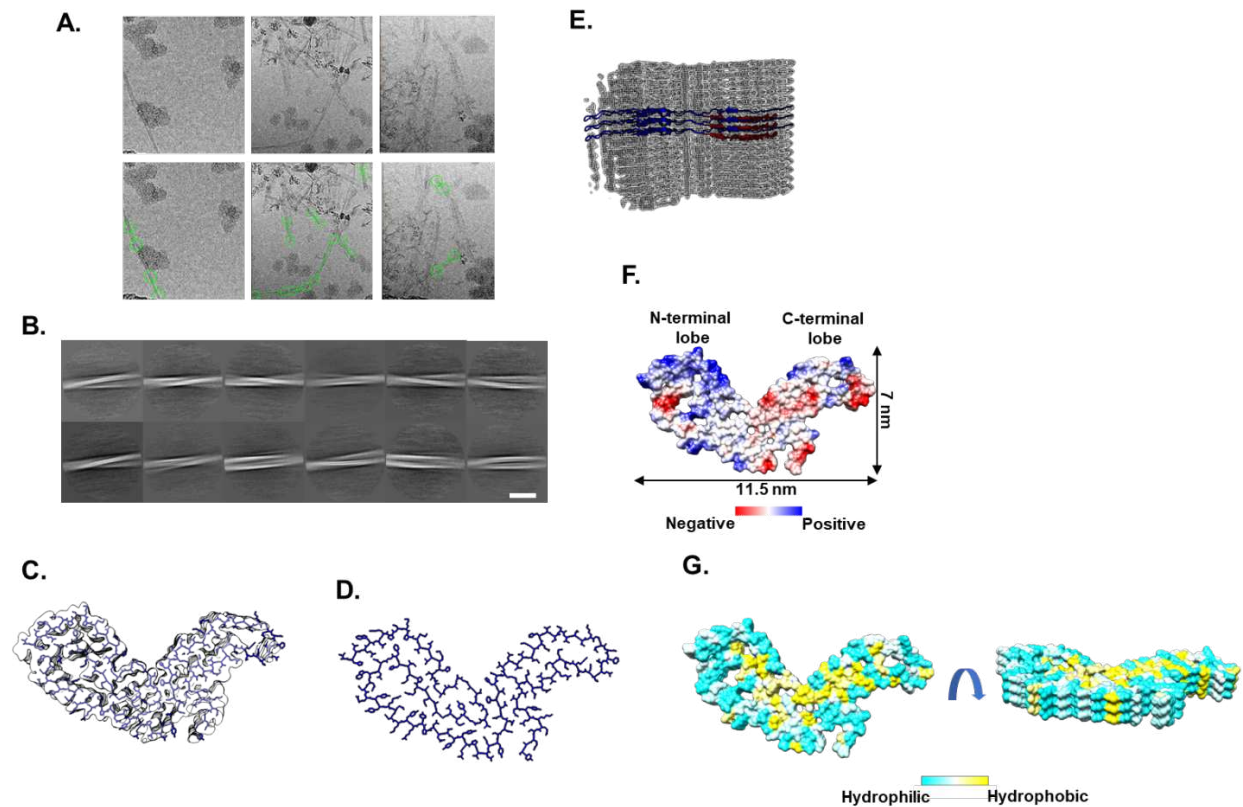


Figure 4. Recombinant PrP fibril morphologies, 2D classification, 3D reconstruction, and atomic model of the recPrP^{res} protofilament core. (A) Representative cryo-EM micrographs showing fibrils in the PMCA-amplified preparation. Green lines indicate manual particle picking along individual fibrils. (B) A subset of 2D class averages displaying fibril crossover patterns of recPrP^{res}. Scale bar, 20 nm. (C) Refined cryo-EM density map of recPrP^{res} sharpened using EMReady. The fitted atomic model is shown in blue within the density. (D) Atomic model of recPrP^{res} viewed from the top, illustrating the overall fold and side-chain organization. (E) Side view of the cryo-EM density map (mesh) with the model fitted into one of the stacked fibrils, showing the repeating cross- β architecture characteristic of amyloid fibrils. The red region corresponds to the residues spanning from G130-P157, which sit slightly lower along the fibril axis and align with the corresponding region of the next subunit. (F) Coulombic surface representation illustrating the distribution of charged residues across the N-terminal and C-

terminal lobes. The fibril exhibits asymmetric polarity, characterized by a predominantly negative N-terminal region and a more positive C-terminal region. **(G)** Hydrophobicity-mapped surface of the recPrP^{res} fibril. Hydrophilic and hydrophobic patches are shown in blue and yellow, respectively, highlighting the hydrophobic spine and the amphipathic nature of recPrP^{res} fibrils.

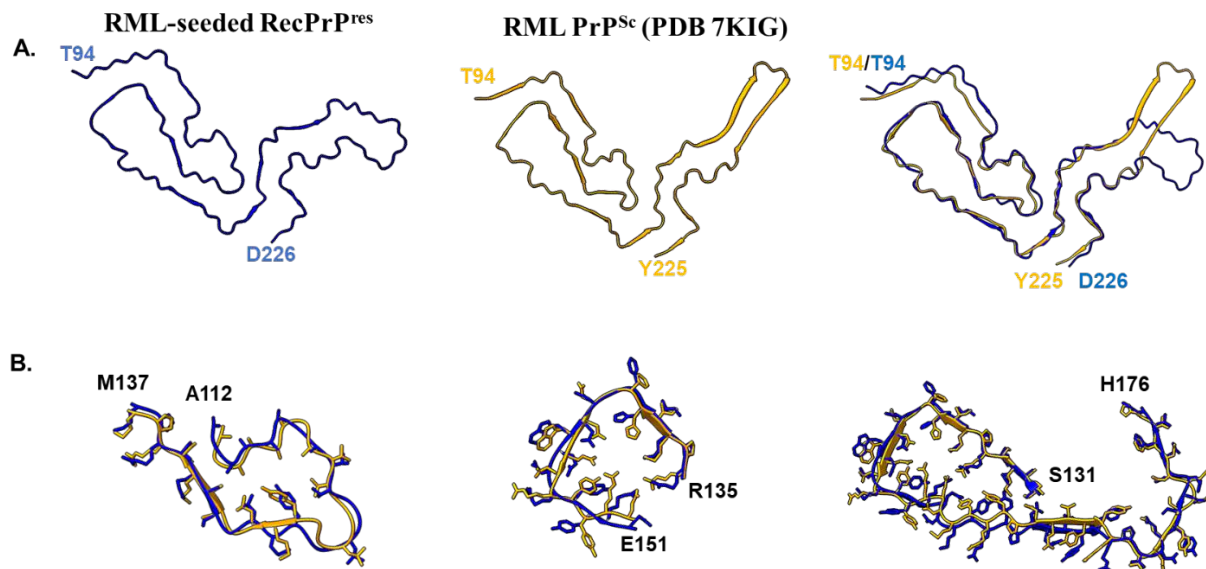


Figure 5. Structural comparison between recPrP^{res} and ex vivo RML PrP^{Sc}. (A) PrP structure between residues T94 and D226 of recPrP^{res} (left panel, blue) and T94 and Y225 of ex vivo RML prion (PDB 7QIG) (middle panel, orange). Right panel: The PMCA-propagated recPrP^{res} fibril structure overlying with the RML fold, showing the V-shaped architecture, a similar curvature at the N-terminal segment, and divergent C-terminal hairpin tips. (B) Local structural comparison. Left panel: PMCA-propagated recPrP^{res} model (blue) showing hydrophobic residues at the head of the β -arch (112-137) project inward toward the fibril core, and the side chain of Y127 adopts the same inward orientation as in the RML fibril (orange). Middle panel: The overall arrangement of hydrophobic residues in the β -arch and the inward-facing pattern of R135, H139, D143, E145, R150, and E151 are preserved in both fibrils. Right panel: Structural superposition of residues S131-H176 reveals close correspondence between the two conformations, with an RMSD of 1.5 Å.

Supplementary Files

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