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1 **Quaternary structural convergence and structural diversification of prion**
2 **assemblies at the early replication stage**

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26

27 **Abstract**

28 Aggregation of misfolded forms from host-encoded proteins is key to the pathogenesis of a
29 number of neurodegenerative disorders, including prion diseases, Alzheimer's disease and
30 Parkinson's disease. In prion diseases, the cellular prion protein PrP^C can misfold into PrP^{Sc}
31 and auto-organize into conformationally distinct assemblies or strains. A plethora of
32 observations reports the existence of PrP^{Sc} structural heterogeneity within prion strains,
33 suggesting the emergence and coevolution of structurally distinct PrP^{Sc} assemblies during prion
34 replication in controlled environment. Such PrP^{Sc} diversification processes remain poorly
35 understood. Although central to prion host-adaptation, structural diversification of PrP^{Sc}
36 assemblies is also a key issue for the formation of PrP conformers involved in neuronal injury.
37 Here, we characterized the evolution of the PrP^{Sc} quaternary structure during prion replication
38 *in vivo* and *in bona fide* cell-free amplification assays. Regardless of the strain studied, the early
39 replication stage conduced to the preferential formation of small PrP^{Sc} oligomers, thus
40 highlighting a quaternary structural convergence phenomenon. Their evolutionary kinetics
41 revealed the existence of a PrP^C-dependent secondary templating pathway in concert with a
42 structural rearrangement. This secondary templating pathway provides, for the first time, a
43 mechanistic explanation for prion structural diversification during replication, a key
44 determinant for prion adaptation on further transmission, including to other host species. The
45 uncovered processes are also key for a better understanding of the accumulation mechanisms
46 of other misfolded assemblies believed to propagate by a prion-like process.

47

48 Keywords: prion / neurodegenerative disorders / polymerization / kinetics / diversification

49

50 **Introduction**

51 In terms of pathogenic mechanisms, the prion paradigm unifies a number of age-related,
52 incurable neurodegenerative disorders that are caused by protein misfolding and aggregation
53 [13,21,22,9]. These disorders include human and animal forms of prion diseases, Alzheimer's
54 disease, Parkinson's disease and Huntington's disease. In principle, host-encoded monomeric
55 proteins or peptides are converted into misfolded and aggregated assemblies, which serve as
56 seeds or templates for further autocatalytic conversion. In prion diseases, the ubiquitously
57 expressed, host-encoded prion protein PrP^C is converted into a misfolded, β -sheet-rich
58 conformer termed PrP^{Sc} [39]. In susceptible host species and in laboratory rodent models, PrP^{Sc}
59 assemblies form stable, structurally distinct PrP^{Sc} conformers [4,8,12,52], known as prion
60 strains, and encode unique stereotypical biological phenotypes [45,47,48,5]. The strain-specific
61 structural differences can be observed at the secondary structural level in terms of local
62 structural variation but also at the quaternary level with strain-specific size distributions
63 [44,49,47]. A large body of evidence supports the view for further structural diversity within
64 specific prion populations and strains: i) some studies highlight the selection of prion substrains
65 during the transmission of natural isolates [11,27,2] or experimental prion strains [29] with a
66 species or transmission barrier, ii) size- or density-fractionation studies support the existence
67 of a heterogeneous spectrum of PrP^{Sc} assemblies with distinct tertiary/quaternary structures
68 [24,44,49,23,50,6,7,41] and biological activity (templating activity and infectivity) [24,44,49],
69 and iii) kinetic studies of prion pathogenesis suggest that the formation of neurotoxic PrP^{Sc}
70 species [46] occurs at the late stage of prion infection but that replicative PrP^{Sc} assemblies are
71 formed at earlier stages [42,43]. The prion replication process thus intrinsically allows the
72 structural diversification of PrP^{Sc} assemblies.

73 While the kinetic aspects of prion replication 'as a whole' have been comprehensively described
74 by measuring infectivity or PrP^{Sc} levels in the brain (see references [25,34] as examples), the

75 processes by which PrP^{Sc} structural diversification and the formation of different
76 subpopulations occur within a given strain remain undescribed and are not mechanistically
77 supported in the actual framework of the prion paradigm. The autocatalytic conversion model
78 proposed by Griffith in 1967 [17], the nucleated-polymerization model described by Lansbury
79 and Caughey in 1995 [26] and other derived models (e.g., [30]) merely assume the existence of
80 structurally homogenous assemblies that have absolutely identical propensity to replicate
81 throughout disease progression. These mechanisms intrinsically reduce PrP^{Sc} heterogeneity due
82 to the best replicator selection process (35, 36). A recent high-resolution structural analysis of
83 the N-terminal domain of the yeast prion Sup35 suggests that conformational fluctuations in
84 natively disordered monomeric Sup35 are responsible for the stochastic, structural
85 diversification of Sup35 aggregates [36]. This idea can be extrapolated to mammalian prion
86 PrP to explain intrastrain structural diversification and strain mutation [12]. However, based on
87 the best replicator selection concept [33,37,35], the aforementioned idea does not explain the
88 coevolution of at least two structurally distinct PrP^{Sc} subassemblies within the same
89 environment [11,28].

90 To examine the molecular mechanisms of PrP^{Sc} replication and structural diversification in
91 depth, we explored, with sedimentation velocity (SV)-based methods, the early stage of prion
92 conversion *in vivo* and in a cell-free system by protein misfolding cyclic amplification (PMCA).
93 PMCA mimics *in vivo* prion replication with accelerated kinetics [40]. By using several prion
94 strains as templates, we demonstrated that the early stage of prion replication invariably
95 generates two subsets of assemblies, termed A_i and B_i, which differ in proportion, size and
96 structure according to their specific infectivity. The analysis of their kinetics of formation
97 during mb-PMCA combined with kinetic data assimilation revealed the existence of two
98 sequential processes during prion replication. The first process corresponds to a quaternary
99 structural convergence, as it tends to reduce the parental quaternary structure polydispersity to

100 generate mostly small-sized assemblies, namely A_i . The second process transforms the A_i into
101 structurally different assemblies, namely, B_i , according to a secondary auto-catalytic pathway
102 requiring PrP^C and where B_i facilitates its own formation. Our findings provide, for the first
103 time, mechanistic insights allowing the generation of structurally distinct assemblies during the
104 prion replication process.

105

106

107 **Results**

108 **Small PrP^{Sc} oligomers are formed at the early stage of prion replication**

109 The early phases of prion replication are commonly thought to consist of an elongation/growing
110 process [16], with the PrP^{Sc} template serving as a base. We studied the size distribution of
111 proteinase K (PK)-resistant PrP^{Sc} (PrP^{res}) assemblies at the early step of prion replication in the
112 brain by SV in an iodixanol gradient using a previously published methodology [24,49,20]. The
113 PrP^{res} size distribution at the disease end stage served as the control. Three different host
114 PrP/strain combinations were studied: the 127S cloned scrapie prion strain in ovine PrP tg338
115 transgenic mice [25], the 139A cloned mouse prion strain in mouse PrP tga20 mice [15] and
116 the vCJD cloned human prion strain in human PrP tg650 mice [3,19]. As shown in Figure 1A-
117 C, small oligomers sedimenting between fractions 1 and 4 were preferentially detected at the
118 early stage of pathogenesis, regardless of the strain considered. A second population of
119 oligomers with a larger size distribution and peaking in fractions 8-10 and 18 was observed for
120 127S. At the disease end stage and for the 3 strains, the small assemblies mostly disappeared at
121 the expense of larger assemblies.

122 To determine whether the formation of small assemblies in the brain at the early stage of
123 replication can be reproduced by an *in vitro bona fide* amplification method, we used a high-
124 throughput variant of PMCA (i.e., mb-PMCA), which generates as much infectivity as in the
125 brain at terminal stage of the disease in one unique round of 48 h, with high reproducibility in
126 terms of limiting dilution and the amplification yield [31,32]. When the size distribution of the
127 amplified products was analyzed by SV after one mb-PMCA round, two discrete distributions
128 were observed for the three strains (Figure 1D). The post-PMCA sedimentograms revealed the
129 existence of a major set of small PrP^{res} assemblies sedimenting between fractions 1 and 3 (peak
130 P₁) and a minor set of larger assemblies with a well-defined Gaussian distribution centered on

131 fraction 15 (peak P₂). The relative proportions of P₁ and P₂ varied among the three strains; P₂
132 was barely detected in the 139A amplicons. These data indicate that during mb-PMCA
133 amplification, two populations of PrP^{Sc} assemblies are generated that differ according to their
134 quaternary structures, with a predominance of small assemblies.

135 The bimodal (i.e., generation of two peaks) and discrete behavior of the size distribution as well
136 as the formation of predominantly small assemblies in P₁ suggest that the mb-PMCA
137 condition(s) can be a consequence of shearing forces during the sonication step [1,38,51] rather
138 than an intrinsic consequence of the replication process. To discriminate between these two
139 possibilities, undiluted 127S seeds (i.e., 20% brain homogenate) were incubated and sonicated
140 in identical mb-PMCA conditions but without the PrP^C substrate (i.e., in PrP^{0/0} brain lysate).
141 As shown in Figure 1E, the size distribution analysis of these sonicated 127S seeds in the PrP^{0/0}
142 substrate revealed mostly the presence of larger-sized assemblies, as observed upon
143 solubilization at 37°C [24], thus ruling out the mb-PMCA conditions being at the origin of the
144 formation of small-size assemblies.

145 Altogether, these observations suggest that *in vivo*, the early phase of replication for the 127S,
146 139A and vCJD prion strains generates mainly small-sized assemblies. Similar to *in vivo*
147 replication, the mb-PMCA amplification condition generates two sets of PrP assemblies that
148 differ in their quaternary structures. The formation of these two groups of assemblies is
149 common to the three strains used here.

150

151 P₁ and P₂ contain two structurally distinct PrP^{res} assemblies

152 We next asked whether the formation of P₂ resulted from a simple condensation of assemblies
153 present in the P₁ peak (Oswald ripening process [53]) or from an alternative templating
154 pathway. To address this question, we first examined the influence of the amplification rate on

155 the formation of these two species by varying the concentration of the seed used as the template
156 for the mb-PMCA reaction. We compared the SV-sedimentograms of the mb-PMCA products
157 seeded with 10^{-3} to 10^{-10} diluted 127S brain homogenate. As shown in Figure 2A, as a function
158 of the seed concentration, the relative amounts of assemblies in P₁ decreased as the amounts of
159 those from P₂ increased. The variation in the P₁ and P₂ peak surface area as a function of the
160 logarithm of the dilution factor revealed a quasi-linear decrease in P₁ when the P₂ peak surface
161 followed a sigmoidal increase (Figure 2B). The sigmoidal increase in P₂ to the detriment of the
162 quasi-linear decrease in P₁ surface indicates that i) the formation of PrP^{res} assemblies present in
163 P₂ follows a seed concentration-dependent cooperative process and that ii) the formation of the
164 P₂ peak does not result from the simple condensation of assemblies present in P₁ as the
165 variations in P₁ and P₂ are uncorrelated (Figure 2B). This observation strongly suggests that
166 assemblies forming the P₁ and P₂ peaks result from distinct polymerization pathways and should
167 therefore be structurally distinct.

168 To further explore the entanglement between the assemblies forming P₁ and P₂, we fixed the
169 mb-PMCA regime to favor the formation of the P₁ peak by fixing high dilutions of the inoculum
170 seed, followed by quiescent incubations at 37°C for variable periods. As shown with the 127S
171 prions, the SV analysis at defined incubation time points post-PMCA reaction revealed a
172 decrease in the population of P₁ in favor of P₂ (Figure 2C). At 4 h postincubation, there were
173 equal proportions of assemblies forming P₁ and P₂. At 24 h, most of the PrP^{res} assemblies were
174 located in the P₂ peak. Comparing the distribution in isopycnic gradients [24] of the PrP^{res}
175 populations at 0h and 24h of quiescent incubation at 37°C revealed a quasi-similar density for
176 PrP^{res} assemblies composing the P₁ and the P₂ peaks (Figure 2D). This observation leads us to
177 conclude that i) the low sedimentation velocity of the assemblies forming P₁ does not result
178 from an interaction with lipids or other low-density molecules and ii) the sedimentation velocity

179 increase of P₂ compared to P₁ results strictly from a quaternary structure rearrangement through
180 size increase rather than change in compactness reducing the hydrodynamic radius.

181 As shown in Figure 2E, the formation of assemblies sedimenting in P₂ exhibited bimodal
182 behavior (i.e., absence of assemblies of intermediate size) without any significant shift in the
183 P₂ peak position, suggesting that the formation of these assemblies resulted from the association
184 with a specific number of assemblies present in P₁. Furthermore, the time-dependent surface
185 variation in P₁ and P₂ showed a sigmoidal shape, indicating that the assemblies present in P₂
186 enhance their own formation according to an autocatalytic process (Figure 2E). Similarly, the
187 139A and vCJD prions showed a bimodal evolution of P₁ to P₂ during a 24-h quiescent phase
188 (Figure 2F), arguing in favor of a generic process of transformation.

189 To determine whether the quaternary structure rearrangement accompanying the transformation
190 of P₁ to P₂ was in concert with a deeper structural rearrangement in the PrP^{Sc} assemblies, we
191 determined the specific infectivity of the P₁ and P₂ assemblies. A 127S-PMCA product was
192 fractionated at the end of the reaction or after 48 h of quiescent incubation. Pools of fractions
193 corresponding to the P₁ and P₂ peaks were inoculated into reporter tg338 mice. The specific
194 infectivity (infectivity per PrP molecule), which is mostly associated to PrP^{res} assemblies
195 [24,49], was calculated from the mean survival time using 127S dose-response curves [49]. As
196 shown in Figure 2G, the specific infectivity of the P₁ peak assemblies was 50-100-fold higher
197 than that of the P₂ peak assemblies. This value did not change over a longer period of quiescent
198 incubation (7 days, Figure 2G).

199 To determine whether the P₂ peak assemblies could further evolve, we extended the quiescent
200 phase up to 30 days. For the 127S, 139A and vCJD prion strains, the sedimentogram curves at
201 7 and 30 days showed a translational shift in the P₂ peak to higher fractions, indicative of an
202 isokinetic increase in their mean average sizes (Figure 3, left curves). The difference in the

203 specific infectivity values of the P₁ and P₂ peak assemblies did not change over a longer period
204 of quiescent incubation (7 days, Figure 2G, 127S strain). This size translation thus contrasts
205 with the bimodal phase and highlights a change in the kinetic regime.

206 Collectively, these observations indicate that the P₁ and P₂ peaks contain structurally distinct
207 sets of PrP^{res} assemblies named A_i and B_i. The index i indicates their sizes, with A_i and B_i being
208 the major constituents of the P₁ and P₂ peaks, respectively. The formation of the B_i assemblies
209 is cooperative and results from a complex kinetic pathway. Upon longer quiescent incubation,
210 a change in the kinetic regime occurs.

211

212 The formation of B_i from A_i assemblies requires the presence of PrP^C during the 213 quiescent phase

214 Our previous studies revealed that only ~30% of the PrP^C substrate was converted into PrP^{Sc}
215 after a complete round of mb-PMCA [31,32]. To determine whether the remaining 70% still
216 participated in the transformation of A_i to B_i assemblies during the quiescent phase, PMCA
217 products from the 139A, 127S and vCJD prions were treated with PK to eliminate PrP^C before
218 quiescent incubation at 37°C. As shown in Figure 3 (right curves), the amount of B_i assemblies
219 generated during the 48-h quiescent incubation was drastically decreased for the three prion
220 strains in the absence of the PrP^C substrate. Further quiescent incubation for 7 and 30 days in
221 the absence of PrP^C allowed the formation of low amounts of B_i assemblies for the 127S and
222 139A prion strains. The fact that the transformation of A_i to B_i assemblies is strongly facilitated
223 by the presence of PrP^C suggests that B_i assemblies result from the integration/conversion of
224 PrP^C into A_i assemblies. The appearance of a low amount of B_i after a long incubation period
225 without PrP^C may result from the leakage of monomers from a conformer cosedimenting with
226 A_i.

227

228 Kinetical scheme describing the transformation of A_i to B_i assemblies

229 To establish a kinetic mechanism and provide a molecular interpretation of the assemblies'
230 dynamics during the quiescent phase, a number of elementary steps were identified based on
231 experimental observations and were used as unavoidable constraints [14]. The first constraint
232 was the existence of two structurally distinct PrP^{Sc} subassemblies, namely, A_i and B_i , with
233 distinct dynamics. Indeed, structurally equivalent assemblies would fail to present a bimodal
234 size distribution, cooperative seed concentration and kinetic evolution or distinct specific
235 infectivity. The second constraint was the existence of a detailed balance between the PrP^{Sc}
236 assemblies and their elementary subunit (suPrP), as previously shown [20] making the size
237 distribution of the PrP^{Sc} assemblies highly dynamic and dependent on the assembly
238 concentration, as shown in Figure 1E. Indeed, SV analysis of the $\text{PrP}^{0/0}$ brains lysates seeded
239 with 30-fold-diluted 127S-infected brains and submitted to PMCA revealed a quaternary
240 structure rearrangement with a shift in lower molecular weight assemblies according to the
241 detailed balance:



243 where PrP_i^{Sc} and $\text{PrP}_{i-1}^{\text{Sc}}$ are the sizes i and $i-1$ of suPrP^{Sc} , respectively.

244 Because the existence of suPrP is a generic property of prion strains [20], the 3rd constraint
245 leads us to assume that the A_i and B_i assemblies are in detailed balance with their respective
246 suPrPs (denoted suPrP^A and suPrP^B) but with distinct equilibrium constants K_{eq}^A and K_{eq}^B .
247 Thus, at any moment of the process of assembly transformation of A_i to B_i , the following
248 equilibrium should be respected:



251 The equilibrium constant $K_{eq}^{A_i}$ and $K_{eq}^{B_i}$ governs the respective size distribution of the A_i and B_i
252 assemblies and, thus, the bimodal aspect of the curve. According to our previous SV
253 calibrations with PrP oligomers and globular mass markers [49], the size distribution of the A_i
254 and B_i subassemblies were fixed: $i_A < 5$ and i_B centered around 20 PrP-mers. Due to the limited
255 resolution of SV fractionation for small assemblies, we assumed that A_i and $suPrP^B$
256 cosedimented. The fourth constraint relies on the fact that the transformation of A to B requires
257 PrP^C and that the kinetic is cooperative, as shown in Figures 1E and 2. This cooperativity
258 implies that B subassemblies facilitate their own formation according to an autocatalytic
259 process. This can be resumed by the following minimalistic autocatalytic process:



262 where C is an active complex reacting with PrP^C that generates B assemblies. Considering that
263 $suPrP^B$ can condense into B_2 [20] and according to detailed balance (2), one can establish the
264 reaction model describing the formation of B_i assemblies from the neo-formed $suPrP^B$:



267 Altogether, these six elementary steps constitute the reaction mechanism that describes the
268 transformation of A_i into B_i subassembly species.

269 To validate the designed mechanism, we translated these elementary reactions into time-
270 dependent differential equations (for more details, see Supplementary text) and performed
271 kinetic simulations using the size distribution of the PrP^{Sc} assemblies immediately after cyclic
272 amplification as the initial condition (blue curve in Figure 2A). According to the model, the
273 simulated size distribution variation as a function of time showed bimodal behavior, as was

274 experimentally observed (Figure 4A). Furthermore, the theoretical size distribution centroid
275 presented similar sigmoidal patterns to those of the experimental data (Figure 4B), arguing in
276 favor of an autocatalytic kinetic model describing the overall quaternary structure evolution of
277 PrP^{Sc} assemblies during the quiescent phase. The analysis of the model (for more details, see
278 Supplementary text) revealed that the autocatalytic formation of B_i species occurs at the
279 expense of A_i species and with PrP^C consumption (Figure 4 C and D). According to this model,
280 when PrP^C is in large excess, A_i constitutes the limiting compound for the formation of B_i
281 assemblies. Therefore, during the quiescent phase, the PrP^C to PrP^{Sc} conversion rate is directly
282 proportional to the amount of A_i assemblies (Figure 4D).

283 Discussion

284 The mechanisms of prion replication and the dynamics responsible for prion structural
285 diversification in the infected host remain unclear and rarely addressed. In the actual framework
286 of the prion paradigm, the templating process is admitted to occur at the prion assembly
287 interface, leading to an increased size of the complex formed by the template:substrate, out of
288 the fragmentation/depolymerization context. The atypical size distribution observed here at the
289 early stage of the replication process for three distinct prion strains, where accumulation of
290 small-size assemblies dominates, contrasts with this canonical templating model and requires
291 an additional process that considers the dynamics of replication. Furthermore, the existence of
292 a multistep conversion process provides an unexpected approach to reconciling the best
293 replicator [35] selection paradigm and diversification process, which is inherent to prion
294 adaptation and evolution.

295 As shown *in vivo* for the vCJD, 127S and 139A prion strains, the early stage of the replication
296 process in the brain is dominated by the accumulation of small assemblies, whereas higher-size
297 subsets are mostly detected at the terminal stage of pathogenesis. Such quaternary structural
298 diversity, - and beyond the existence of structurally distinct types of assemblies, as defined by
299 their specific infectivity ([24,49] and Supplemental Figure 1), can be exclusively explained by
300 the existence of a balance between at least two kinetic modes taking place at different stages of
301 the pathogenesis. Both can be governed by evolution or a fluctuation in the replication
302 microenvironment due to the physio-pathological state of the infected animal and/or to the
303 sequential involvement of specific prion-replicating cell types. However, another possibility
304 can lie in the intrinsic and deterministic properties of the PrP replication process to generate
305 structurally distinct types of assemblies. Discriminating between these two nonmutually
306 exclusive hypotheses is technically difficult *in vivo*. The mb-PMCA as a *bona fide* amplification
307 method in a more simplified and kinetically controlled context constitutes a relevant method

308 for investigating the intrinsic propensity of the replication process to generate structurally
309 distinct assemblies. Interestingly, and against common belief, the size distribution of the PrP^{Sc}
310 assemblies used as seeds was relatively insensitive to mb-PMCA sonication cycles when a
311 simple dilution displaced the assemblies towards a smaller size (Figure 1E), as previously
312 reported [20]. These two observations exclude the contribution of the fragmentation process
313 during the mb-PMCA sonication cycles to the size distribution pattern of PrP^{Sc} assemblies and
314 emphasize the existence of a constitutional dynamic between the PrP^{Sc} subpopulation [20],
315 which should be considered during the replication process. We showed that two sets of PrP^{Sc}
316 assemblies, A_i and B_i, were generated during the mb-PMCA reaction. The A_i and B_i assemblies
317 constitute two structurally distinct PrP^{Sc} subpopulations, as supported by their distinct specific
318 infectivity; the bimodal size distribution instead of a continuum; the effect of initial seed
319 concentration on the respective proportions of A_i and B_i; and the role of PrP^C in the
320 transformation of A_i to B_i that indicates that B_i assemblies do not result from simple
321 condensation of A_i assemblies. Therefore, the prion replication process *per se* intrinsically
322 generates structurally diverse PrP^{Sc} subassemblies.

323 According to our SV experiments, small-sized PrP^{Sc} assemblies were mainly formed at the early
324 stage of prion replication in the brain and during the mb-PMCA reaction. This was observed
325 with three distinct prion strains (127S, 139A, vCJD) on 3 different PrP genetic backgrounds.
326 Considering that the PrP^{Sc} assemblies that constitute each strain are structurally distinct, one
327 can ask how distinct PrP^{Sc} assemblies can all generate A_i assemblies that harbor strain structural
328 information while showing the same quaternary structure (at the SV resolution). The first
329 explanation can be the existence of a common narrow subpopulation of PrP^{Sc} (with respect to
330 their quaternary structure) within the three strains that serves as the best replicator and
331 participates in the formation of A_i assemblies. However, the PrP^{Sc} quaternary structure subset
332 that exhibits the highest specific infectivity *in vivo* (i.e., the best replicator) can be associated

333 with either small-size assemblies (i.e., 127S and 139A [24,49] and Supplemental Figure 1A,
334 respectively) or high-molecular-weight assemblies (i.e., vCJD, Supplemental Figure 1B) and is
335 therefore strain-dependent. The existence of a structurally common PrP^{Sc} subpopulation is thus
336 unlikely to be at the origin of the generic formation of a small-size subset in the brain or A_i
337 assemblies in the mb-PMCA condition. Intrinsically, the early steps of the replication process
338 favor the emergence of mainly one subspecies A_i with a highly narrowed size distribution,
339 arguing in favor of a quaternary structural convergence phenomenon during these steps. This
340 structural convergence concerns the PrP domain that governs polymerization (the size of
341 assemblies). However, as the A assemblies harbor the strain structural determinant, one can
342 conclude that A_i assemblies present a certain degree of structural variability, allowing the
343 encoding of strain structural information (Supplemental Figure 1).

344 All along the quiescent phase and for the three prion strains studied, the A_i assemblies constitute
345 the precursor species in the formation of B_i assemblies. Furthermore, the presence of PrP^C is
346 required for the evolution of A_i into B_i assemblies, and according to the kinetic model
347 describing the autocatalytic formation of B_i during the quiescent phase, A_i is the limiting species
348 for conversion when large amounts of PrP^C are present (Figure 4C and D and Supplementary
349 text). The bimodal quaternary structure evolution during the quiescent phase is in concert with
350 a specific infectivity decrease that is indicative of a structural rearrangement of species present
351 in P₁ and P₂ and thus during the transformation of A_i to B_i. Even if the first event conducing to
352 the formation of B_i assemblies remains undetermined, we can assume that A_i can have the
353 intrinsic propensity to spontaneously evolve into B_i assemblies in the presence of PrP^C (Figure
354 5). The cooperative disappearance of P₁ in favor of P₂ strongly suggests an autocatalytic process
355 for the transformation of A_i to B_i (reactions 3 and 4). This last phenomenon shows the existence
356 of a secondary autocatalytic process, undescribed until now, in the canonical prion replication
357 process [26]. One can be reasonably envisage that A_i can have the intrinsic propensity to

358 generate B_i assemblies in the presence of PrP^C assemblies with a very low efficiency. This
359 parallel pathway to the autocatalytic process can then explain how the first set of B_i assemblies
360 is generated (Figure 5). The existence of a secondary autocatalytic process can be crucially
361 important for maintaining PrP^{Sc} structural diversity throughout the evolution of the pathology.
362 In the absence of this secondary autocatalytic process, e.g., in the absence of PrP^C , the system
363 selects the best replicator and the most thermodynamically stable assemblies. In the presence
364 of PrP^C , the system escapes this rule, allowing the specific accumulation of the autocatalytic
365 product (here, the B_i assemblies) rather than the assemblies that are the most
366 thermodynamically stable or have highly specific infectivity. This phenomenon can explain
367 why, for certain prion strains, the most infectious assemblies represent a minor population,
368 while those with the lowest specific infectivity mostly accumulate [24,49].

369

370 Conclusion

371 The early step of prion replication for at least three distinct prion strains leads to the formation
372 of small assemblies. The mb-PMCA approach clearly demonstrates the intrinsic properties of
373 the *bona fide* replication process to generate at least two structurally distinct PrP^{Sc}
374 subassemblies. The deterministic aspect of the replication process to generate a structurally
375 diverse set of assemblies contrasts with the widespread idea that considers the prion
376 diversification process within a given strain (often referred to as the creation of prion quasi-
377 species) as a stochastic event and as a process that is cogoverned by environmental fluctuations
378 [52]. The secondary autocatalytic pathway leading to the formation of B_i subassemblies can
379 participate in prion adaptation during transmission events with species barriers. Considering
380 that the transmitted inoculum initially contains A_i and B_i assemblies, the autocatalytic
381 conversion process of B_i can kinetically drive the adjustment and integration of the new-host

382 PrP^C to generate host-adapted B_i assemblies. This hypothesis is supported by our recent
383 observations in which complementation between A_i and B_i subassemblies is required to cross
384 existing species barriers (submitted article).

385

386 **Methods**

387 **Ethics**

388 Animal experiments were conducted in strict accordance with ECC and EU directives 86/009
389 and 2010/63 and were approved by the local ethics committee of the author's institution
390 (Comethea; permit numbers 12/034 and 15/045).

391 **Transgenic mouse lines and prion strains**

392 The ovine (tg338 line; Val136-Arg154-Gln171 VRQ allele), human (tg650 line; Met129 allele)
393 and mouse (tga20) PrP transgenic lines have been described previously [3,15,25]. The mouse
394 lines were homozygous and overexpressed approximately 8-, 6-, and 10-fold amounts of
395 heterologous PrP^C on a mouse PrP-null background. PrP^{0/0} mice were the so-called Zürich-I
396 mice [10]. Cloned 127S scrapie, human vCJD and mouse 139A prion strains were serially
397 passaged in tg338, tg650 and tga20 mice, respectively [31,32]. These strains were used as pools
398 of mouse-infected brains and prepared as 20% wt/vol homogenates in 5% glucose by use of a
399 tissue homogenizer (Precellys 24 Ribolyzer; Ozyme, France).

400 **Time course analysis of prion accumulation**

401 Eight-week-old female tg338, tg650 and tg20 mice were inoculated intracerebrally in the right
402 cerebral hemisphere with 127S, vCJD or 139A prions (20 µl of a 10% brain homogenate dose).
403 Infected animals were euthanized by cervical column disruption in triplicate at regular time
404 points and at the terminal stage of disease. Brains were removed and kept for PrP^{Sc} size
405 fractionation.

406 Miniaturized bead-PMCA assay

407 The miniaturized bead-PMCA assay [11,20,32] was used to amplify prions. Briefly, serial ten-
408 fold dilutions of 127S, vCJD and 139A prions (mouse brain homogenates diluted in PMCA
409 buffer) were mixed with brain lysates (10% wt/vol) from healthy tg338, tg650 and tga20 mice
410 as respective substrates and subjected to one round of 96 cycles of 30-s sonications (220-240
411 Watts) followed by 29.5 min of incubation at 37°C. With a $>10^4$ dilution of the seeds, input
412 PrP^{Sc} is not detected in the mb-PMCA products. PMCA was performed in a 96-well microplate
413 format using a Q700 sonicator (QSonica, USA, Delta Labo, Colombelles, France). For
414 quiescent incubation, the samples were left in the incubator at 37°C for the indicated period of
415 time, without any sonication. To eliminate residual PrP^C present in the PMCA products before
416 quiescent incubation, the samples were treated with PK (80 µg/ml final concentration). The
417 treatment was stopped by adding 2 mM Pefabloc and 1x EDTA-free protease inhibitor cocktail.
418 All final products were kept for PrP^{Sc} size fractionation, and aliquots were PK-digested (115
419 µg/ml final concentration, 0.6% SDS, 1 h, 37°C) prior to immunoblot analyses, as described
420 below.

421 Sedimentation velocity fractionation

422 SV experiments were performed as described previously [24,49,20]. Mouse brain homogenates
423 or PMCA products were solubilized by adding an equal volume of solubilization buffer (50
424 mM HEPES pH 7.4, 300 mM NaCl, 10 mM EDTA, 2 mM DTT, 4% wt/vol dodecyl- β-D-
425 maltoside (Sigma)) and incubated for 45 min on ice. Sarkosyl (N-lauryl sarcosine; Fluka) was
426 added to a final concentration of 2% wt/vol, and the incubation continued for an additional 30
427 min on ice. A total of 150 µl of solubilized samples was loaded atop a 4.8-ml continuous 10–
428 25% iodixanol gradient (Optiprep, Axys-Shield), with a final concentration of 25 mM HEPES
429 pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5% Sarkosyl. The gradients were
430 centrifuged at 285,000 g for 45 min in a swinging-bucket SW-55 rotor using an Optima LE-

431 80K ultracentrifuge (Beckman Coulter). Gradients were then manually segregated into 30 equal
432 fractions of 165 μ l from the bottom using a peristaltic pump and analyzed by immunoblotting
433 or bioassay for PrP^{Sc} or infectivity, respectively. To avoid any cross-contamination, each piece
434 of equipment was thoroughly decontaminated with 5 M NaOH followed by several rinses in
435 deionized water after each gradient collection [24].

436 **Isopycnic sedimentation**

437 The entire procedure was performed as described previously [24]. Mouse brain homogenates
438 or PMCA products were solubilized as described above. For mouse brain homogenates,
439 solubilization was performed at 37°C to mimic PMCA conditions. A total of 220 μ l of
440 solubilized material was mixed to reach 40% iodixanol, 25 mM HEPES pH 7.4, 150 mM NaCl,
441 2 mM EDTA, 1 mM DTT, 0.5% Sarkosyl final concentration and loaded within a 4.8 ml of 10–
442 60% discontinuous iodixanol gradient with a final concentration of 25 mM HEPES pH 7.4, 150
443 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5% Sarkosyl. The gradients were centrifuged at 115
444 000 g for 17 hours in a swinging-bucket SW-55 rotor using an Optima LE-80K ultracentrifuge
445 (Beckman Coulter). Gradients were then manually segregated into 30 equal fractions of 165 μ l
446 from the bottom using a peristaltic pump and analyzed for PrP^{Sc} content by immunoblotting.

447 **Analysis of PrP^{Sc} content by immunoblotting**

448 Aliquots of the SV-fractionated PMCA samples were treated with PK (50 μ g/ml final
449 concentration, 1 h, 37°C) before mixing in Laemmli buffer and denaturation at 100°C for 5
450 min. The samples were run on 12% Bis-Tris Criterion gels (Bio-Rad, Marne la Vallée, France)
451 and electrotransferred onto nitrocellulose membranes. In some instances, denatured samples
452 were spotted onto nitrocellulose membranes using a dot-blot apparatus (Schleicher & Schuell
453 BioScience (Whatman)). Nitrocellulose membranes were probed for PrP with 0.1 μ g/ml
454 biotinylated anti-PrP monoclonal antibody Sha31. Immunoreactivity was visualized by
455 chemiluminescence (GE Healthcare). The protein levels were quantified with ImageLab

456 software after acquisition of chemiluminescent signals with a Chemidoc digital imager (Bio-
457 Rad, Marnes-la-Coquette, France). For all SDS-PAGE analyses, a fixed quantity of human
458 recombinant PrP was employed for consistent calibration of the PrP signals in different gels.

459 **Bioassays**

460 The pool of fractions of interest was extemporarily diluted ten-fold in 5% glucose and
461 immediately inoculated via the intracerebral route into reporter tg338 mice (20 μ l per pool of
462 fraction, n = 5 mice per pool). Mice showing prion-specific neurological signs were euthanized
463 at the end stage. To confirm prion disease, brains were removed and analyzed for PrP^{Sc} content
464 using the Bio-Rad TsSeE detection kit [27] prior to immunoblotting, as described above. The
465 survival time was defined as the number of days from inoculation to euthanasia. To estimate
466 what the difference in mean survival times means in terms of infectivity, strain-specific curves
467 correlating the relative infectious dose to survival times were used, as previously described
468 [49].

469 **Kinetic simulation**

470 The details of the kinetic simulation are reported in the Supplementary text. Briefly, two distinct
471 sets of assemblies were considered (A_i and B_i). Based on experimental observations, a set of
472 constraints was retained to build biochemical reactions describing the evolution of the
473 quaternary structure of PrP^{res} assemblies. The ordinary differential equations of the biochemical
474 reactions 1 to 6 (in the manuscript) were established and coded in MATLAB for simulations.

475 **Legends**

476 **Figure 1. Size distribution of PrP^{Sc} assemblies from different prion strains at the early** 477 **and late stages of pathogenesis *in vivo* and after the PMCA reaction**

478 The size distribution of proteinase K (PK)-resistant PrP^{Sc} assemblies present in the brain *in vivo*
479 (A-C) and in PMCA products (D-E) was examined by sedimentation velocity (SV).

480 (A-C) For the *in vivo* sedimentograms, brains from ovine (tg338), murine (tga20) and human
481 (tg650) transgenic mice inoculated with 127S scrapie prions (A), 139A mouse prions (B) and
482 vCJD human prions (C) were collected (in triplicate) at the early stage (15 days postinfection
483 (127S), 11 days postinfection (139A) and 120 days postinfection (vCJD), blue curves) and at
484 the end stage of the disease (60 days postinfection (127S), 55 days postinfection (139A), 495
485 days postinfection (vCJD), red curves). The brains were solubilized and SV-fractionated. The
486 collected fractions (numbered from top to bottom) were analyzed for PK-resistant PrP^{Sc} content
487 by immunoblotting.

488 (D-E) For the sedimentograms from the PMCA products with PrP^C substrate (D), the same
489 strains were subjected to a single round of mb-PMCA by using 10⁻⁵ (139A) or 10⁻⁶ (vCJD,
490 127S) diluted brain homogenates as seed for the reaction. Thirty minutes after the last
491 sonication, the amplified products were solubilized and SV-fractionated. The mean levels of
492 PK-resistant PrP^{Sc} per fraction were obtained from the immunoblot analysis of $n=4$ independent
493 fractionations of PMCA reactions. The peaks containing PrP^{Sc} assemblies sedimentating in the
494 top and middle fractions were termed P₁ and P₂, respectively. For the sedimentograms from the
495 PMCA products without PrP^C substrate (E), undiluted 127S-infected tg338 brain (20% w/v, red
496 curve) or a 1:32 dilution in PMCA buffer (blue curve) was used as seed, mixed with brain
497 homogenate from PrP^{0/0} mice as substrate and subjected to a single round of mb-PMCA before
498 SV fractionation (mean levels from $n=3$ independent fractionations).

499

500 **Figure 2. Seed concentration- and time-dependent dynamic evolution of the PMCA-**
501 **generated PrP^{Sc} assemblies**

502 **(A-B)** SV profile of mb-PMCA products seeded with ten-fold dilutions from 127S-infected
503 brain homogenates, as indicated. Thirty minutes after the last sonication, the amplified products
504 were solubilized and SV-fractionated. The mean relative levels of PK-resistant PrP^{Sc} per
505 fraction **(A)** were obtained from the immunoblot analysis of $n=4$ independent fractionations of
506 PMCA reactions (representative dot-blot shown). Variation in the P₁ and P₂ peak surface areas
507 as a function of the logarithm of the seed dilution factor **(B)**.

508 **(C)** PK-resistant PrP^{Sc} sedimentograms from the PMCA products generated with 127S prions
509 (10^{-5} dilution) and further incubated at 37°C during the indicated quiescent phase (t), i.e.,
510 without sonication. At each time point, the collected products were frozen. All collected
511 samples were then thawed, fractionated in parallel by SV and analyzed by immunoblot **(C, $n=3$)**
512 independent experiments, representative dot-blot shown).

513 **(D)** PK-resistant PrP^{Sc} isopycnic sedimentograms from PMCA products generated with 127S
514 prions (10^{-5} dilution) and immediately fractionated at the end of the PMCA reaction (blue line
515 and symbol) or after a 24h-quiescent incubation at 37°C (red line and symbol). At each time
516 point, the collected samples were frozen. All collected samples were then thawed, fractionated
517 in parallel by sedimentation at the equilibrium [24] and analyzed by immunoblot (the mean
518 levels of PK-resistant PrP^{Sc} per fraction were obtained from the immunoblot analysis of $n=3$
519 independent fractionations of PMCA reactions). As control, the density profile of PK-resistant
520 PrP^{Sc} assemblies from the brain of terminally sick tg338 mice (solubilization at 37°C to mimic
521 the PMCA conditions) is shown (gray line and symbol).

522 **(E)** Evolution of the percentage of P₁ and P₂ peaks as a function of the quiescent phase post-
523 PMCA reaction **(C)**.

524 (F) PK-resistant PrP^{Sc} sedimentograms from the PMCA products generated with 139A and
525 vCJD prion seeds (10^{-5} dilution) and further incubated for a quiescent period of 24 h at 37°C.
526 (G) Specific infectivity of the P₁ and P₂ peaks post-PMCA reaction and after quiescent
527 incubation. Fractions corresponding to P₁ (fractions 1-3) and P₂ (fractions 14-16 (days 0 and 2)
528 or 16-18 (day 7)) were pooled and inoculated into groups of reporter tg338 mice at two different
529 dilutions (1:10 and 1:1000) for better accuracy. The specific infectivity of the assemblies was
530 calculated from the mean survival time of the mice using a 127S dose-response curve. *:
531 incomplete attack rate.

532

533 **Figure 3. PrP-dependent generation of B_i assemblies from A_i assemblies**

534 PMCA products from 127S, 139A and vCJD prions (10^5 , 10^4 and 10^4 diluted seeds,
535 respectively) were treated with or without PK to eliminate PrP^C before quiescent incubation at
536 37°C for 24 h, 7 days or 30 days, as indicated. At each time point, the collected products were
537 frozen. All collected samples were then thawed, SV-fractionated in parallel and analyzed by
538 immunoblotting ($n=3$ independent experiments).

539

540 **Figure 4. Mathematical modeling of the time-dependent dynamic evolution of the PMCA-** 541 **generated PrP^{Sc} assemblies**

542 (A) The size distribution evolution of a structurally distinct set of assemblies A_i and B_i
543 dimensioned on gradient fraction numbers was simulated based on the kinetic scheme described
544 in the results section (equations 1 to 6) and the Supplementary text.

545 (B) The time dependency evolution of the simulated centroid (black line) and centroid
546 calculated from experimental sedimentograms of Figure 2D (red circle) show a similar shape,
547 supporting the cooperativity hypothesis of the transformation of A_i into B_i.

548 (C) The simulation of time dependency evolution of the total amount of A_i assemblies ($\sum iA_i$
549 in black), B_i assemblies ($\sum iB_i$ in blue) and the monomer (in red) revealed that A_i assemblies
550 constitute the limiting species for the conversion of PrP^C during the quiescent phase. In the
551 present simulation framework (for more details, see Supplementary text), only 14% of PrP^C is
552 consumed.

553

554 **Figure 5. Quaternary structural convergence and secondary autocatalytic pathway at the**
555 **root of the formation of B_i assemblies**

556 (A) Different prion strains (S_1 , S_2 and S_3) give rise to the formation of common oligomeric
557 assemblies, termed A_i , with a narrowed size distribution during mb-PMCA reactions. This
558 common quaternary structural convergence at the early stage of the replication process suggests
559 the existence of a common conversion pathway and a common oligomerization domain that is
560 independent of the strain structural determinant (SSD, represented in red).

561 (B) According to the dilution experiments (see Figure 1E), an equilibrium exists between PrP^{Sc}
562 assemblies and the suPrP from each subpopulation [20]. Based on the constraints imposed by
563 the experimental observations, the best model to account for the cooperative and PrP^C
564 dependency transformation of A_i into B_i assemblies implicates a secondary templating pathway
565 where the transformation of suPrP^A to suPrP^B is assisted by suPrP^B, rendering the process
566 autocatalytic.

567

568 **Supplemental Figure 1. PK-resistant PrP^{Sc} and the infectivity sedimentation profile of**
569 **139A and vCJD prion strains**

570 Brain homogenates from tga20 mice infected with 139A prions (A) and tg650 mice infected
571 with vCJD prions (B) were solubilized and SV-fractionated. The collected fractions were
572 analyzed for PK-resistant PrP^{Sc} content (black line) and for infectivity (red bars or line) with an

573 incubation time bioassay in reporter tga20 and tg650 mice. The mean survival time values of
574 these mice were reported as standard dose-response curves ([18] and unpublished) to determine
575 relative infectious dose values. A relative infectious dose of 0 corresponds to animals inoculated
576 with 2 mg of infectious brain tissue.
577

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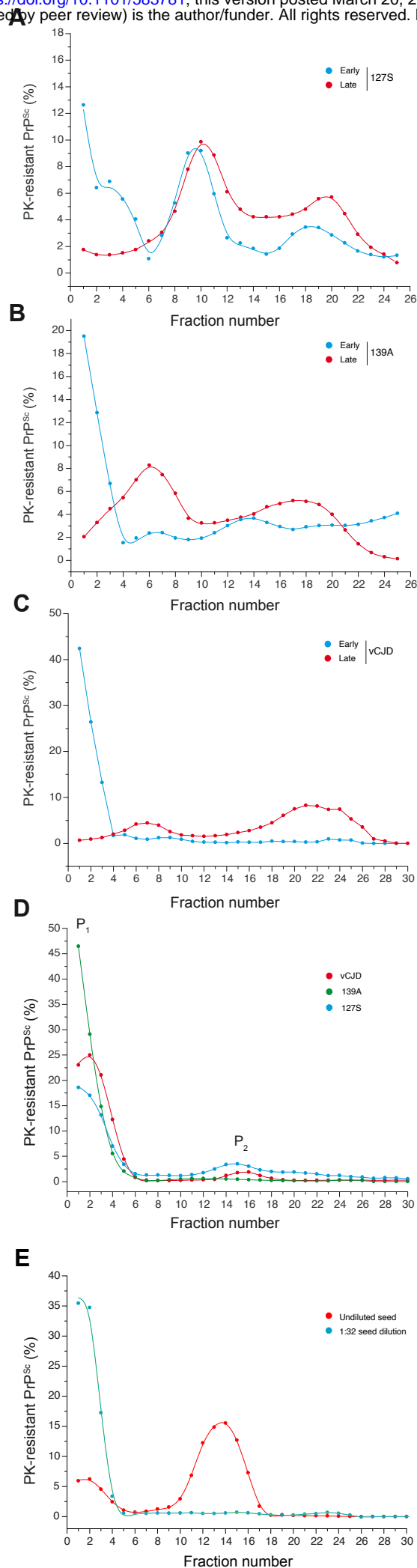


Figure 1

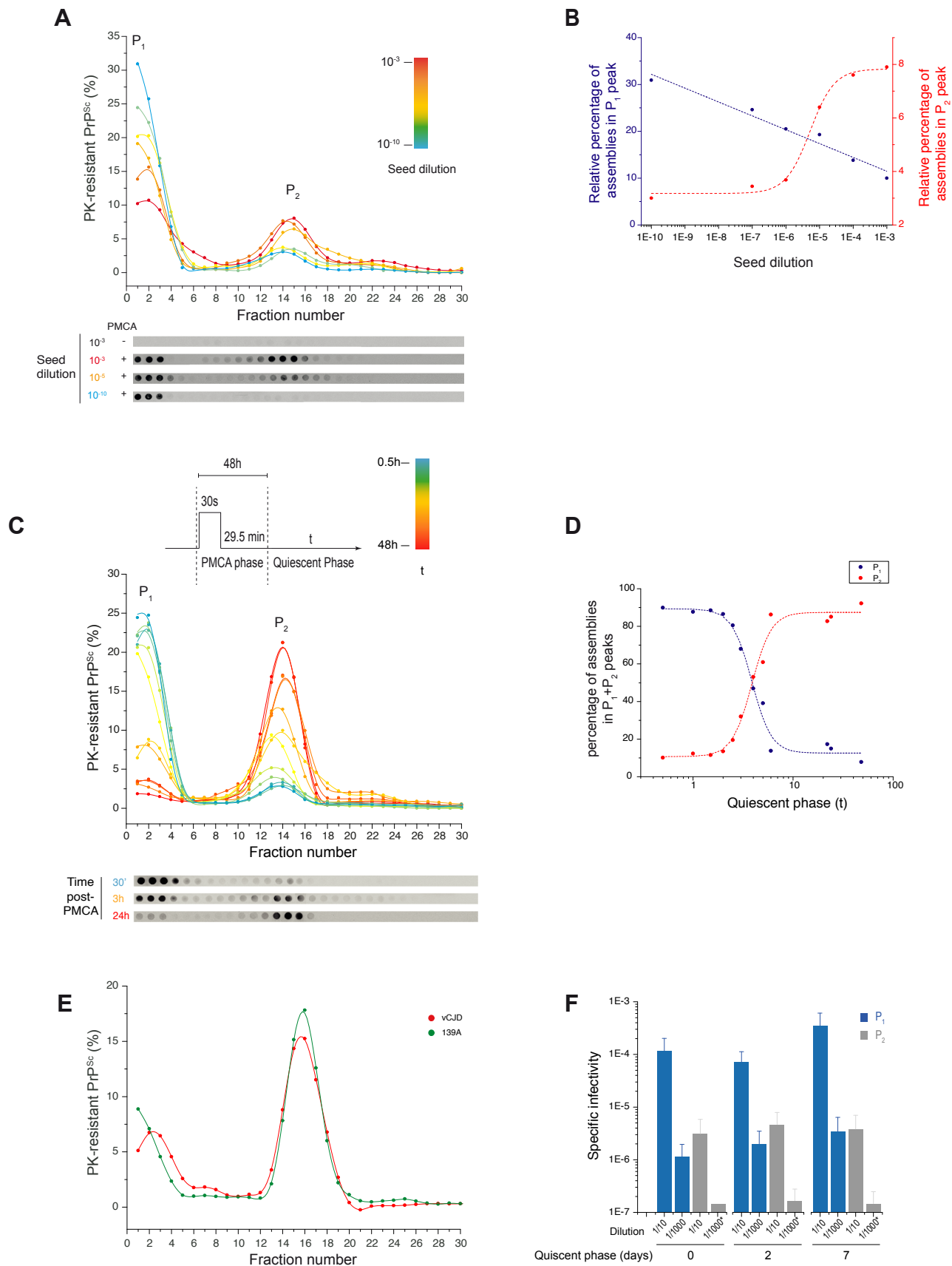


Figure 2

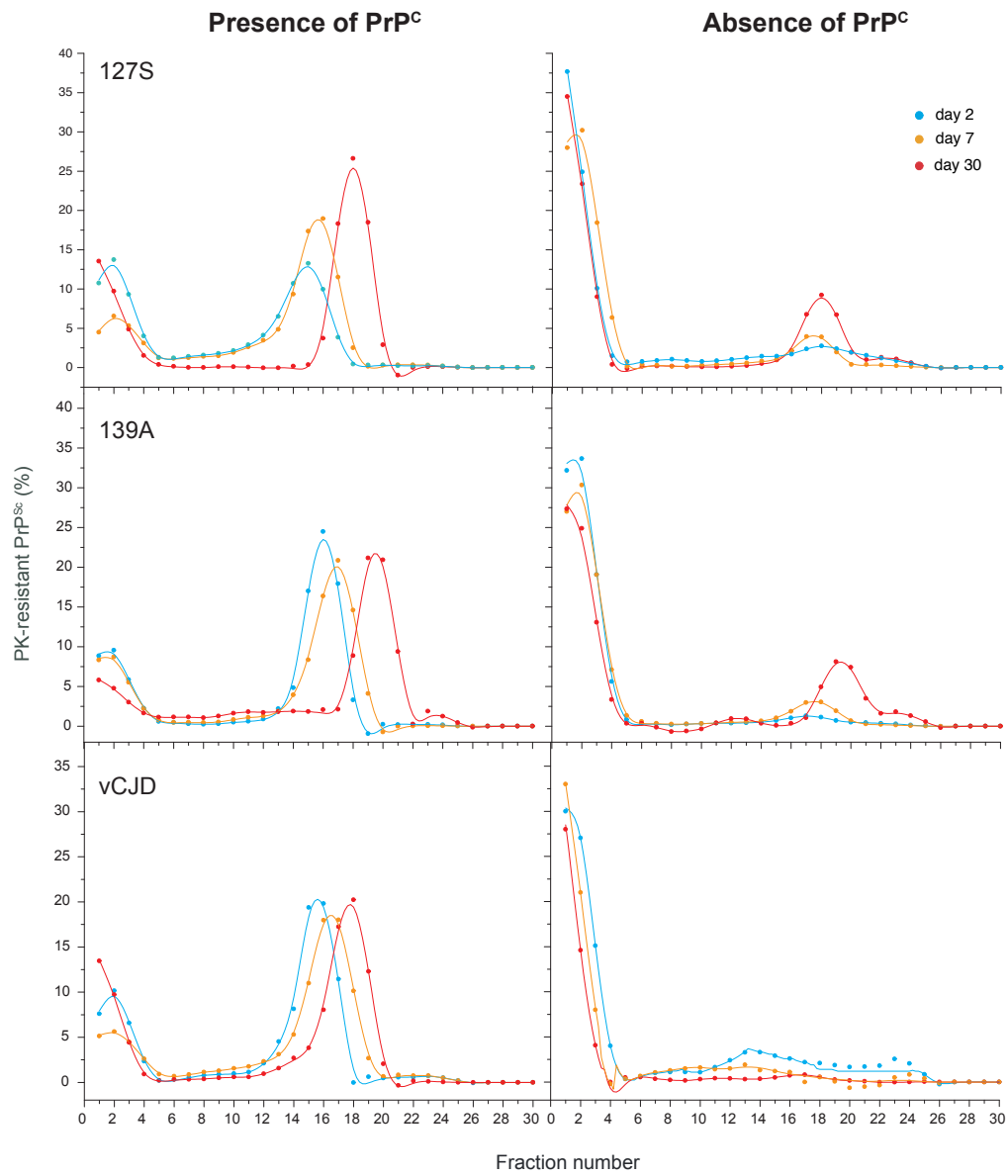


Figure 3

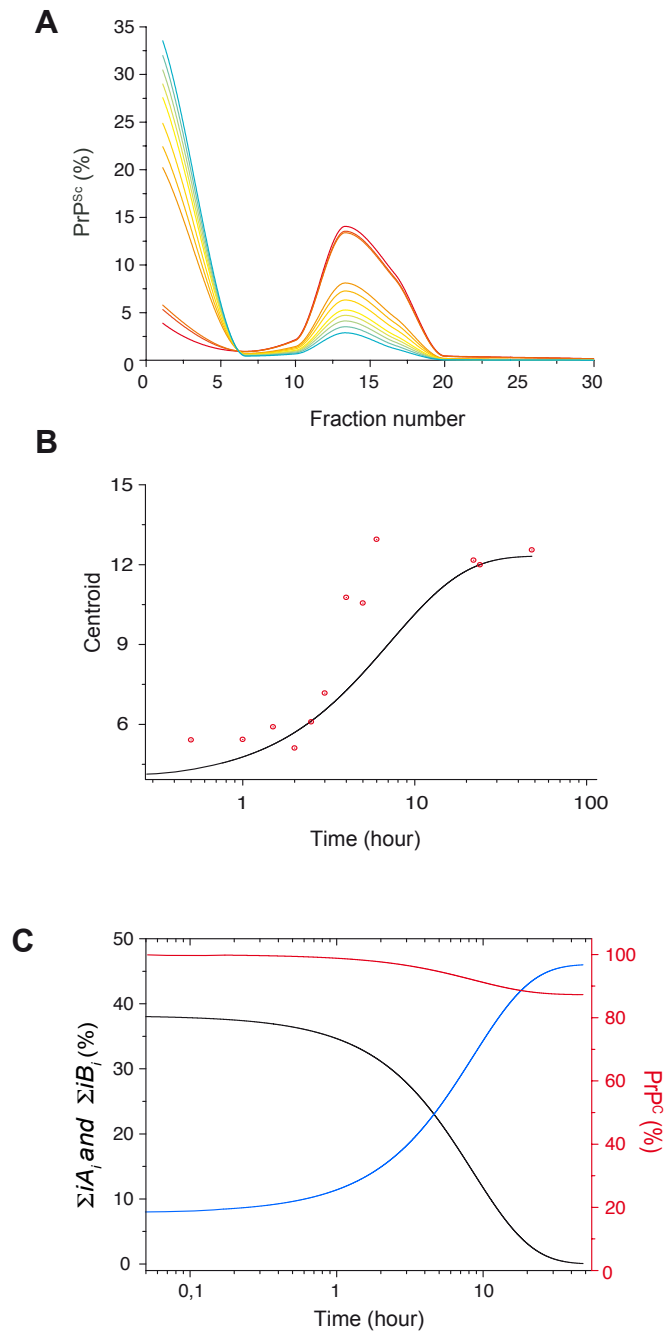
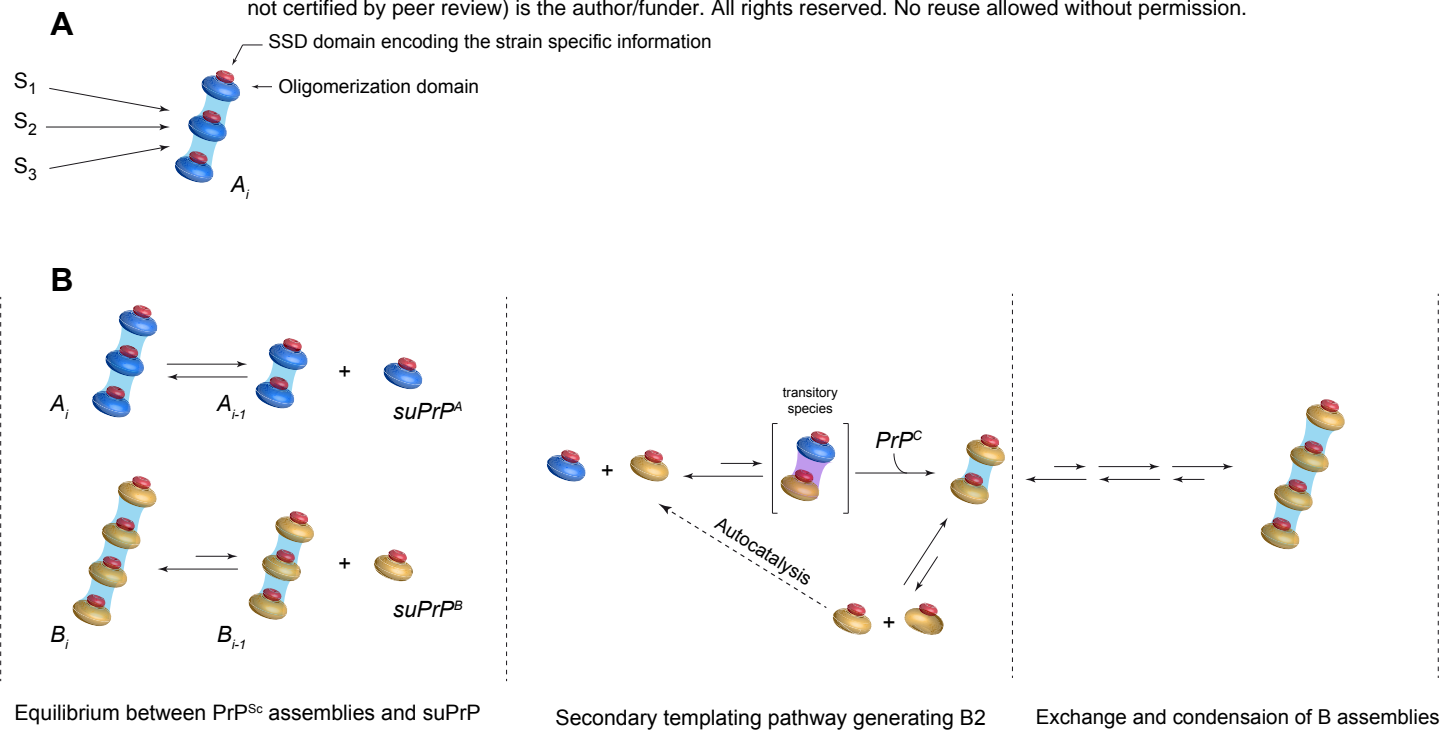


Figure 4



Equilibrium between PrP^{Sc} assemblies and $suPrP$

Secondary templating pathway generating B2

Exchange and condensation of B assemblies