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1 **Complementation between pathological prion protein subassemblies to cross**
2 **existing species barriers**

3 Angélique Igel-Egalon^{1¶}, Florent Laferrière^{1¶#}, Philippe Tixador^{1¶∇}, Mohammed Moudjou¹,
4 Laetitia Herzog¹, Fabienne Reine¹, Juan Maria Torres², Hubert Laude¹, Human Rezaei^{1*}, and
5 Vincent Béringue^{1*}

6

7 ¹VIM, INRA, Université Paris-Saclay, 78350, Jouy-en-Josas, France,

8 ²Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, Spain

9 [#]Current address: Institute of Neurodegenerative diseases, CNRS UMR5293, University of
10 Bordeaux, Bordeaux, France

11

12 ^{*}Corresponding authors:

13 E-mail: vincent.beringue@inra.fr (VB), human.rezaei@inra.fr (HR)

14 [¶]Equal contributors

15

1 **Abstract**

2 **Background:** prion replication results from the autocatalytic templated assisted conversion of
3 the host-encoded prion protein PrP^C into misfolded, polydisperse PrP^{Sc} conformers. Structurally
4 distinct PrP^{Sc} conformers can give rise to multiple prion strains. Within and between prion
5 strains, the biological activity (replicative efficacy and specific infectivity) of PrP^{Sc} assemblies
6 is size-dependent and thus reflects an intrinsic structural heterogeneity. The contribution of such
7 PrP^{Sc} heterogeneity across species prion adaptation, - which is believed to be based on fit-
8 adjustment between PrP^{Sc} template(s) and host PrP^C -, has not been explored.

9 **Methods:** to define the structural-to-fitness PrP^{Sc} landscape, we measured the relative capacity
10 of size-fractionated PrP^{Sc} assemblies from different prion strains to cross mounting species
11 barriers in transgenic mice expressing foreign PrP^C.

12 **Results:** in the absence of a transmission barrier, the relative efficacy of the isolated PrP^{Sc}
13 assemblies to induce the disease is superimposable to the efficacy observed in the homotypic
14 context. However, in the presence of a transmission barrier, size fractionation overtly delays
15 and even abrogates prion pathogenesis in both neural and extraneural, prion-permissive tissues,
16 for reason independent of the infectivity load of the isolated assemblies. This suggests that a
17 synergy between structurally distinct PrP^{Sc} assemblies in the inoculum is requested for crossing
18 the species barrier. We further strengthen this hypothesis by showing that altering, by serial
19 dilution, PrP^{Sc} assemblies content of unfractionated inocula reduce their specific infectivity in
20 an aberrant manner, solely in the presence of a transmission barrier.

21 **Conclusions:** our data support a mechanism whereby overcoming prion species barrier requires
22 complementation between structurally distinct PrP^{Sc} assemblies. This work provides key insight
23 into the “quasi-species” concept applied to prions, which would not necessarily rely on prion
24 sub-strains as constituent but on structural PrP^{Sc} heterogeneity within prion population.

25

1 **Keywords**

2 Prion / species barrier / transgenic mice / quasi-species / sedimentation velocity / assemblies

3

4 **Background**

5 Mammalian prions are proteinaceous pathogens formed from misfolded assemblies (PrP^{Sc}) of
6 the host-encoded prion protein PrP^C. Prions self-replicate by templating the conversion and
7 polymerization of PrP^C [1]. Prions cause inexorably fatal neurodegenerative diseases such as
8 human Creutzfeldt-Jakob disease (CJD), sheep scrapie, bovine spongiform encephalopathy
9 (BSE) and chronic wasting disease of cervids [2].

10 The prion strain phenomenon which is due to a structural polymorphism of PrP^{Sc} assemblies is
11 defined by the physiopathological and biochemical characteristics of prion disease within these
12 host species and in experimental models [3-10]. The strain-specified physiopathological
13 differences include duration of disease in the challenged or affected species, vacuolation
14 distribution and pattern of PrP^{Sc} deposition in the brain, tropism for the lymphoid tissue and
15 biochemical properties of PrP^{Sc}, including resistance to denaturation and to proteases [3]. How
16 PrP^{Sc} structural polymorphism causes such distinct phenotypes remains poorly understood.

17 A broad panel of experimental observations, including by size fractionation supports the
18 existence of structurally different PrP^{Sc} subsets within a given prion strain [11-19]. This intra-
19 strain structural heterogeneity results from the intrinsic and deterministic properties of the prion
20 replication process to generate structurally diverse PrP^{Sc} subsets [20]. Structural diversity can
21 be observed at different levels. Size-fractionation studies by sedimentation velocity (SV)
22 indicate that variability in PrP^{Sc} quaternary structure is strain-specific [15,18,17,21,22]. Within
23 a given prion strain, PrP^{Sc} assemblies with differing quaternary structure exhibit markedly
24 different templating and biological activities, which are a hallmark of the existence of
25 structurally distinct PrP^{Sc} subpopulations, as previously discussed [23]. The biological and

1 biochemical consequences of PrP^{Sc} structural heterogeneity during prion replication and
2 propagation are poorly understood.

3 Maybe one of the most intriguing and unpredictable change in prion replicative environment is
4 the interspecies transmission. In certain host/strain combinations, prions will propagate readily
5 as in an intraspecies transmission. In other host/strain combinations, only a fraction of the
6 exposed animals will develop the disease with variable incubation periods, reflecting a ‘species’
7 or ‘transmission’ barrier. Attaining full attack rate and minimal incubation periods will require
8 iterative transmissions. At that stage, prions are considered to be adapted to the new host [3].

9 Two main theoretical models have been developed to describe the transmission barrier at the
10 molecular level. Both propose that the species barrier is governed by a misfit between PrP^{Sc}
11 contained in the infecting prion and host PrP^C folding landscape [5,24]. Schematically, the first
12 one called ‘deformed templating’ consider that PrP^C will progressively adopt, -due to its
13 specific conformational dynamic-, the “quasi-right” conformation to be selected during the
14 templating process [24]. As a consequence, a switch to a new strain structural determinant can
15 occur, which combines structural information from the inoculated PrP^{Sc} assemblies with the
16 folding landscape of the new host PrP^C [25-29]. A major limitation of this model resides in the
17 assumption that all PrP^{Sc} assemblies are structurally equivalent, despite experimental counter-
18 evidence. The second model called ‘conformational selection’ considers the existence of
19 structurally distinct PrP^{Sc} subsets within a strain or isolate, the species adaptation resulting from
20 the selection of the best replicator [5]. The two models are not mutually exclusive. They at least
21 have the merit to highlight the role of structural diversity of PrP^C and/or PrP^{Sc} in prion
22 adaptation and evolution.

23 Here, we aim at defining the contribution of PrP^{Sc} (quaternary) structure polydispersity to across
24 species prion fitness. We compare the relative capacity of size-fractionated PrP^{Sc} assemblies to
25 propagate in transgenic mice expressing homotypic versus heterotypic PrP^C, used as proxy of

1 mounting species barriers (one with prion ‘mutation’). We show that fractionating PrP^{Sc}
2 assemblies strengthens existing species barrier, to a degree of magnitude independent of their
3 infectivity load. Coexistence of heterogeneous PrP^{Sc} quaternary structure in the inoculum may
4 thus be requested to cross species barriers. We strengthen this hypothesis by showing that
5 altering, by serial dilution, PrP^{Sc} assemblies content overtly reduces the specific infectivity from
6 unfractionated inocula during cross-species transmission events. Our data support a mechanism
7 whereby overcoming prion species barrier requires complementation between structurally
8 distinct PrP^{Sc} subsets within the inoculum, refining the current prion adaptation models.

9 **Methods**

10 **Transgenic mouse lines**

11 The transgenic mouse lines expressing ovine, hamster and bovine PrP have been described
12 previously [30-33]. The bovine PrP tg540 [30] and tg110 [31] lines showed equivalent
13 susceptibilities to classical BSE prions [30].

14 **Prion sources**

15 LA19K, LA21K *fast* and 127S scrapie prions are cloned prion strains. They have been obtained
16 by serial transmission and subsequent biological cloning by limiting dilutions of classical field
17 scrapie isolates to tg338 transgenic mice expressing the VRQ allele of ovine PrP [32,18]. Pooled
18 tg338 mouse brain homogenates (20% wt/vol. in 5% glucose) were used in centrifugation
19 analyses, as indicated.

20 The transmission properties of the L-type BSE isolate (designated BASE by the authors [34])
21 in tgBov mice (tg540 line) and tgOv mice have been previously described [26]. The same brain
22 material from the same isolate was transmitted to tgBov line (tg110) line by intracerebral route
23 by using 20 µl of a 10% (wt/vol. in 5% glucose) brain homogenate.

1 **Fractionation by sedimentation velocity**

2 The sedimentation velocity procedure has been previously and comprehensively described
3 [15,18,20,13]. Briefly, detergent-solubilized brain homogenates were loaded atop a continuous
4 10–25% iodixanol gradient (Optiprep, Axys-shield). The gradients were centrifuged at 285 000
5 g for 45 min in a swinging-bucket SW-55 rotor using an Optima LE-80K ultracentrifuge
6 (Beckman Coulter). Gradients were then manually segregated into 30 equal fractions of 165µl
7 from the bottom using a peristaltic pump. Fractions were aliquoted for immunoblot and
8 bioassays. Gradient linearity was verified by refractometry. To avoid any cross-contamination,
9 each piece of equipment was thoroughly decontaminated with 5 M NaOH followed by several
10 rinses in deionised water after each gradient collection.

11 **Bioassays**

12 Fractions were diluted extemporarily in 5% glucose (1:5) in a class II microbiological cabinet
13 according to a strict protocol to avoid any cross-contamination. Individually identified 6- to 10-
14 week-old mice were inoculated intracerebrally with 20 µl of the solution, using a 27-gauge
15 disposable syringe needle inserted into the right parietal lobe. At terminal stage of disease or at
16 end-life, mice were euthanized and analyzed for proteinase K (PK) resistant PrP^{Sc} (PrP^{res})
17 content in brains and spleens tissues (as indicated), using the Bio-Rad TsSeE detection kit [26]
18 before immunoblotting, as described below.

19 **Endpoint titration**

20 For titration purposes, groups of indicator mice were inoculated intracerebrally (20 µl) with
21 serial ten-fold dilutions of the indicated brain homogenates. Animals inoculated with the initial
22 dose at 10% (w/v) solution were assigned an infectious dose of 0. The mice were monitored
23 daily, euthanized at terminal stage and analyzed as above for PrP^{res} content. The survival times
24 of tgBov or tgHa reporter mice was measured for each tenfold dilution tested and the relative
25 infectious dose / survival time relationship was reported, when available. It allows translating

1 survival times of the inoculated fractions in infectious dose (i.e., equivalent to that found in
2 brain homogenate dilutions) as estimate of infectivity.

3 **Immunoblots**

4 Aliquots of the collected fractions were treated with a final concentration of 50 µg/ml PK (1
5 hour, 37°C). Samples were then mixed in Laemmli buffer and denatured at 100°C for 5 min.
6 The samples (15 µl) were run on 12% Bis-Tris Criterion gels (Bio-Rad, Marne la Vallée,
7 France) and analyzed by immunoblots, using the Sha31 anti-PrP antibody (human PrP epitope
8 at residues 145 to 152 [35]). Immunoreactivity was visualized by chemiluminescence (GE
9 Healthcare). The amount of PrP present per fraction and the PrP^{sc} glycoforms ratios were
10 determined with the GeneTools software after acquisition of chemiluminescent signals with a
11 GeneGnome digital imager (Syngene, Frederick, Maryland, United States).

12 **Histoblots**

13 For histoblotting procedure, brains were rapidly removed from euthanized mice and frozen on
14 dry ice. Cryosections were cut at 8–10 µm, transferred onto Superfrost slides and kept at -20°C
15 until use. Histoblot analyses were performed on 3 brains per experiment, using the 12F10 anti-
16 PrP antibody (human PrP epitope at residues 145 to 160 [36]).

17 **Results**

18 **Transgenic modelling of prion species barrier**

19 We used a previously developed SV protocol to separate different populations of PrP^{sc}
20 assemblies according to their quaternary structure [13,15,18]. To determine the potential role
21 of the SV-isolated PrP^{sc} subpopulations in prion adaptation and evolution, we included in the
22 present study three well-characterized ovine prion strains termed LA19K, LA21K *fast* and
23 127S. These cloned strains markedly differ according to the specific infectivity of their
24 respective PrP^{sc} subpopulations in the homotypic PrP transmission conditions. For LA21K *fast*
25 and 127S strains, a discrete population of small oligomers (<pentamers) exhibit the highest

1 specific infectivity values. For LA19K, the highest specific infectivity values are associated
2 with larger-size oligomers (>40 PrP-mers) [23]. We also included atypical L-BSE prions in the
3 study, because of their mutability on cross-species transmission [37].
4 We transmitted SV fractionated-PrP_{Sc} assemblies from the aforementioned strains to transgenic
5 mouse models expressing heterotypic PrP. The models were chosen according to the
6 transmission barrier observed with unfractionated material, as examined by gold-standard
7 criteria [38-40], including disease attack rate on primary passage, reduction of incubation
8 durations (ID) on serial passaging, and establishment of prion strain properties. The same
9 analyses were performed on back-passage to mice expressing the parental host PrP_C. For the
10 sake of clarity, these data are comprehensively summarized as [Additional file 1, supplementary](#)
11 [text and supplementary Fig. 1-4](#). However, to provide a straightforward estimator of the
12 stringency of the species barrier, we used the reduction factor between the mean ID on first to
13 second passage in the heterotypic context and on retrotransmission [40]. These data are shown
14 as [Fig. 1a](#). A magnitude of 1 signifies prion straight adaptation. As model of prion transmission
15 without species barrier, LA19K scrapie prions from ovine PrP (tgOv) mice was passed to
16 bovine PrP mice (tgBov). As model of prion transmission with species barrier and ‘mutation’,
17 we passed L-BSE prions onto tgOv mice. As models of prion transmission with strong species
18 barrier, we passed LA21K *fast* and 127S scrapie prions onto hamster PrP mice (tgHa).

19 **Impact of size-fractionation on prion pathogenesis in the heterotypic PrP context**

20 Brain homogenates from tgOv mice containing LA19K prions, LA21K *fast* or 127S prions and
21 from cattle containing L-BSE prions were solubilized and fractionated by SV [15,18]. The
22 fractions were then intracerebrally inoculated in the heterotypic PrP_C context to determine the
23 specific transmission propensity of each fraction. ([Fig. 1b](#)). As controls, some fractions were
24 transmitted in the homotypic PrP context and confirmed our previous results ([18]; *vide infra*).

1 In the absence of any apparent species barrier, as determined for LA19K inoculated to tgBov
2 mice, the infectivity sedimentograms in the homotypic and heterotypic passage conditions,
3 tended to superimpose, in terms of distribution and infectivity values amongst the fractions
4 (Fig. 2a). Each LA19K isolated PrP_{sc} assembly thus exhibited quasi-equivalent specific
5 infectivity in both transmission contexts. The strain-specified PrP_{res} electrophoretic profile and
6 pattern of cerebral PrP_{res} deposition [33] were conserved amongst the tgBov mice inoculated
7 with the different fractions or unfractionated LA19K brain material (Fig. 2b and additional file
8 1, supplementary Fig. 5), suggesting phenotypic invariance of the isolated LA19K assemblies
9 on heterotypic transmission.

10 In the presence of a strong species barrier, as determined for LA21K *fast* and 127S inoculated
11 to tgHa mice, SV-fractionation had a strong negative impact. None of the mice inoculated with
12 the fractions from three independent gradients developed any neurological symptoms up to end-
13 life. Only 3 out of the 221 tested mice (i.e., 1%) accumulated PrP_{res} in the brain. These 3 mice
14 were inoculated with fractions 10 and 13 from one LA21K *fast* gradient (Fig. 2, Table 1). In
15 particular, none of the tgHa mice inoculated with the PrP_{sc} assemblies with the highest specific
16 infectivity values from fractions 1-2 accumulated any detectable PrP_{res} (n=43, Table 1). These
17 results were confirmed by a second passage with individual or pooled (by 2) tgHa brains,
18 covering the entire LA21K *fast* gradient. All the secondary transmissions with PrP_{res}-negative
19 brains were negative (neurological signs and PrP_{res}; Table 2), indicating absence of a subclinical
20 disease [41,42] and/or of infectious, PK-sensitive PrP_{sc} species [16,43] in the non-responder
21 mice. Oppositely, serial transmissions from PrP_{res}-positive brains led to isolation of prions with
22 strains properties identical to those obtained on adaptation of unfractionated LA21K *fast* prions
23 to tgHa mice (Table 2, Fig. 2b, Additional file 1, supplementary Fig. 3).

24 To discard the possibility that the loss of tgHa-transmissibility of the isolated PrP_{sc} was due to
25 an insufficient infectivity load, we estimated the infectivity levels of the top and middle

1 fractions of one LA21K *fast* gradient. Fractions 2 and 12 induced disease in tgOv mice in $68 \pm$
2 3 days (5/5) and 82 ± 1 days (5/5), respectively (Fig. 3a), meaning, according to LA21K *fast*
3 dose/response curve (Fig. 3a, Table 3), that their infectivity levels were equivalent to $10^{-1.2}$ and
4 $10^{-3.2}$ dilutions of LA21K *fast* brain material, respectively. These values were fully consistent
5 with the mean (\pm SEM) relative infectious dose contained in LA21K *fast* most infectious upper
6 fractions and middle fractions established from seven independent bioassays (top fractions:
7 mean: $10^{-1.36}$ ($10^{-1.08}$ to $10^{-1.64}$); middle fractions: between $10^{-2.9}$ and $10^{-4.65}$ depending on the
8 middle fractions). To estimate the infectivity load of these fractions in the heterotypic PrP
9 context, we established a dose/response curve of LA21K *fast* in tgHa mice by transmitting by
10 intracerebral route serial tenfold dilutions of a LA21K *fast* tgOv-brain to reporter tgHa mice.
11 The limiting dilution value of LA21K *fast* prions in tgHa mice was surprisingly low (*vide infra*),
12 establishing at 10^{-2} (Table 3). Nevertheless, this value was below the infectivity value of the
13 top fractions of the gradient. Those were thus sufficiently infectious *per se* to induce or transmit
14 disease, at least partly in tgHa mice. This was not observed in 3 independent experiments with
15 a sufficient number of inoculated mice. Almost counterintuitively, the sole fractions eliciting
16 asymptomatic replication in a very low number of mice had infectivity values below the limiting
17 dilution. This suggests a stochastic transmission process and lends support to the view that
18 infection is possible at doses below the limiting dilution [44].
19 Together, these observations indicate that PrP^{Sc} subassemblies segregation by SV-fractionation
20 alters their replication efficiency in the PrP transmission barrier context, for reasons
21 independent of their infectivity load. This suggests synergetic interactions between PrP^{Sc}
22 subsets for an efficient heterospecies transmission.

1 **Dilution of LA21K *fast* prion-infected brain homogenate strengthens existing**
2 **transmission barriers**

3 LA21K *fast* prions are composed of at least two structurally PrP^{Sc} subsets in different
4 proportion, each with distinct specific infectivity [23,15,18]. Dilution experiments constitute a
5 relevant method to explore the contribution of each PrP^{Sc} subtype. Over a certain dilution factor,
6 the minor population will be quasi-eliminated from the inoculum leading to explore the effect
7 of the major species. The dilution approaches should also allow dissociating biochemical
8 complex(es) between different PrP^{Sc} subsets due to equilibrium displacement toward
9 dissociation [13].

10 We thus further analyzed the titration of LA21K *fast* prions in tgHa mice. Neat brain
11 homogenate (20 μ L 10%) containing LA21K *fast* prions induced disease in all tgHa mice with
12 a mean ID of 153 days (Table 3; Additional file 1., supplementary Fig. 3a). At the 10⁻¹ dilution,
13 the mean ID increased to 181 days (6/6 mice affected). The limiting dilution value resulting in
14 positive transmission established at the 10⁻² dilution, with 2 out of 6 tgHa mice developing the
15 disease at 192 and 230 days (Table 3). In homotypic transmission, LA21K *fast* limiting dilution
16 value established at 10⁻⁷ (Table 3). There was thus a considerable 10⁵-fold reduction of this
17 value in the heterotypic PrP transmission context. For comparison, in the absence of a
18 transmission barrier, as for LA19K prions in tgBov mice, there was no significant variations in
19 the limiting dilution value between the homotypic and heterotypic contexts (Table 3).

20 The inefficacy of LA21K *fast* diluted material to infect tgHa mice appeared further discrepant
21 when considering the fold increase between the IDs at the lowest and at the limiting dilution.
22 There was here a 1.37-fold increase for LA21K *fast* in tgHa mice (from 153 to 211 days, Table
23 3) whereas the mean \pm SD fold increase value observed in all the titrations we performed so far
24 in our laboratory in homotypic conditions, including in tgHa mice, was statistically higher at
25 2.17 \pm 0.32 (p<0.05, One-sample z test; Fig. 4a). Applying this value to LA21K *fast* titration in

1 tgHa mice would result in a theoretical limiting dilution value at $\sim 10^{-5}$ and a ~ 330 days ID (Fig.
2 4b).

3 Collectively, these data suggest that the dilution leads to the quasi elimination of at least one
4 compound and/or the dissociation of a complex mandatory for the efficacy of the heterotypic
5 transmission and consolidate our SV-based observations.

6 **PrP_c level and/or tissue environment imposes the species barrier stringency**

7 When isolated L-BSE SV-fractions were transmitted to tgOv mice, only fractions 8 to 14
8 induced disease at complete or near-complete attack rate, with an overt delay compared to
9 unfractionated material (Fig. 5a, Table 4). Mice inoculated with the top and bottom fractions
10 transmitted the disease only erratically, suggesting unfavorable transmission conditions (Fig.
11 5a, Table 4). Isolated L-BSE assemblies had therefore altered transmission capacities in the
12 heterotypic PrP transmission context, as observed with LA21K *fast* and 127S. The western blot
13 profile on primary passage (Fig. 5b) and the strain phenotype obtained on 2nd passage with
14 PrP_{res}-positive mouse brains indicated that C-BSE like prions has emerged from all positive
15 transmissions (Additional File 1, supplementary Fig. 5; supplementary Table 1).

16 Previously, we reported that lymphotropic prions replicated easier in the spleen than in the brain
17 in both homotypic and heterotypic PrP context, despite 20-fold lowered PrP_c levels [27,45].
18 This makes bioassays based on prion detection in the mouse spleen a highly sensitive method
19 to detect low dose of lymphotropic prions [45]. We thus examined further the capacity of SV-
20 fractionated L-BSE prions to propagate in the heterotypic PrP context by examining tgOv
21 spleens for PrP_{res} content after inoculation with the different fractions. Of the 49 tgOv spleens
22 analyzed, only 3 (6%) accumulated low levels of PrP_{res} (Table 4). Of the 13 mice for which
23 both the brains and spleens were analyzed for PrP_{res} content and the brain was positive (notably
24 fractions 10-12 with 100% attack rate), only 3 had also positive spleens (23%). For comparison,

1 all the spleens analyzed after inoculation of unfractionated L-type prions were strongly PrP_{res}-
2 positive from the first passage onward ([Table 4](#), [46]).
3 Strikingly, prion replication in the spleen was still altered on secondary passage of SV-
4 fractionated L-BSE assemblies. While all the spleens analyzed (n=15) were PrP_{res}-positive, the
5 levels of accumulation were still reduced by ~4-fold as compared to those observed on serial
6 transmission of unfractionated material ([Fig. 6a-b](#)). It may be noted that in homotypic
7 transmissions, fractionating brain material had no significant influence on spleen colonization
8 by prions, with regards to the number of positive spleens and PrP_{res} accumulation levels ([Fig.](#)
9 [6b](#)), indicating that, alone, the size of the infectious particles or PrP_{sc} subpopulation segregation
10 were not causal. Therefore, we can conclude from the impairment observed over two serial
11 passages that the low barrier penetrance of the isolated fractions is not due to a low infectivity
12 load and that size fractionation impairs L-BSE replication in the spleen to an extent that is
13 higher than in the brain.

14

15 **Discussion**

16 As conventional pathogens, prions are subject to adaptation and evolution. The concept of
17 molecular quasispecies, as defined by Eigen in 1979 [47], has been applied to prions [5,48] to
18 reconcile the structural diversity of prion assemblies (i.e. prion structural landscape) to the
19 preferential selection based on the ‘best replicator’ selection concept of certain subassemblies
20 during prion adaptation to new host or to a new environment/replicative conditions [49,27]. To
21 refine this ‘best replicator’ hypothesis [50], we tested the capacity of isolated PrP_{sc}
22 subassemblies within a given prion strain to adapt to a new host. We show here with multiple
23 PrP transgenic mouse models inoculated with both field-derived and biologically cloned prions,
24 that cross-species prion transmission does not strictly involve the selection of an existing

1 subpopulation of optimized PrP_{Sc} assemblies. Rather, our observations suggest that the key
2 determinant is a synergy between structurally distinct PrP_{Sc} subassemblies.
3
4 There was a positive correlation between the magnitude of the transmission barrier and the
5 difficulty for SV-individualized PrP_{Sc} assemblies to transmit the disease in the heterotypic PrP
6 conditions. L-BSE prions fractionation significantly delayed priogenesis in the brain and even
7 more negatively in the spleen of the challenged tgOv mice. Fractionated LA21K *fast* scrapie
8 prions replicated asymptotically in a very small proportion of tgHa mice and fractionated
9 127S prions failed to propagate in these mice. As shown previously and also demonstrated here,
10 LA21K *fast* and 127S scrapie prion strains are composed of at least two structurally distinct
11 PrP_{Sc} subpopulations according to their specific infectivity values (Fig. 3a and [15,18,23]). The
12 loss of transmissibility of separately taken PrP_{Sc} assemblies to tgHa mice cannot be attributed
13 to a global decrease of infectivity levels due to the solubilization/fractionation method as we
14 previously reported that the cumulated infectivity of the SV fractions did not differ significantly
15 from that present in the loaded material [18]. Further, for LA21K *fast*, the relative infectivity
16 titer of the top ‘most infectious’ fractions was sufficiently high to induce disease in tgHa. The
17 fact that PrP_{Sc} assemblies segregation affects prion heterospecies transmission suggests a
18 synergy between PrP_{Sc} subassemblies to overcome the species barrier. This hypothesis
19 implicates an unprecedented notion of complementation between structurally distinct prion
20 assemblies.
21 While the molecular mechanisms of such synergy between different PrP_{Sc} subsets remain to be
22 elucidated, some simple biochemical considerations can bring basements on the biochemistry
23 of the process. It implicates interactions between structurally distinct PrP_{Sc} subsets in order to
24 create a new structural information absent in each individual PrP_{Sc} population and leading to
25 heterologous PrP_C integration. During the early stage of prion replication, we showed that two

1 sets of structurally distinct PrP_{Sc} assemblies are generated, in fractions 1-5 and 10-18 [20].
2 These sets are defined by their elementary subunit [13] as suPrP_A and suPrP_B, respectively [27].
3 During the early step of prion replication, suPrP_A and suPrP_B form a suPrP_A:suPrP_B
4 heterocomplex involved in a secondary autocatalytic templating process generating *de novo* the
5 formation of suPrP_B, in a PrP_C-dependent manner [27]. The synergetic effect between different
6 PrP_{Sc} subsets can reside in the formation of this complex, making therefore this secondary
7 templating process a pivot of prion adaptation to a new host. In the heterotypic PrP transmission
8 context, the suPrP_A:suPrP_B complex (likely present in the inoculum) may incorporate
9 heterologous PrP_C, leading to the formation of a *de novo* suPrP_B* formed by the heterologous
10 PrP_C with a new templating interface different from the inoculum suPrP_B (Fig. 7). This first
11 event would be a limiting step of the adaptation process. The entrance of suPrP_B* in the
12 autocatalytic cycle makes its formation highly cooperative. Based on these assumptions, if the
13 stability of the complex is high enough (i.e., low dissociation constant), the reconstitution of
14 the initial composition by mixing different PrP_{Sc} subsets should allow recovering the
15 transmission efficiency. This approach has been unsuccessfully attempted in the case of LA21K
16 *fast*. This fail could be due to the weakness of the suPrP_A:suPrP_B complex, as previously
17 observed [20]. Indeed, size exclusion chromatography analysis of the 127S suPrP_A:suPrP_B
18 complex revealed that this last is highly labile and can be observed only in conditions where
19 the concentration of suPrP_A and suPrP_B were high.

20 The existence of a complex such as suPrP_A:suPrP_B within the inoculum is further supported by
21 the striking observation that endpoint dilutions aberrantly impaired LA21K *fast* prions transfer
22 to tgHa mice (1000-fold decreased efficacy); the dilution steps would make certain PrP_{Sc}
23 subpopulations disappearing given their ratio/amount and/or induce the dissociation of an
24 existing complex [51,15]. While cross-species transmission of prions has rarely been done at
25 high dilution, it may be noted that comparing the limiting dilution values of MM1 sporadic CJD

1 prions (homozygous for Met at codon 129) in transgenic mice expressing human PrP with either
2 Met or Val at codon 129 resulted in 10³-fold decrease [52], a negative impact quantitatively
3 comparable to our observations.
4 If the synergy between structurally different PrP_sc subsets involves the formation of a
5 suPrP_A:suPrP_B heterocomplex and the PrP_c-dependent secondary templating pathway, the
6 magnitude of the species barrier would thus depend both on the strength of the heterocomplex
7 and on the amount of heterotypic PrP_c which defines the rate of the secondary templating and
8 its cooperativity aspect [20]. Such prominent role of PrP_c is experimentally supported by the
9 observation that L-BSE prion adaptation is more impaired in tg338 mouse spleen than brain,
10 despite a potentially favored local environment for heterotypic conversion as prion species
11 barrier is lower in the spleen than in the brain [27]. The lower concentrations of PrP_c [53] in
12 the spleen [27] and/or the different structure (including the post-translational modifications)
13 may impact the secondary templating pathway and its autocatalytic nature.

14

15 **Conclusion**

16 Together, our data would expand the prion quasispecies concept to an ensemble of
17 macromolecular assemblies, -not necessarily associated to different substrains-, that
18 complement each other to adapt tissue-specific selection pressure and extend prion host range.
19 Such ‘epistatic’ behavior may provide new fundamental principles for understanding prion
20 distinctive properties to transfer between species [54] and can explain the relative inability of
21 recombinant PrP to generate high-titer prions [55], unless submitted to intense mechanisms of
22 polymerization/fragmentation which may generate aggregate polydispersity and/or a larger
23 portfolio of conformations [56] for complementation.

24

1 **List of Abbreviations**

2 Bov: bovine

3 BSE: bovine spongiform encephalopathy

4 CJD; Creutzfeldt-Jakob disease

5 Ha: hamster

6 Ov: ovine

7 PrP: prion protein

8 PK: proteinase K

9 PrP_{res}: proteinase K resistant PrP_{sc}

10 PrP_{sc}: pathological/abnormal form of the prion protein

11 suPrP: PrP elementary brick

12 SV: sedimentation velocity

13 tg: transgenic

14

15 **Declarations**

16 **Ethics approval**

17 All the experiments involving animals were carried out in strict accordance with EU directive
18 2010/63 and were approved by INRA local ethics committee (Comethea; permit numbers 12-
19 034 and 15-056).

20 **Consent for publication**

21 Not applicable.

22 **Availability of data and material**

23 All data supporting our findings are presented in the main paper and additional files.

24 **Competing interests**

25 The authors declare no competing financial interests.

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8 **Author's contribution**

9 Conceptualization, AIE, FL, PT, MM, HL, HR, and VB; Data curation and Analysis, AIE, FL,
10 PT, MM, LH, FR, JMT, HL, HR, and VB; Resources, HL, JMT, HR and VB; Writing – Original
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14

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5

6 **Figures**

7 **Figure 1. Magnitude of the species barrier on transmission of scrapie prions (LA19K,**
8 **LA21K *fast*, 127S) and cattle prions (L-BSE) to transgenic mice expressing heterotypic**
9 **PrP**

10 (a) Scrapie LA19K, LA21K *fast*, 127S prions and cattle L-BSE prions were transmitted
11 iteratively to mice expressing bovine PrP (tgBov), hamster PP (tgHa) and ovine PrP (tgOv),
12 respectively, before reisolation in transgenic mice expressing the parental PrP ([Additional file](#)
13 [1](#)). The magnitude of the transmission barrier in transgenic mice expressing heterotypic PrP
14 (blue color) and on reisolation in transgenic mice expressing the parental PrP (green color) was
15 calculated as the ratio of the mean incubation durations (ID) on first to second passage in the
16 new host PrP and on reisolation in mice expressing the parental PrP. A magnitude of 1 signifies
17 prion straight adaptation.

18 (b) Overview of the bioassays made with PrP^{Sc} assemblies fractionated by sedimentation
19 velocity.

20

21 **Figure 2. Unaltered capacity of SV-fractionated PrP^{Sc} assemblies to propagate onto a new**
22 **host PrP sequence in the absence of a transmission barrier**

23 (a) SV profiles of PrP^{res} (black line) and infectivity in the homotypic PrP (green line) and
24 heterotypic PrP (blue line) transmission context. Brain homogenates from tgOv mice inoculated

1 with LA19K prions were solubilized before fractionation by SV. The collected fractions were
2 analyzed for PrP_{res} content by immunoblot and for infectivity by an incubation time bioassay in
3 tgOv and tgBov mice (tg110 line). In the homotypic context, plain and dotted symbols/lines
4 refer to this study and to previous reports [18], respectively. The right logarithmic brown scale
5 provides the LA19K-specific reciprocal relation between survival time in tgOv and tgBov mice
6 and infectious dose, as established by limiting dilution titration (as from [Table 3](#) and [18]).
7 Animals inoculated with 10% infectious brain material are assigned an infectious dose of 0.
8 **(b)** PrP_{res} electrophoretic profiles in the brains of tgBov mice inoculated with size-fractionated
9 LA19K-tgOv PrP_{Sc} assemblies. The profile obtained with unfractionated (U) material is shown
10 for comparison.

11

12 **Figure 3. Altered capacity of size-fractionated PrP_{Sc} assemblies to propagate onto a new**
13 **host PrP sequence in the presence of a ‘strong’ transmission barrier**

14 **(a)** SV profiles of PrP_{res} (black line) and infectivity in the homotypic PrP (green line) and
15 heterotypic PrP (blue dots) transmission context. Brain homogenates from tgOv mice
16 inoculated with LA21K *fast* prions were solubilized before fractionation by SV. The collected
17 fractions were analyzed for PrP_{res} content by immunoblot and for infectivity by an incubation
18 time bioassay in tgOv and tgHa mice. In the homotypic context, plain and dotted symbols/lines
19 refer to this study and to previous reports [18], respectively. Because of the reduced penetrance
20 of the disease in tgHa mice, the mean individual ID of the PrP_{res} positive mice are shown as
21 blue dots. The right logarithmic brown scale provides the LA21K *fast*-specific reciprocal
22 relation between survival time in tgHa and tgOv mice and infectious dose, as established by

1 limiting dilution titration (as from [Table 3](#) and [18]). Animals inoculated with 10% infectious
2 brain material are assigned an infectious dose of 0.

3 **(b)** PrP_{res} electrophoretic profiles in the brains of tgHa mice inoculated with size-fractionated
4 LA21K *fast* prions at the 1st (p1) and 3rd (p3) passage. The profiles obtained with unfractionated
5 (U) LA21K *fast* and 127S material in tgOv and tgHa mice are shown for comparison.

6

7 **Figure 4. Aberrant titration of LA21K *fast* prion in hamster PrP mice as compared to**
8 **other prion titrations in PrP transgenic mice**

9 **(a)** Fold increase (x) between the mean IDs at the lowest and at the limiting dilution during
10 prion titrations. The prions titrated and the reporter mice are indicated on the graph. The
11 mean±SD value observed for all but the LA21K *fast*→tgHa titrations established at 2.17±0.32
12 (mean ± SD, dotted vertical line:mean; shadow square: SD). The 1.37-fold increase for LA21K
13 *fast* in tgHa mice is significantly lower (*p<0.05, One-sample z test).

14 **(b)** Titration curve of LA19K prions in tgOv and tgBov mice as compared to that of LA21K
15 *fast* in tgOv and tgHa mice. The theoretical curve of LA21K *fast* prions in tgHa mice is the
16 result of the 2.17-fold increase in the IDs at the lowest and at the limiting dilution.

17

18 **Figure 5. Capacity of size-fractionated PrP_{Sc} assemblies to propagate onto a new host PrP**
19 **sequence in the presence of an ‘intermediate’ transmission barrier and with mutation**

20 **(a)** SV profiles of PrP_{res} (black line) and infectivity in the homotypic PrP (green line) and
21 heterotypic PrP (blue dots) transmission context. Brain homogenates from cattle infected with
22 L-BSE prions were solubilized before fractionation by SV. The collected fractions were
23 analyzed for PrP_{res} content by immunoblot and for infectivity by an incubation time bioassay in
24 tgBov and tgOv mice. The disease incidence in tgOv mice is presented on the right red graph.

1 Because of the reduced penetrance of the disease in tgOv mice, the mean individual ID of the
2 PrP_{res} positive mice are shown as blue dots.

3 **(b)** PrP_{res} electrophoretic profiles in the brains of tgOv and tgBov mice inoculated with size-
4 fractionated L-BSE PrP_{Sc} assemblies. The profiles obtained with unfractionated (U) material
5 from L-BSE or classical BSE (C-BSE) are shown for comparison.

6

7 **Figure 6. Capacity of size-fractionated L-BSE prions to colonize the spleens of mice**
8 **expressing homotypic or heterotypic PrP_C**

9 **(a)** PrP_{res} detection in the spleens of tgOv mice inoculated with unfractionated L-BSE or C-
10 BSE prions (primary passage) or with fractionated L-BSE prions (second passage, fractions
11 used for passaging are indicated).

12 **(b)** PrP_{res} accumulation levels in the spleens of tgOv mice inoculated with unfractionated (2nd
13 passage) or SV-fractionated L-BSE ((all fractions combined), 2nd passage) prions as compared
14 to unfractionated or SV-fractionated 127S prions. (* $p < 0.05$, Mann-Whitney test).

15

16 **Figure 7. The formation of heterocomplex between structurally distinct PrP_{Sc} subsets may**
17 **drive the conversion of heterologous PrP_C by a secondary templating pathway process**

18 **(a)** In the homotypic PrP transmission context, prion replication involves two templating
19 pathways: a primary templating pathway where structurally distinct PrP_{Sc} subsets, taken
20 individually (A_j and B_i), are able to perpetuate the strain information and a PrP_C-dependent
21 autocatalytic secondary templating pathway contributing to structural diversification, which

1 requires the formation of a heterocomplex between the elementary subunits of two structurally
2 distinct sets of PrP^{Sc} assemblies (suPrP_A and suPrP_B) [20].

3 **(b)** This autocatalytic secondary templating pathway could drive the incorporation of
4 heterologous PrP^C during the species barrier passage, leading to *de novo* generation of suPrP_B
5 (in red) more prone to replicate/propagate in the new host. PrP^{Sc} assemblies segregation or
6 dilution could drastically affect the formation of the suPrP_A:suPrP_B complex, compromising
7 this secondary templating pathway.

1 **Tables**

2

3 **Table 1. Survival time of hamster PrP mice inoculated with LA21K *fast* and 127S prions**

4 **fractionated by sedimentation velocity**

5

Fractions	LA21K <i>fast</i>				127S	
	n/n ₀₁	Survival ₂ (days ± SEM)	n/n ₀₁	Survival ₂ (days ± SEM)	n/n ₀₁	Survival ₂ (days ± SEM)
1	0/12	399-627	0/5	384-631	0/6	365-552
2	0/13	520-692	0/6	318-576	0/5	341-496
3	0/5	389-651	0/6	401-636	0/5	405-538
4	0/5	507-647	0/6	384-535	0/6	317-506
6	0/6	386-780				
8	0/6	375-713				
10	2/6	353; 386	0/6	461-531	0/6	331-510
11	0/5	536-937	0/6	378-517		
12	0/11	347-687	0/6	322-426	0/6	382-587
13	1/6	394				
14	0/6	556-720	0/6	344-629	0/6	481-631
15	0/6	323-854				
16	0/6	614-710				
18	0/6	325-839				
20	0/6	406-877				
22	0/6	371-751				
24	0/5	543-927				
26	0/6	413-602				
28	0/6	345-749				
30	0/6	383-665				
U₃	8/8	157 ± 6			8/8	168 ± 7

6 n/n₀: Number of mice with neurological disease or positive for PrP_{res} in the brain by
7 immunoblotting/number of intracerebrally inoculated mice.

8 ²For mice negative for PrP_{res} accumulation, only the range of survival time is given.

9 ³U: unfractionated material ([Additional File 1, supplementary Fig. 3](#)).

10

1 **Table 2. Serial passage of SV fractionated LA21K *fast* prions in hamster PrP mice**
 2

Fractions (mouse no.1)	LA21K <i>fast</i>					
	2 nd passage		3 rd passage		4 th passage	
	n/n ₀₂	Survival ₃	n/n ₀₂	Survival ₃	n/n ₀₂	Survival ₃
1 (no. 2+3)	0/6	382-524				
2 (no.4+7)	0/6	301-605				
10 (no.1+2)	6/6	54 ± 1	6/6	50 ± 1	6/6	48 ± 1
11 (no.1+4)	0/6	353-544				
12 (no.1+2)	0/6	347-687				
13 (no.1)	6/6	58 ± 1	6/6	48 ± 1		
20 (no.2+5)	0/6	361-518				
26 (no.1+3)	0/6	405-587				
U₄	6/6	48 ± 1	6/6	47 ± 1	6/6	46 ± 1

3 ¹mouse identification number inoculated as pools.

4 ²n/n₀: Number of mice with neurological disease or positive for PrP_{res} in the brain by
 5 immunoblotting/number of intracerebrally inoculated mice (the number of the mouse
 6 inoculated is indicated).

7 ³For mice negative for PrP_{res} accumulation, only the range of survival time is given.

8 ⁴U: unfractionated material ([Additional File 1, supplementary Fig. 3](#)).

1 **Table 3. Endpoint titration of LA19K and LA21K *fast* scrapie prions in transgenic mice**
 2 **expressing homotypic and heterotypic PrP**

Dilution ¹	Survival time ² (n/n ₀) ³			
	LA19K		LA21K <i>fast</i>	
	Ovine PrP	Bovine PrP ⁴	Ovine PrP	Hamster PrP
10 ⁻⁰	<i>128 ± 2 (6/6)</i>	196 ± 3 (6/6)	<i>60 ± 2 (5/5)</i>	153 ± 6 (6/6)
10 ⁻¹	<i>151 ± 3 (6/6)</i>	213 ± 5 (6/6)	<i>64 ± 1 (5/5)</i>	181 ± 11 (6/6)
10 ⁻²	<i>167 ± 4 (6/6)</i>	240 ± 5 (6/6)	<i>71 ± 1 (5/5)</i>	192; 230 (2/6)
10 ⁻³	<i>188 ± 4 (6/6)</i>	272 ± 19 (6/6)	<i>79 ± 1 (6/6)</i>	371-591 (0/6)
10 ⁻⁴	<i>223 ± 15 (4/6)</i>	361 ± 90 (4/6)	<i>85 ± 1 (5/5)</i>	399-518 (0/6)
10 ⁻⁵	<i>245 (1/6)</i>		<i>99 ± 2 (5/5)</i>	367-636 (0/6)
10 ⁻⁶			<i>112 ± 2 (5/5)</i>	
10 ⁻⁷			<i>148 (1/5)</i>	

3 ¹10% brain material used as 10⁻⁰ dilution.

4 ²Days ± SE of the mean. For mice negative for PrP_{res} accumulation, only the range of survival
 5 time is given.

6 ³n/n₀: Number of mice with neurological disease or positive for PrP_{res} in the brain by
 7 immunoblotting/number of inoculated mice.

8 ⁴tg110 line.

9 Data in italic are from [18].

1 **Table 4. Survival time and PrP_{res} detection in the brain and spleen tissues of ovine PrP**
 2 **mice intracerebrally inoculated with L-BSE prions fractionated by sedimentation velocity**

3

Fractions	n/n ₀₁	Survival ²	PrP _{res} detection	
			Brain	Spleen
1	1/6	802	1/6	0/5
2	2/5	519 ; 739	2/5	0/3
3	0/5	532-711	0/5	0/4
4	1/6	496	1/6	0/3
6	1/6	472	1/6	0/2
8	3/6	542 ± 67	3/6	0/2
10	5/6	625 ± 64	5/6	1/5
12	6/6	575 ± 38	6/6	1/4
14	4/6	577 ± 76	4/6	0/1
16	2/6	530 ; 655	2/6	1/4
18	2/6	621 ; 681	2/6	0/1
20	0/6	406-877	0/6	0/3
22	2/6	479 ; 538	2/6	0/3
24	1/6	452	1/6	0/2
26	0/6	381-754	0/6	0/3
28	0/6	357-739	0/6	0/2
30	0/6	465-884	0/6	0/1
U₃	9/9	423 ± 7	8/8	8/8

4 ¹n/n₀: Number of mice with neurological disease or positive for PrP_{res} in the brain by
 5 immunoblotting/number of inoculated mice.

6 ²Days ± SE of the mean. For mice negative for PrP_{res} accumulation, only the range of survival
 7 time is given.

8 ³U: unfractionated material; data from [26,46] are in italic.

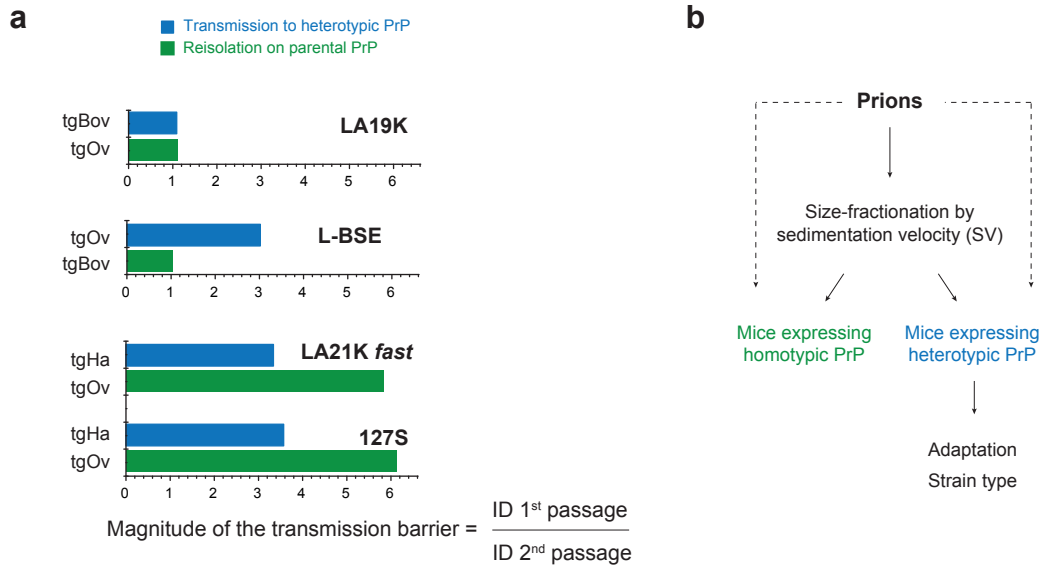


Figure 1

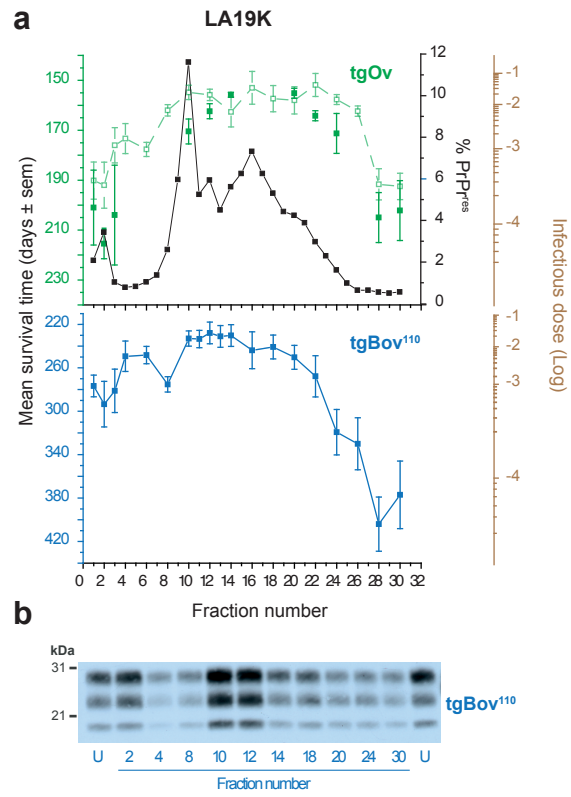


Figure 2

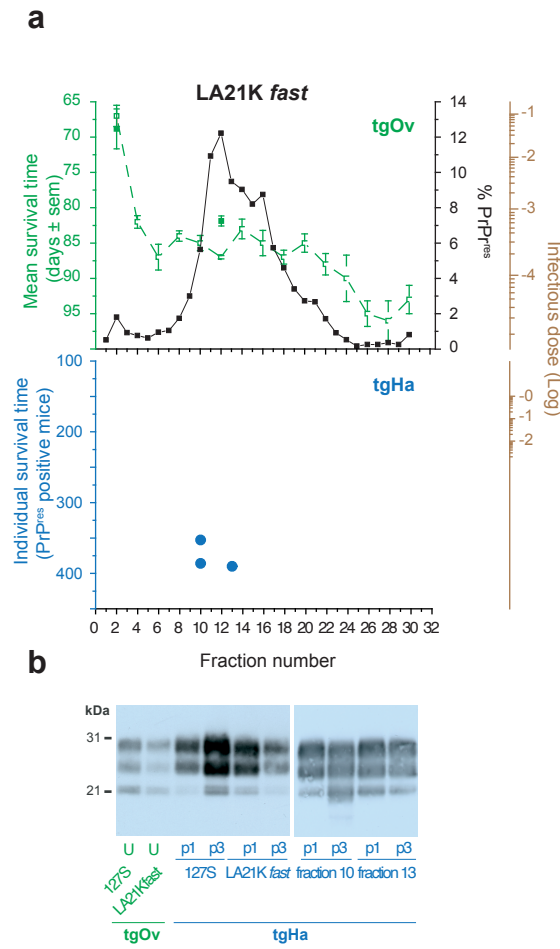
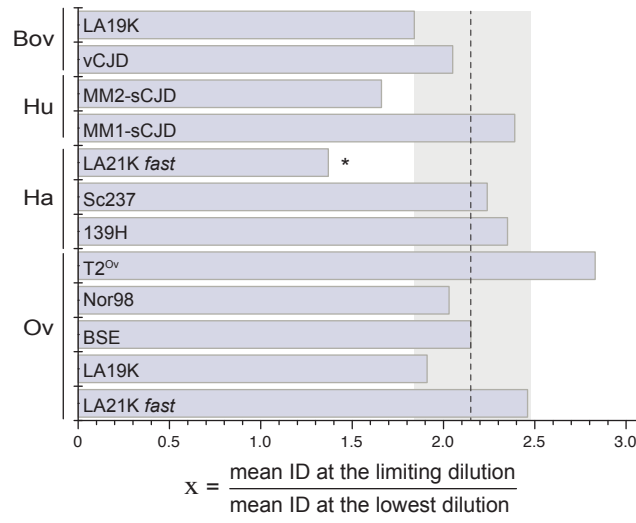


Figure 3

a

Host PrP Prions



b

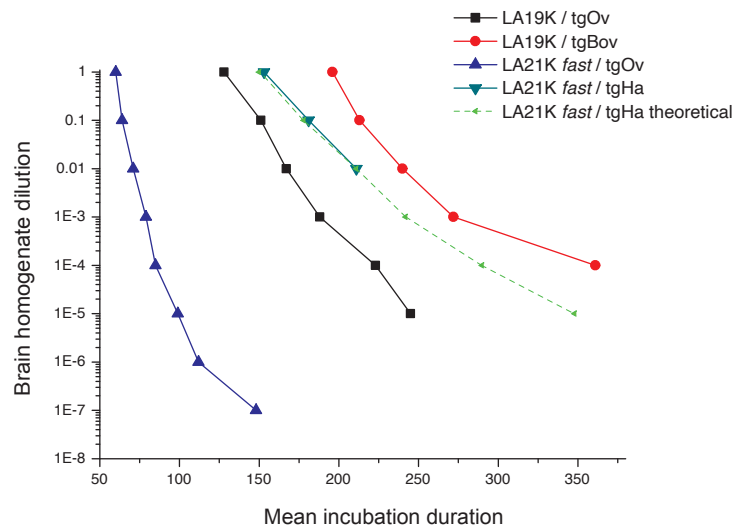


Figure 4

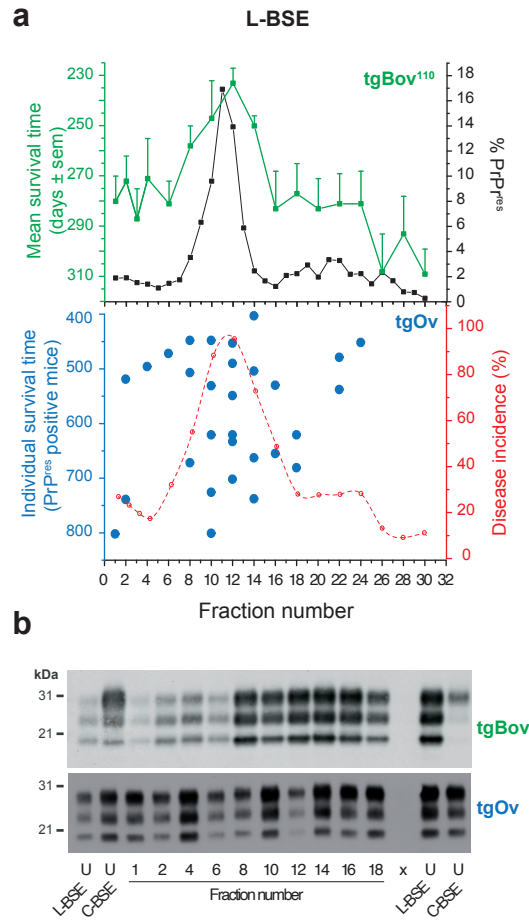


Figure 5

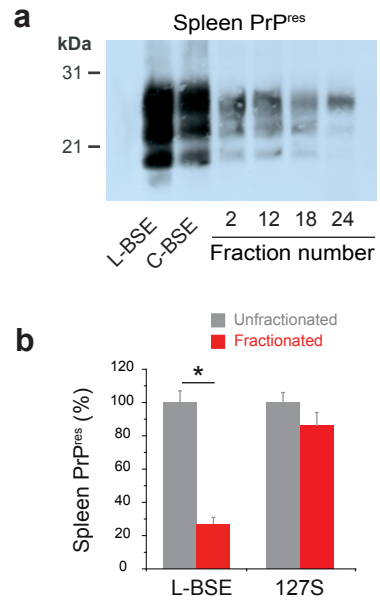


Figure 6

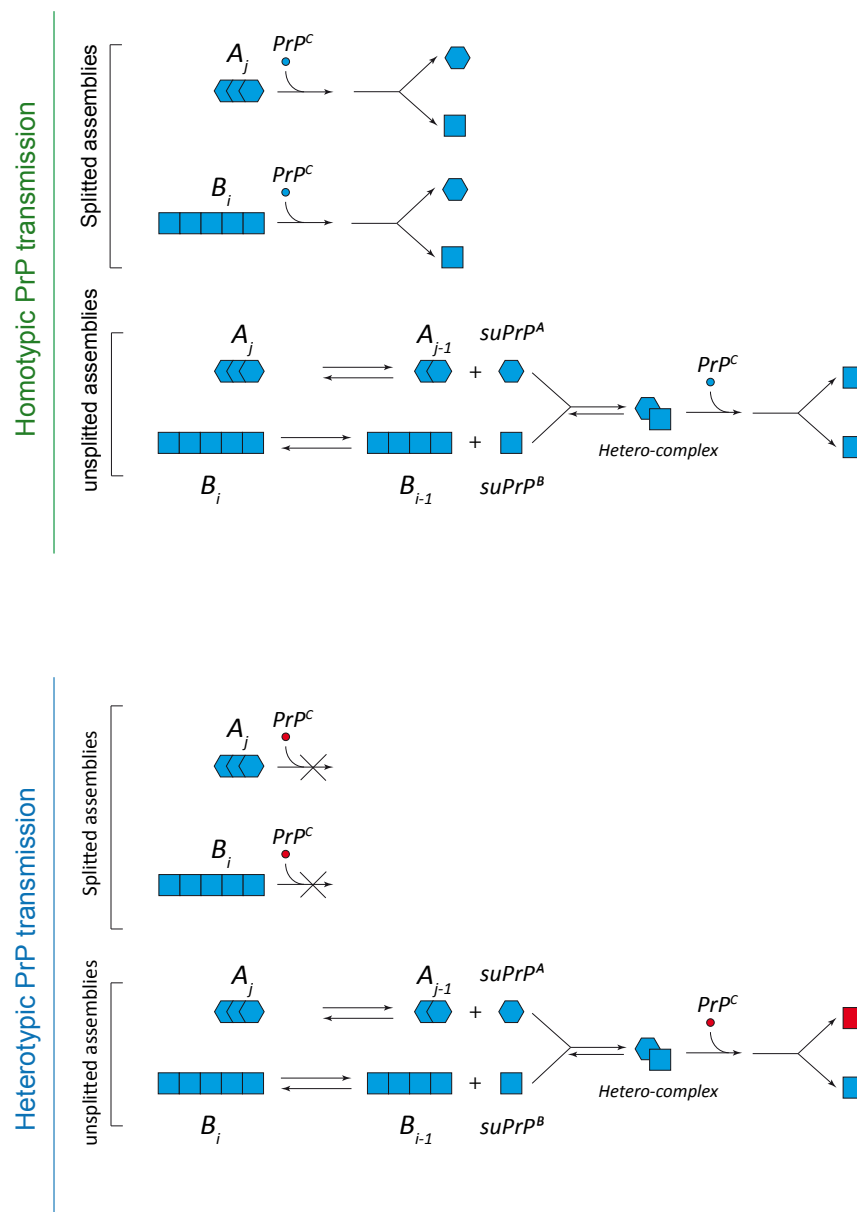


Figure 7