Small critical RNAs in the scrapie agent

Steve Simoneau[1], Marie-Madeleine Ruchoux[1], Nicolas Vignier[2], Pierre Lebon[3], Sophie Freire[1], Emmanuel Comoy[1], Jean-Philippe Deslys[1], Jean-Guy Fournier[1]

1. CEA-Institute of Emerging Diseases and Innovative Therapies, Route du Panorama, Bat. 60, F-92265 Fontenay-aux-Rose, France

2. U974 INSERM, UMRS 7215 CNRS, Institut Myologie, Hôpital Pitié-Salpêtrière, 75013 Paris, France

3. Laboratoire Virologie, Hôpital Saint Vincent de Paul, 75014 Paris, France

Unconventional infectious agents cause transmissible spongiform encephalopathy (TSE) diseases including scrapie and bovine spongiform encephalopathy (BSE) in animals and Creutzfeldt-Jakob disease in humans. The protein only hypothesis claims that the TSE agent is composed solely of the protein called prion (PrP^{sc})[1]. This protein is the misfolded form of a host-encoded cellular protein, PrP^{c} exerting presumably a vital role at the synapse[2]. Even though now widely accepted, the prion concept fails to provide in certain circumstances[3-6], a satisfying interpretation of the infectious phenomenon. Using the 263K scrapie-hamster model, we conducted a transmission study to search for a putative prion-associated factor indispensable for infectivity. Here we show that innocuous recombinant prion protein (recPrP) was capable, in a reproducible manner, of transmitting scrapie disease when the protein was β–sheet converted in a solution containing PrP^{sc}-derived RNA material. Analysis of the PrP-RNA mixture revealed the association of recPrP with two prominent populations of small RNA molecules having an average length of about ~27 and ~55 nucleotides. We conclude that the nature of the TSE agent seems to be composed of a nucleoprotein molecular

complex, in which informative RNA molecules of small sizes are associated with the misfolded prion protein (PrP^{sc}).

More than a decade ago, experiments of BSE transmission in mice alerted the scientific community on the possibility that TSEs could be transmitted in the absence of detectable PrP^{sc} [5]. Subsequent studies studies have also shown the absence of a strict correlation between infectivity and PrP^{sc}, suggesting that another component could be required for transmissibility of the TSE agent[3,4,6]. Here, we have reconsidered the fundamental question of the nature of TSE agent making use of recent technological developments in the prion field such as recombinant protein engineering and in vitro protein conversion. We set up the following strategy to search for a putative nucleic acid partner of PrP^{sc}. After extensive destructive treatments, the native PrP^{sc} pathological conformer eliminated from infectious preparations (called SAF for scrapie-associated fibrils which corrrespond to the amyloid filament form of the PrP^{sc}), was replaced by an equivalent PrP molecule consisting of β-sheeted recombinant PrP. As such, the recombinant protein constituted an appropriate tool to probe the conjectured nucleic acid previously released by the native PrP^{sc}[7]. To demonstrate its presence, we reasoned that recPrP, during β-sheet conversion, could interact specifically with this nucleic acid to form a nucleoprotein complex that would transmit scrapie to recipient animals.

Infectious SAF solution was prepared from 263K-infected hamster brain and exposed to a harsh protein destruction procedure involving phenol-chloroform and PK digestion. The resulting PrP^{sc}-free SAF-derived solution was either used as such or treated with DNAse I (as a control see Supplementary Methods) before it was mixed with various versions of recPrP. The different mixes were then inoculated to hamsters and their infectious potential investigated up to 545 days post-inoculation (PI)(see Table 1). Unexpectedly, we achieved successful infection with the DNAse I-treated sample (inoculum 8; see Table 1). Two sick animals out of five (40%) succumbed to disease with clinical symptoms of scrapie, at 151 and 246 days PI. None of the other animals from either

the PrP^{sc}-free SAF-derived (inocula 2, 6, 7 and 11) or the controls (inocula 12 and 13) developed scrapie during an observation period of 545 days PI.

We then characterized the nucleic acid content of our samples, using the Agilent 2100 Bioanalyzer. To have a more acurate view of the nucleic acids present before and after DNAse I digestion, we examined the parent solution of inoculum 8 (inoculum 2). This inoculum contains the crude nucleic acids extracted from PrP^{sc} preparation. When subjected to DNAse I, we found that inoculum 2 contained two distinct populations of small RNA of sizes averaging ~27 and ~55 nucleotides (Fig. 1a). This solution showed a complete absence of contaminating or degraded large cellular RNA (Fig. 1b). By contrast, the DNAse I untreated original solution (inoculum 2) contained a large amount of cellular DNA (heterogenous mixture of sizes <300 nucleotides) masking the small RNA populations (Fig. 1c). We also investigated the molecular characteristics of inoculum 8. A fraction of the small RNA interacted with the recPrP as shown by its liberation from the protein after PK treatment (Supplementary Fig. 1a, c). Moreover, it was sensitive to RNAse A treatment (Supplementary Fig. 1a, b). Electron microscopy (EM) analysis of the recPrP in the inoculum 8 (subjected to thermal β-sheet conversion) displayed occasional ovoidal and spherical aggregates (diameter ~7-40nm,; Fig. 1d). These supramolecular structures of recPrP were not observed in the control inoculum 7 containing alpha-recPrP (Fig. 1e), and no filament-like structures were observed in any of the solutions.

To confirm these first results, we initiated a second experiment (ongoing > 360 days) with new solutions (see Table 1). The results up to now appear so convincing that they can be reasonably incorporated in this study. Here, three animals out of nineteen (16%) died of scrapie disease (148, 178 and 207 days PI) after injection with inoculum D which was prepared and treated with DNAse I in the same way as inoculum 8. Again, none of the negative control animals (29 : inocula A, B and C) are showing any clinical signs of scrapie (Table 1). Inoculum D also contained the two small RNA populations observed in the first experiment (Supplementary Fig. 1d).

To verify the animals infected with inoculum 8 died from an authentic scrapie disease, we performed neuropathological and PrP-immunohistochemistry analysis of their brains (Fig. 2 and Supplementary Fig. 2). Results of histopathology revealed spongiosis typical of TSE infection (Fig. 2d,e and Supplementary Fig. 2b). The spongiosis lesion profile exhibited a much higher score than the 263K profile with particular emphasis on 11 and 9 brain regions (score difference (Sdf) $\geq 1^{8}$) in animals 8.1 and 8.2 respectively (Fig. 3a). The brain of animal 8.1 and 8.2 also showed a more intense PrP staining (Fig. 3b). In the case of the second experiment, we analyzed one of the infected animals and found a neuropathology and a lesion profile distinct to the 263K strain (supplementary Fig. 3). Despite their lesion profile divergences, all infected animals showed a PrP^{sc} western blot profile indistinguishable from the reference 263K scrapie strain (Supplementary Fig. 4a).

In the present study, we were able to reveal RNA isolated from scrapie agent, as a factor essential for the acquisition of scrapie infectivity. Our strategy was based on the presumption that artificial recPrP, in its β-sheeted converted form, could become infectious upon binding to a natural putative RNA originating from PrP^{sc}, thus demonstrating the existence of this genetic element and its contribution for infectivity. As expected, we observed that the PrP^{sc}-free SAF preparation, obtained after protease and phenolic treatments was not infectious, but nevertheless still contained a component indispensable for infectivity released by the proteolysed PrP^{sc}. This was demonstrated by injecting, into hamster brain, the DNAse I treated inoculum 8. Initially included as a control, this digestion became an essential supplementary step to completely eliminate the residual cellular DNA and allow the RNA material to associate with recPrP. Therefore, the lack of infectivity on the non DNAse I-treated sample (inoculum 6), can be explained by the inhibitory effect of the DNA (due to its large excess) that most likely hampered the formation of the ribonucleoprotein complex. Unlike the incomplete digestion of cellular DNA from the DNAse I treatment performed during the initial PrP^{sc}purification step, the RNAse A pre-treatment appeared to eliminate all traces of cellular RNA. This suggested that the highly purified RNA recovered after PK digestion of PrP^{sc}, was protected by this protein during the nuclease digestion. This RNA material of sizes averaging ~27 and ~55

nucleotides, was RNAse A sensitive indicating that it was constituted of single-strand molecules. Their interaction with recPrP (inoculum 8), was confirmed by their release after PK digestion. The β-sheeted recPrP in inoculum 8 adopted various supramolecular structures of ovoidal and spherical shapes (7-40 nm), not seen in the unconverted recPrP sample (inoculum 7), when observed by EM as previously shown[9]. Such small aggregates of PrP^{sc} would be, according to recent data, the most infectious forms of prions[10]. In future experiments it will be interesting to further dicepher the molecular arrangement between β-sheeted PrP and its ribonucleic partner. A clear understanding of the role played by these intriguing RNA species in the infectious process will only be possible after identification of their sequences (work in progress). One crucial question is whether they are in any way related to recently discovered cellular ribo-regulators designated miRNA, siRNA and piRNA[11,12]. The possibility of such RNAs interacting with PrP has been evoked previously, but was immediately rejected because of the absence of data establishing a link between the RNA substrate and the infectious phenomenon [13,14].

We have created in test tube a macromolecular complex consisting of hamster recPrP and RNA material extracted from 263K prions. The pathogenicity of this novel coupling was validated, after brain inoculation, by the development of neurological disease reflecting typical experimental scrapie in 5/24 (20%) wild-type animals taking into account the two experiments. However on first passage, a remarkable divergence between the infectious β recPrP-RNA complex created *in vitro* and the natural 263K agent was observed in three tested animals. The lesions obtained with this new infectious entity were clearly more severe, characterized by intense spongiosis, important neuronal loss and gliosis. So the two most important points of our study, namely the absence of residual contamination of our samples and the infectious nature of the *in vitro*-generated biological entity were observed in both experiments.

It is clear that the infectivity level of our preparations remains modest in that we chose to use minimal conditions essential to ensure the required effect. In future experiments it will be of great interest to explore variables able to improve the infectious nature of these preparations. It will also

be important to verify whether our biomacromolecular assemblage, able to generate an infraviral particle, satisfies the virino hypothesis, which dictates that the genome, protected by a coating cellular protein (PrP), is host-independent[15-17]. The two structural pieces composing the novel infectious entity, β-recPrP and PrP^{sc}-associated RNAs, are by themselves not infectious. It is through their molecular synergy that infectivity manifests. However we cannot exclude the possibility that in exceptional circumstances, RNA alone might be able to initiate the infectious process[4]. Information encoded by the RNA sequences constitute a prerequisite for elucidating the replicative modalities of this new structural version of the scrapie agent. In keeping with the 'paradoxical' prion, it is conceivable that the genetic moiety, as was discovered for PrP, might be host-encoded. Although it can be considered at the rough state, the present work provides serious clues to ultimately resolve in a near future the enigmatic nature of the TSE infectious agent [18].

Methods summary

Animals and SAF preparation.

Brains of hamsters infected with the 263K scrapie strain were collected at the terminal stage of the disease (~80 days; titer of $2x10^9$, 50% lethal dose/ml) and pooled together to be used for SAF purification. PrP^{sc} was purified using the BioRad TeSeE purification kit according to the manufacturer's instructions with one exception. Cellular DNA and RNA in the homogenate were digested prior to the PK step of the protocole. The BioRad purification was used to purify PrP^{sc} to avoid too much compaction and facilitate subsequent digestion by PK.

PrP^{sc}destructive procedures.

Two lines of attack were adopted to simultaneously suppress infectivity of SAF preparations, by destroying PrP^{sc} and releasing the hypothetical PrP^{sc}-associated nucleic acids. One, using phenol-chloroform, and the other involving a high concentration of guanidinium-HCl which profoundly denatures PrP^{sc}. In both cases, extensive proteinase K digestion completed the PrP^{sc} destruction

protocole (see Supplementary) . The GdnHCl method was not successful and the data is not shown in this paper.

Preparation of inocula

The two types of treated SAF solutions constituted the reactive medium in which the conversion process of hamster recombinant alpha-PrP (AllPrion) took place. This was carried out using thermal conditions as previously described[9] and two other in vitro conversion methods were also tested (see Supplementary Methods).

Histopathology

Formalin-fixed brains were embedded in paraffin and processed for histopathology and PrP immunocytochemistry as previously described[19]. The anti-PrP antibody used was SAF 32. Sections were analysed in double-blind fashion.

Nucleic acid analysis

One microliter of the solution was deposited in wells of a Lab-on-a-Chip device (2100 Bioanalyser, Small RNA Assay, and RNA 6000 Nano, Agilent Technologies) according to the manufacturer's recommendations.

Electron microcopy

Five microliters of the solution were deposited on grids and negatively stained as previously described[9]

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Author Contributions : J.G.F. conceived the project and performed electron and light microscopy observations. J.G.F. and S.S. designed the experiments and wrote the manuscript. S.S. performed all biochemical and bioassays experiments. M.M.R. performed histo-immunopathological studies, and discussed the data. N.V. performed the nucleic acid assays, P.L. performed negative staining and

electron microscopy analysis, S.F. performed tissue sectioning and staining. E.C. discussed the data. J.P.D.supervised the study and the manuscript.

Correspondance and requests for materials should be addressed to J.G.F. (jean-guy.fournier@cea.fr).

Tables

Table 1. Results of bioassays of phenol/chloroform group – First and second experiments

Inocula[a]	Description	Scrapie after IC	Death from other causes	Residual infectivity	WB PrP^{sc}	Incubation periods[b]
1	BioRad purified SAF	4/4	0/4	N/A	+	85,85,85,85
Experiment 1: SAF treated with PK and Phenol/Chloroform (duration : 545 days)						
2	Treated SAF solution (SaS)	0/5	1/5$^{\phi}$	No$^{\phi}$	+	413$^{\phi}$
6	SaS+ β-recPrP (72°C)	0/7	2/7	No	-	300*, 424*
7	SaS + α-recPrP	0/5	1/5	No	-	310*
8	SaS+ Dnase I + β-recPrP (72°C)	2/5	1/5	No	+	151, 246, 452*
11	SaS + β-recPrP (urea)	0/7	3/7	No	-	424*, 508*, 515*
12	α - recPrP in MOPS	0/4[c]	0/4	No		545[c]
13	β- recPrP (72°C) in MOPS	0/2	1/2	No		410*
Experiment 2: SAF treated with PK and Phenol/Chloroform (ongoing > 280 days)						
A	Treated SAF solution 2 (SaS2)	0/7	0/7	No		
B	SaS2+DNAse I	0/17	1/17	No	-	279*
C	SaS2+DNAse I + α- recPrP	0/5	0/5	No		
D	SaS2 + DNAse I + β-recPrP (72°C)	3/19	0/19	No	+	148, 178, 207

a, The order of inoculation was done randomly, excluding cross contamination; for example, the positive inoculum 8 followed the two negative inocula 6 and 7. **b,** Delay between injection and death/sacrifice at the time when the animal show severe clinical signs (ataxia, rachitism,tremor). **c,** All surviving animals were sacrified at 545 days post inoculation **ϕ,** Aberrant scrapie infection observed in one control animal that can be clearly attributed to enviromental contamination. The animal (death at 413 days) lived in a cage with three animals of another experimental group injected with contaminated inoculum from an unsuccessful PrP^{sc} removal protocole involving GnHCl (data not shown). These animals died at 231, 247 and 372 days and it is known that feces and urine are very efficient sources of infectivity[20,21]. This was confirmed by WB (Supplementary Fig. 4b, lane 3) and histopathology giving a lesion profile identical to 263K (Supplementary Fig. 5). ***,** These animals died of causes other than scrapie disease showed no clinical signs and no PrP^{sc} accumulation (some are shown in Supplementary Fig. 4b).

Figures

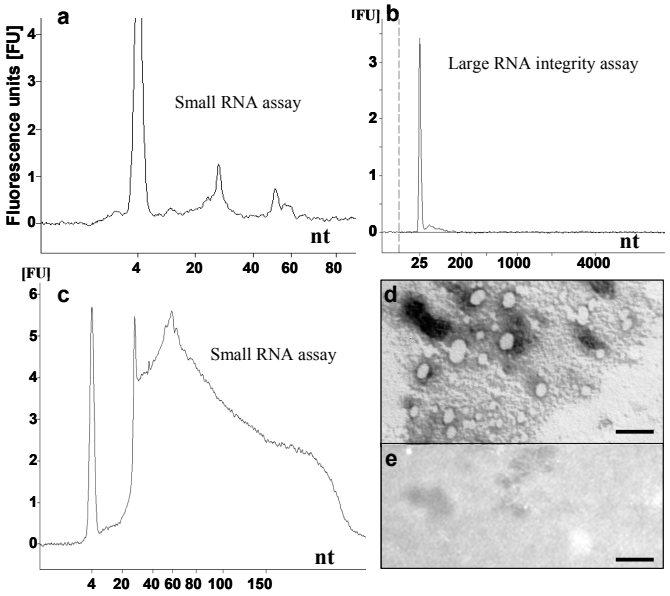

Figure 1: Nucleic acid analysis and recPrP nanostructural formation. a, Electropherogram of DNAse I digested inoculum 2 analysed with a small RNA assay chip, showing two distinct populations of RNA having an average length of about 27 and 55 nucleotides. **b**, Electropherogram of DNAse I digested inoculum 2 analysed with a large RNA integrity assay chip showing the absence of large cellular RNA contamination and degradation products. **c**, Inoculum 2 sample analysed with a small RNA assay chip, showing the presence of contaminating cellular DNA (sizes ≤ 300 nucleotides). **d**, Electron microphotography of inoculum 8 after negative staining showing the presence of ovoidal and spherical aggregated forms of PrP with a diameter range of 7 - 40 nm. **e**, Microphotography of control inoculum 7 showing the absence of PrP aggregates (scale bar100 nm).

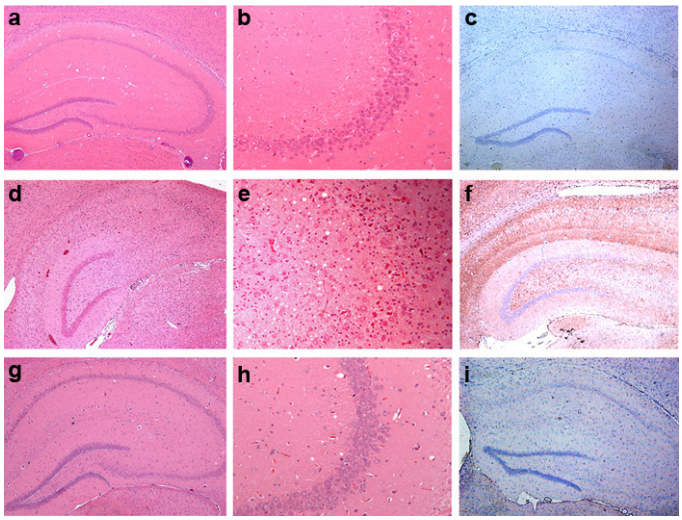

Figure 2: Histopathology and PrP-Immunochemistry of infectious inoculum 8. Hematoxylin and eosin (H&E)-stained (two left columns) and PrP immunohistochemistry with SAF32 antibody (right column). Control hamster (**a, b, c**), inoculum 8.1- infected hamster (**d, e, f**) and SAF-infected hamster (**g, h, i**). The inoculum 8.1-infected hamster brain showed an intense destruction of the hippocampus, cumulating extensive spongiosis, severe gliosis and neuronal loss (**d, e**). PrP^{sc}immuno-detection displayed a diffuse and strong distribution (**f**) never seen in the SAF hamster group.

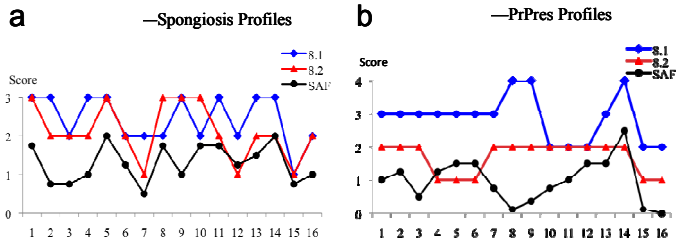

Figure 3: Spongiosis and PrP lesion profiles of infectious inoculum 8. a, Spongiosis profiles of hamsters inoculated with SAF (n=4; black). Two hamsters inoculated with inoculum 8 (8.1 blue, 8.2 red). Spongiosis was scored on a scale of 0-3. The score of 0: no vacuoles, 3; highest degree of spongiosis observed. Eleven areas (1, 2, 3, 4, 5, 7, 9, 11, 13, 14, 16) showed a score difference ≥ 1[8] between the SAF and 8.1 animals and nine (1, 2, 3, 4, 5, 8, 9, 10, 16) between the SAF and 8.2 animals. **b**, PrP lesion profile (SAF32 antibody) scored on a scale of 0 to 4 with 0: no PrP^{sc}deposition, 1: focal slight deposition, 2: diffuse slight, 3: focal strong, 4: diffuse strong deposition. Brain areas examined were: 1, pyriform cortex; 2, motor cortex; 3, septal nuclei; 4, striatum; 5, thalamus; 6, hypothalamus; 7, dentate gyrus; 8, CA1-2; 9, CA3-4; 10, superior colliculus; 11, pons/mesencephalon; 12, cerebellum; 13, dentate nucleus; 14, olivary nuclei; 15 subcortical white matter; 16, cerebellum white matter.

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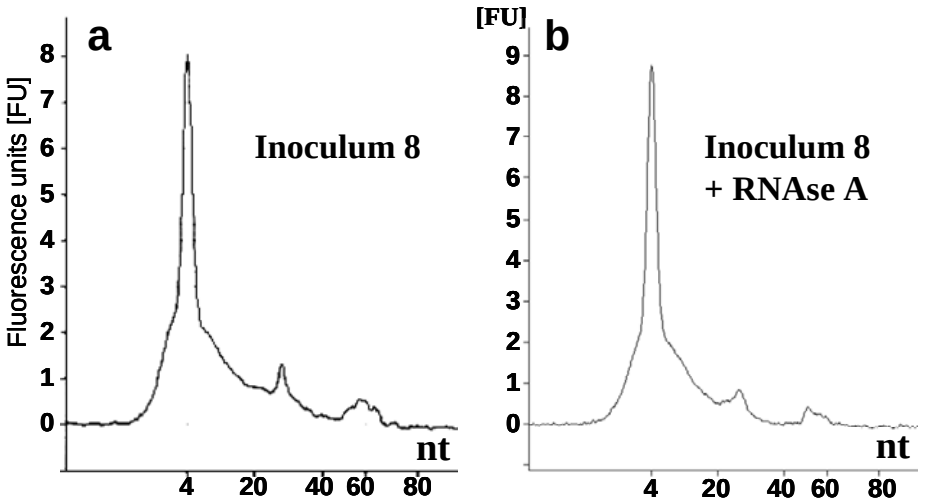
Supplementary Data

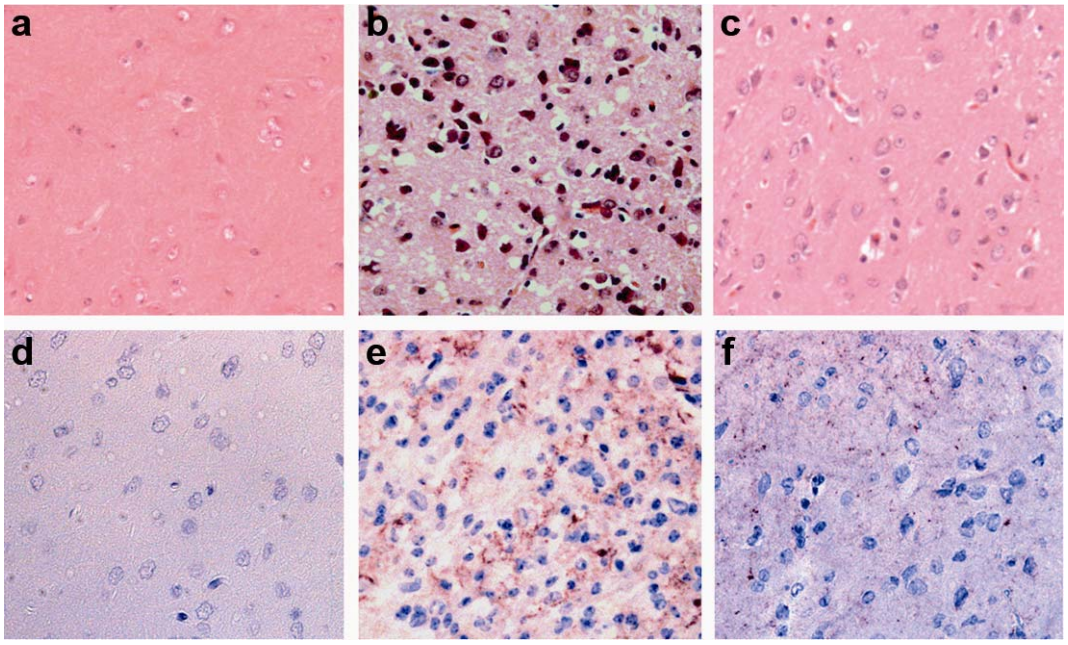
1. Supplementary Figures
2. Supplementary Methods



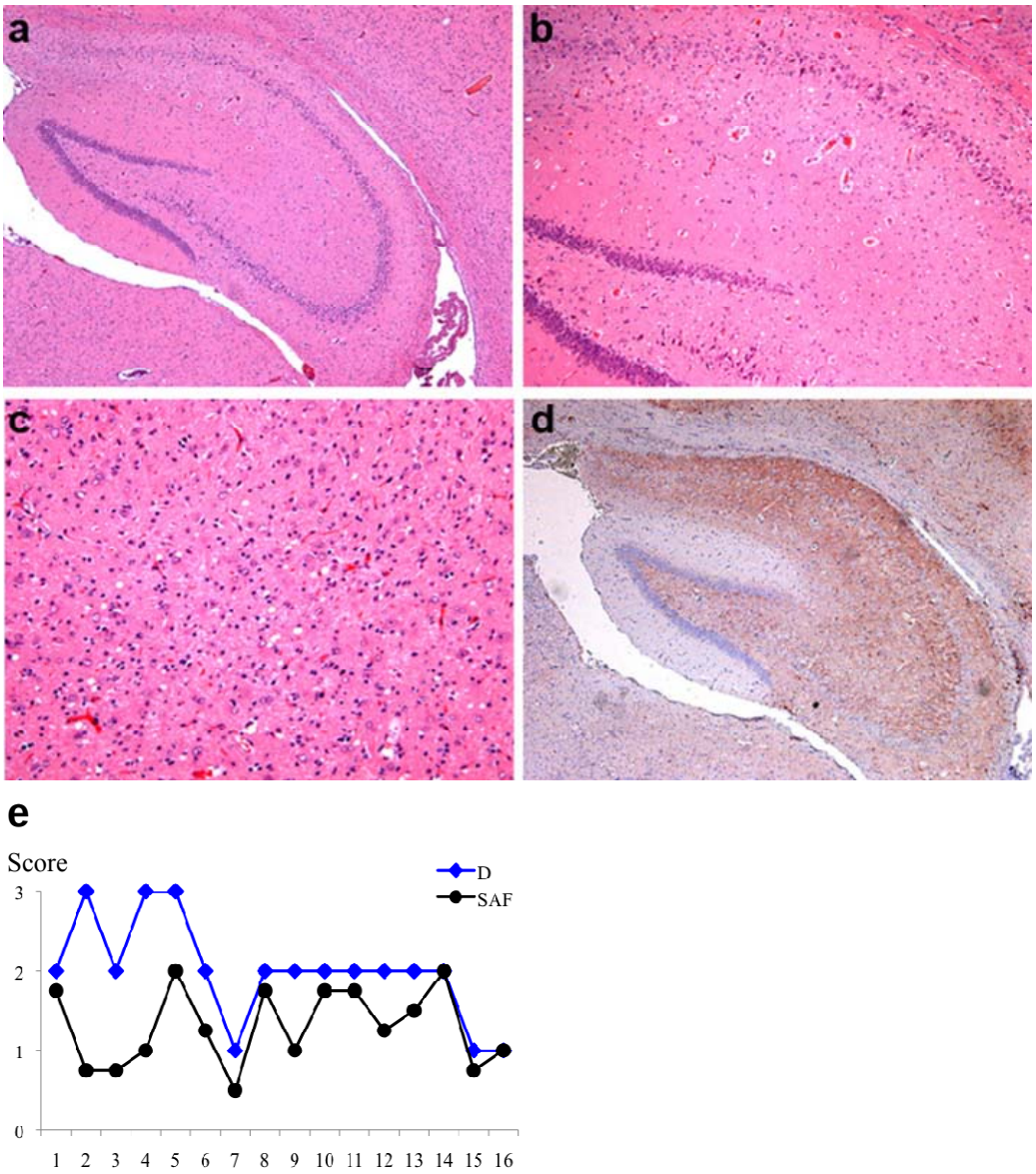
e

Sample	Total free RNA (pg/µl)
Fig. a: Inoculum 8	900
Fig. b: Inoculum 8 + RNAse A	450
Fig. c: Inoculum 8 + PK	1200
Values of a - b	450
Values of c - b	300

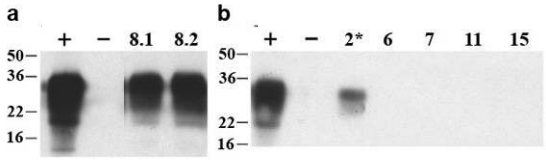
Supplementary Figure 1. Nucleic acid analysis of infectious inocula – 1st and 2nd experiments. a, Infectious inoculum 8 showing the presence of the two RNA populations free in solution. **b**, Inoculum 8 treated with RNAse A. The total amount of free RNA is reduced when compared with **a** (see values in **e**). **c**, Inoculum 8 treated with PK to eliminate recPrP. The total amount of free RNA is increased when compared with **a** indicating its liberation from recPrP (see values in **e**). **d**, Infectious inoculum D showing the same two small RNA populations than in inoculum 8. Several measures performed independantly gave the following averages: **a**: 750 pg/µl (n=3) **b**: 320 pg/µl (n=2) and **c**: 1100 pg/µl (n=2).



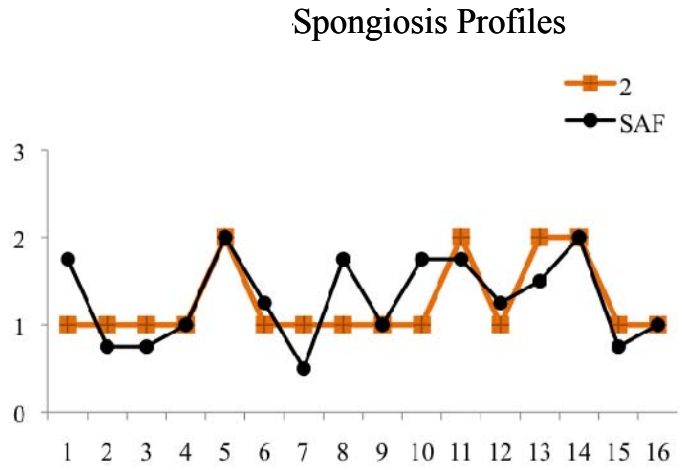
Supplementary Figure 2. Histopathology of Septum – 1st experiment. Hematoxylin and eosin (H&E)-stained (top) and proteinase K-resistant prion protein (PrP^{sc}) (bottom) from a healthy hamster (**a, d**), an inoculum 8-infected hamster (8.1) (**b, e**) and a SAF-infected hamster (**c, f**). The inoculum 8.1 infected hamster showed extensive spongiosis, severe gliosis and neuronal loss (**b**) and a focal and strong PrP^{sc}distribution (**e**) in the Septum, never seen in the SAF hamster group.



Supplementary Figure 3. Histopathology and lesion profiles – 2nd experiment. Hematoxylin and eosin (H&E)-stained from one animal of the inoculum D group that developped scrapie. **a** and **b**, Hippocampus showing lesions distinct to the 263K strain. **c**, Thalamus presenting extensive lesions similar to those observed in the first experiment. **d**, Hippocampus PrP^{sc} deposition (with SAF32 antibody) showing a diffuse and strong distribution. **e**, Spongiosis profiles of hamsters inoculated with i) SAF (n=4; circles) and ii) inoculum D (diamonds). See Figure 3 legend for score scale and brain areas description.



Supplementary Figure 4. PrP^{sc} detection in hamsters – 1st experiment. a, PrP^{sc}from brains of animals inoculated with inoculum 8 (lanes 3; 151 days, lane 4; 246 days). Lane 1: 263K positive control and lane 2: healthy control. **b**, PrP^{sc}from brains of animals inoculated with inocula 2* (lane 3; environmentally contaminated animal), 6 (lane 4), 7(lane 5), 11 (lane 6) and 15 (lane 7). Lane 1: 263K positive control and lane 2: healthy control. A selection of animals which died of a cause other than scrapie has been represented.



Supplementary Figure 5. Lesion profile of environmentally contaminated animal – 1st experiment. Spongiosis lesions profile of the environmentally contaminated animal (inoculum 2; squares) that died at 413 days compared with the SAF group (n=4; circles) showing a profile matching the 263K strain.

PrPsc purification from 263K-infected brains. Brain homogenate (20%) was prepared from 263K-infected hamster brains collected at terminal stage of the disease (~80 days). The homogenate was incubated with 20μg/ml of RNAse A (Ambion) at 37°C for 2hrs. Then, $CaCl_2$ was added to a final concentration of 5mM and the homogenate was incubated for an additional 2hrs with 20U/ml of DNAse I (Roche). PrP^{sc} was then purified from this homogenate using the BioRad TeSeE purification kit according to the manufacturer's instructions (500 μl of homogenate per Bio-Rad reaction tube).

Phenol/Chloroform PrP^{sc} elimination method. Bio-Rad pellets (see PrP^{sc} purification) were resuspended into 50 μl of PBS containing 0.5 % SDS and sonicated for 1 minute at highest setting. The pellet solutions from 10 tubes (total : 500 μl) were pooled together and kept at 4°C. The following day, the solution was mixed with 2500 μl of PBS – 0.5% SDS containing 500μg/ml of PK and incubated overnight at 37°C (~24 hrs). The next day, the PK reaction was stopped by heating the sample at 70°C for 5 minutes. Nucleic Acids were then purified from this solution by mixing with Phenol – chloroform – isoamyl alcohol (25:24:1) (# 77617 Sigma) and extracting the aqueous phase. Extreme caution was taken to avoid touching the meniscus containing the denatured proteins and potentialy residual PrP^{sc}. This protocole results in a solution devoid of PrP^{sc}. 1 μl /ml of SUPERase-In (Ambion) solution was then added to protect from the action of unwanted RNAses. From this stage on, all reagents and material were prepared RNAse-free. This 'A solution' was then mixed with various recombinant PrP preparations (see below).

Normalization of samples. Based on previous experience in the laboratory with the 263K model, we estimated the PrP^{sc} quantity in the Bio-Rad pellets (see PrP^{sc} purification) to be between 5 and 10 μg. We designated this initial estimated quantity as the 'equivalent PrP^{sc}'. The initial quantity was not so important however, but was simply used as a measure for normalizing the different samples throughout the experiment and also as an indicator of how

much recombinant PrP needed to be added to the different samples. All samples were normalized throughout the experiments using the 'equivalent PrP^{sc}' values.

Recombinant hamster PrP. The recombinant PrP used for these experiment was hamster PrP (23-231) from Allprion (P00025). The powder was resuspended in Milli-Q H_2O at a concentration of 1 μg/μl. Once diluted in water, the protein is stable for at least two years at -20 °C.

β-sheeted oligomeric recPrP preparations. The 'A' solution was dialysed against 20 mM MOPS Na^+ pH 7.2 (MOPS buffer) using Slide-A-Lyzer 2K MWCO dialysis cassettes (Pierce) according to manufacturer's instructions. The buffer was replaced with fresh buffer after 3 hours and the dialysis was left to continue overnight. The next day, the 'equivalent PrP^{sc}' of the solution was calculated (taking into account the initial volume and final volume after dialysis) and used with the following preparations:

For 72°C β-sheeted recPrP ('A': Inoculum 6), 4 μg of 'equivalent PrP^{sc}' from MOPS dialysed solution 'A' was combined with MOPS buffer to a final volume of 400 μl. In parallel, 4 μl (1 μg/μl) of recombinant hamster PrP (recPrP; allprion) was added to 96 μl of 20 mM MOPS Na^+ pH 7.2 and heated at 72°C for 20 minutes and mixed immediately with the 400 μl prepared. In these conditions, the recPrP forms β-sheeted PrP oligomers [9].

For 72°C β-sheeted recPrP + DNAse I ('A' : Inoculum 8), 4 μg of 'equivalent PrP^{sc}' from MOPS dialysed solution 'A' was combined with MOPS buffer to a final volume of 400 μl and 1μl of 2M CaCl2 (final concentration 4mM) and 1 μl of DNAse I was added. The solution was then heated at 37°C for 60 minutes and subsequently 75°C for 10 minutes to inactivate the DNAse. In parallel, 4 μl (1 μg/μl) of recPrP was added to 96 μl of 20 mM MOPS Na^+ pH 7.2 and heated at 72°C for 20 minutes and mixed immediately with the 400 μl prepared initially.

β-sheeted recPrP fibrils preparations. The 'A' solution was dialysed against 0.2 M NaCl, 50mM sodium acetate pH 5 (sodium acetate buffer) using Slide-A-Lyzer 2K MWCO dialysis cassettes (Pierce). The buffer was replaced with fresh buffer after 3 hours and the dialysis was left to continue overnight. The next day, the 'equivalent PrP^{sc}' of the solution was calculated and used with the following preparations:

For 'A': Inoculum 11, 4 µl (4 µg) of hamster recPrP and 1 µl of SUPERase-In was added to 140 µl of sodium acetate buffer containing 10M urea. In parallel, 4 µg of 'equivalent PrP^{sc}' of the sodium acetate dialysed solution 'A' was brought to a final volume of 350 µl in sodium acetate buffer and then mixed with the 145 µl. The final solutions were incubated at 37°C for ~48hrs. In these conditions, the recPrP should form PrP fibrils[22]. The resulting mix was dialysed against PBS using Slide-A-Lyzer 2K MWCO dialysis cassettes. The PBS was replaced after 3 hours and the dialysis left to continue overnight. The next day, the 'equivalent PrP^{sc}' was calculated and the solution diluted and injected in hamsters (see injection protocole below)

Controls. Several controls were injected in hamsters. The details of the preparation of these is described here.

For inoculum 1 (Bio-Rad purified SAFs), 20ul of the pellet solution (100 ng/µl of 'equivalent PrP^{sc}') was diluted in 480 µl of MOPS buffer.

For inoculum 2, 2 µg of 'equivalent PrP^{sc}' from solution 'A' was diluted to a final volume of 500 µl of MOPS buffer.

For inoculum 12 (alpha recPrP alone) 2 µg of hamster recPrP was diluted directly in a final volume of 500 µl of MOPS buffer the day of the injection.

For inoculum 13 (β- recPrP (72°C) in MOPS), 4 µg (1 µg/µl) of recPrP was added to 96 µl of 20 mM MOPS Na^+ pH 7.2 and heated at 72°C for 20 minutes and mixed immediately with

400 µl of MOPS buffer. This sample was then diluted to the proper dilution the day of the injection (see injection protocole below).

For inoculum 7, 4 µg of 'equivalent PrP^{sc}' from solution 'A' was combined with MOPS buffer to a final volume of 400 µl and then mixed with 100 µl of alpha recPrP solution (4 µg).

Injection into golden syrian hamsters. All samples were kept at 4°C until injection day (no longer than 3 days) and prepared for injection (diluted) on the day of the injection. Samples were diluted to a final 'equivalent PrP^{sc}' of 4 ng/µl with either PBS or MOPS buffer depending on the buffer of the solution injected. Each hamster received a total of 50µl in the right hemisphere of the brain. Gloves were changed in between each inoculum to avoid contaminations. The order of injection was: inocula 1, 2, 7, 8, 6, 11, 12, 13.

For the second experiment, the injections were performed in the same way as in the first experiment.