A single amino acid substitution, found in mammals with low susceptibility to prion diseases, delays propagation of two prion strains in highly susceptible transgenic mouse models

Autores: Alicia Otero[1], Carlos Hedman[1], Natalia Fernández-Borges[2], Hasier Eraña[2], Belén Marín[1], Marta Monzón[1], Manuel A. Sánchez-Martín[3,4], Romolo Nonno[5], Juan José Badiola[1], *Rosa Bolea[1] and *Joaquín Castilla[2,6]

***Corresponding authors**

[1] Centro de Encefalopatías y Enfermedades Transmisibles Emergentes, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain.

[2] CIC bioGUNE, Parque Tecnológico de Bizkaia, Derio, Bizkaia, Spain.

[3] Servicio de Transgénesis, Nucleus, Universidad de Salamanca, Salamanca, Spain

[4] IBSAL, Instituto de Investigación Biomédica de Salamanca, Salamanca, Spain

[5] Department of Food Safety and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy.

[6] IKERBASQUE, Basque Foundation for Science, Bilbao, Bizkaia, Spain.

*** Corresponding authors:**

E-mail: rbolea@unizar.es, castilla@joaquincastilla.com

Abstract

Specific variations in the amino acid sequence of prion protein (PrP) are key determinants of susceptibility to prion diseases. We previously showed that an amino acid substitution specific to canids confers resistance to prion diseases when expressed in mice, and demonstrated its dominant-negative protective effect against a variety of infectious prion strains of different origins and characteristics. Here, we show that expression of this single amino acid change significantly increases survival time in transgenic mice expressing bank vole cellular prion protein (PrP^{C}), which is inherently prone to misfolding, following inoculation with two distinct prion strains (the CWD-vole strain and an atypical strain of spontaneous origin). This amino acid substitution hinders the propagation of both prion strains, even when expressed in the context of a PrP^{C} uniquely susceptible to a wide range of prion isolates. Non-inoculated mice expressing this substitution experience spontaneous prion formation, but showing an increase in survival comparable to that observed in mutant mice inoculated with the atypical strain. Our results underscore the importance of this PrP variant in the search for molecules with therapeutic potential against prion diseases.

Keywords: Prions; Prion propagation; transmissible spongiform encephalopathies; canine PrP; bank vole PrP

Introduction

Prions are self-propagating infectious proteins that cause fatal neurodegenerative disorders known as transmissible spongiform encephalopathies (TSE) or prion diseases. Characterized by spongiform changes, gliosis, and neuronal degeneration in the central nervous system (CNS), these diseases include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, and Creutzfeldt-Jakob disease (CJD) in humans [1,2]. While the underlying trigger can be sporadic, genetic, or infectious in origin [3], the characteristic event in the pathogenesis of these diseases is misfolding of normal cellular prion protein (PrP^{C}), giving rise to a protease-resistant, β-sheet-rich isoform known as PrP^{Sc}, which accumulates in the CNS leading to neurodegeneration [4].

The presence of aspartic acid (D) at codon 163 of PrP^{C}, a polymorphism exclusive to the Canidae family [5], may account for the unusual resistance of canid species to prion diseases [6]. Studies of recombinant proteins exposed to denaturing agents and *in vitro* and *in vivo* prion propagation studies assessing the susceptibility of species historically considered prion-resistant (leporids, equids, and canids) have demonstrated that canid PrP^{C} shows the greatest resistance to misfolding [7-11]. Bank voles (*Myodes glareolus*), by contrast, are highly susceptible to prion infection, and have been used widely in prion research owing to their ability to efficiently propagate a broad spectrum of prion strains [12-16]. Bank vole PrP^{C} is polymorphic, and either methionine (M) or isoleucine (I) can be expressed at codon 109 [17]. The adaptation of CWD in voles expressing isoleucine at codon 109 led to the isolation of the fastest prion strain (survival time, ~35 days) identified to date [16]. Interestingly, overexpression of bank vole I109 PrP in transgenic mice leads to the development of spontaneous TSE, providing a very useful model for the study of sporadic prion diseases [18]. Given that sporadic forms are the most common

prion diseases in humans, and their origin remains unknown, the generation of these models is essential for research. It has been suggested that sporadic prion diseases may be caused by random and stochastic misfolding of PrP^{C}, resulting in the accumulation of PrP^{Sc} and consequent clinical and neuropathological features associated with prion disorders [19].

The canid D163 PrP^{C} polymorphism is likely the main determinant of Canidae resistance to prion diseases. Indeed, the presence of this single substitution in mouse PrP^{C} (N158D substitution in mouse PrP^{C} numbering) prevents prion propagation both *in vitro* and *in vivo* [6]. We previously demonstrated that *in vivo* coexpression of the N158D PrP^{C} variant and wild-type mouse PrP^{C} significantly delays disease onset in transgenic mice inoculated with several prion strains of different origins and characteristics [20], suggesting that N158D PrP^{C} is a promising candidate in the search for proteins with dominant-negative effects against a broad spectrum of prion strains. In the present study, we investigated whether this substitution could also prevent or delay the onset of prion disease in a highly susceptible model. Transgenic mice overexpressing bank vole I109 PrP and carrying this specific residue (TgVole-N159D mice), were inoculated with two prion isolates, and the resulting survival times compared with those of transgenic mice expressing comparable levels of bank vole PrP^{C} (TgVole mice) but lacking this PrP^{C} residue. To corroborate the ability of this amino acid substitution to confer protection against prion propagation, we used two distinct strains with very different neuropathological and biochemical features. For both inoculated prion strains, survival periods in TgVole-N159D mice were 52–108% longer than those of TgVole mice. These results are in good agreement with our previous findings demonstrating that expression of this specific amino acid substitution, even when expressed in a PrP^{C} highly susceptible

to misfolding, interferes with prion propagation and delays the onset of disease caused by multiple, distinct prion strains.

Materials and Methods

Ethics statement

All procedures involving animals were approved by the University of Zaragoza's Ethics Committee for Animal Experiments (permit number PI32/13) and were performed in accordance with recommendations for the care and use of experimental animals and with Spanish law (R.D. 1201/05).

Inoculation of transgenic mice and sample processing

Two different transgenic mouse models were used in the present study: transgenic mice expressing ~3-4x the I109 polymorphic variant of bank vole PrP and carrying the critical dog amino acid substitution (I109-N159D PrP^{C}), hereafter referred to as TgVole-N159D mice, and mice overexpressing ~3-4x bank vole I109 PrP^{C}, hereafter referred to as TgVole mice, which were used as controls. The murine *PRNP* promoter was used for both I109-N159D and I109 PrP^{C} expression, in a murine $Prnp^{0/0}$ background. Mice were generated and characterized as previously described [21].

Mice were anesthetized with isoflurane and intracerebrally inoculated (right cerebral hemisphere) with 20 µl of a 1 % brain homogenate. Both TgVole-N159D and TgVole mice were inoculated with one of two different isolates: CWD-vole, a CWD strain adapted to bank voles that contains I109 PrP and is characterized by very short survival times [16]; and an atypical prion isolate (Sp-TgVole isolate) of spontaneous origin. Sp-TgVole inoculum was obtained from brain homogenates from TgVole mice [22] that were sacrificed at 182±5 days of age after developing a spontaneous

neurodegenerative disorder linked to the overexpression of the I109 variant of bank vole PrP [18] (Supplementary Fig. 1). Intracerebral injections were performed using a 50-µl precision syringe and a 25-G needle. After inoculation, mice received a subcutaneous injection of buprenorphine (0.3 mg/kg) to induce analgesia.

Mice were monitored for the onset of neurologic signs after inoculation, sacrificed by cervical dislocation upon detection of clinical signs of terminal disease (*i.e.*, severe locomotor disorders, poor body condition, and any signs of impaired feeding ability), and their brains collected. Coronal sections were cut at the level of the frontal cortex and medulla oblongata and immediately stored at -80 ºC for biochemical analyses. The remaining brain tissue was stored in 10 % formalin fixative for histological analyses.

Histopathological evaluation

Formalin-fixed brains were sectioned transversely at four standard levels for neuropathological analyses of the following brain areas: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellar cortex (Cbl), and medulla oblongata (Mo) [23]. Formalin-fixed brain tissues were embedded in paraffin wax, and 4-µm-thick tissue sections were mounted on microscope slides for hematoxylin-eosin staining. The intensity and distribution of spongiform changes were blindly evaluated using an optical microscope (Zeiss Axioskop 40) and semiquantitatively scored on a scale of 0 (absence of lesions) to 5 (high intensity lesions) in each of the aforementioned brain regions.

Analysis of PrP^{Sc} deposition

The detection of PrP^{Sc} deposition in paraffin-embedded brains was performed using the paraffin-embedded tissue (PET) blot technique, as previously described [24,25]. PrP^{Sc} was detected by incubation with the Sha31 primary monoclonal antibody (1:8,000;

SPI-Bio), followed by an alkaline phosphatase-coupled goat anti-mouse antibody (1:500; Dako). Immunolabeling was visualized using the NBT/BCIP substrate chromogen (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate; Sigma-Aldrich). The presence, intensity, and distribution of PrP^{Sc} aggregates were evaluated using a Zeiss Stemi DV4 stereomicroscope and semiquantitatively scored as described for spongiform lesions.

The distribution of PrP^{Sc} deposition was also analyzed by immunohistochemistry using a previously described protocol [26], with some modifications. Paraffin-embedded sections were pre-incubated with 98 % formic acid for 5 min and underwent hydrated autoclaving in citrate buffer for 20 min at 96 ºC. Peroxidase activity was blocked for 5 min using a peroxidase blocking reagent (Dako). Immunodetection of PrP^{Sc} was achieved by incubation with 6H4 monoclonal antibody (1:100, Prionics) followed by an anti-mouse Envision polymer (Dako). Sections were subsequently incubated with DAB (diaminobenzidine, Dako) and counterstained with hematoxylin.

Biochemical analysis of inoculated strains

Proteinase K (PK) resistant PrP^{Sc} was detected and characterized in both the CWD-vole and Sp-TgVole inocula before inoculation. To this end, 10 % brain homogenates from CWD-vole I109 inoculated animals (CWD-vole strain) were incubated with 80 μg/ml PK for 1 h at 42 ºC with constant agitation (450 rpm), as previously described [27]. Biochemical characterization of spontaneously generated TgVole I109 prions (Sp-TgVole strain) was also carried out as reported previously [28]. Briefly, brain homogenates (20% w/v) from clinically diseased animals were mixed with an equal volume of 100 mM Tris-HCl + 4 % sarkosyl and incubated for 30 min at 37 ºC. Homogenates were then digested with 200 μg/ml PK (Sigma-Aldrich) for 1 h at 55 ºC with gentle agitation. Aliquots of samples were mixed with an equal volume of

isopropanol/butanol (1:1 v/v) and centrifuged for 5 min at 20,000 × g. Supernatants were discarded and pellets were re-suspended in denaturing sample buffer (NuPage). Both CWD-vole and Sp-TgVole inocula were analyzed by Western blotting using the 12B2 antibody (1:2,500), which recognizes the 89–93 epitopes of bank vole PrP.

Histological analysis of PrP distribution

The histological localization and distribution of cellular PrP in the brains of both TgVole-N159D and TgVole mice was analyzed by immunohistochemistry as previously described [20]. Briefly, paraffin-embedded brain sections were incubated with a 1% peroxidase solution for 20 min followed by hydrated autoclaving at 100°C in citrate buffer for 30 min. PrP immunodetection was performed overnight at 4°C using SAF84 (1:1,000; Cayman Chemical) antibody. The anti-mouse Envision polymer (Dako) was used as the visualization system and DAB (diaminobenzidine, Dako) as the chromogen.

The cellular localization of PrP was further analyzed in the brains of TgVole-N159D and TgVole mice using immunofluorescence and confocal imaging. The immunofluorescence staining was performed as described previously [20]. Paraffin-embedded tissue sections from TgVole-N159D and TgVole mice were pre-treated with 1% peroxidase for 30 min. Sections were subsequently permeabilized with 0,1% Triton X-100 for 3 h at room temperature and subjected to hydrated autoclaving. Immunodetection was carried out with SAF84 antibody (1:1000) followed by a goat anti-mouse IgG biotin conjugate (1:100; Invitrogen) and an Alexa fluor 594 streptavidin conjugate (1:1000; Invitrogen). Sections were observed under a Zeiss laser-scanning confocal microscope LSM 510 (Carl Zeiss MicroImaging).

Data analysis

Survival times were analyzed using the Kaplan-Meier method and the survival curves for mice carrying the N159D substitution were compared with those of controls using the log rank test (α= 0.05). Differences in histopathological and PrP^{Sc} deposition profiles between transgenic mouse models were analyzed using the nonparametric Mann-Whitney U-test, with p-values <0.05 considered significant. GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA) was used for data analysis and to generate Kaplan Meier curves and histopathology graphs.

Results

Expression of the N159D PrP^{C} substitution markedly increases survival time in mice challenged with the CWD-vole and Sp-TgVole strains

Transgenic mice expressing bank vole I109 PrP^{C} and carrying the prion resistance-associated N159D amino acid substitution (TgVole-N159D mice) were intracerebrally inoculated with either the classical CWD-vole strain or the atypical Sp-TgVole strain (Supplementary Fig. 1). As controls, TgVole mice expressing comparable levels of bank vole I109 PrP^{C} (~3-4×) were challenged with the same isolates. Both transgenic lines present a normal cellular distribution of PrP through the brain and show a good expression of PrP in the cellular membrane (Supplementary Fig. 2). After challenge with the CWD-vole strain, survival time in TgVole-N159D mice was 108 % longer than that of control TgVole mice, in which the mean survival time was 61±4 days post-inoculation (dpi). In TgVole-N159D mice inoculated with the Sp-TgVole isolate, survival time was 52 % longer than that of control TgVole mice (Table 1).

In both the TgVole and TgVole-N159D transgenic mice, overexpression of bank vole I109 PrP^{C} (~3-4x) leads to the development of a spontaneous neurodegenerative disorder. However, TgVole-N159D mice develop the spontaneous form of the disease

showing an increase in survival time of 60 % relative to non-inoculated TgVole animals (Table 1). This relative increase in survival is comparable to that observed in TgVole-N159D mice inoculated with the Sp-TgVole strain. For both inoculated strains, survival time was significantly longer in TgVole-N159D versus TgVole mice (Fig. 1). Significant differences in survival were obtained between TgVole-N159D and TgVole mice for both strains inoculated and between non-inoculated, spontaneously sick TgVole-N159D and TgVole mice (Fig. 1).

Despite the significant delay in disease onset, TgVole-N159D mice exhibited clinical signs of neurodegeneration identical to those seen in TgVole mice. Animals inoculated with the CWD-vole strain exhibited dorsal kyphosis, circling behavior, cachexia, and tremor. By contrast, those inoculated with the Sp-TgVole isolate showed mild kyphosis and rapidly progressing ataxia.

Table 1. Survival periods of TgVole and TgVole-N159D mice.

Inoculum	Model	Attack rate[a]	Survival time (mean ± SEM)[b]	Age at which animals succumbed to disease[c]	Relative increase in survival time (%)[d]
CWD-vole	TgVole	5[e] /5 (100%)	61 ± 4	143 ± 5	-
	TgVole-N159D	6/6 (100%)	127 ± 13	205 ± 12	108%
Sp-TgVole	TgVole	7/7 (100%)	120 ± 9	178 ± 6	-
	TgVole-N159D	5 /5 (100%)	182 ± 3	247 ± 6	52%
Non-inoculated	TgVole	10/10 (100%)	-	182 ± 5	-
	TgVole-N159D	12/12 (100%)	-	292 ± 10	60%

[a] Data based on PrP^{Sc} detection.

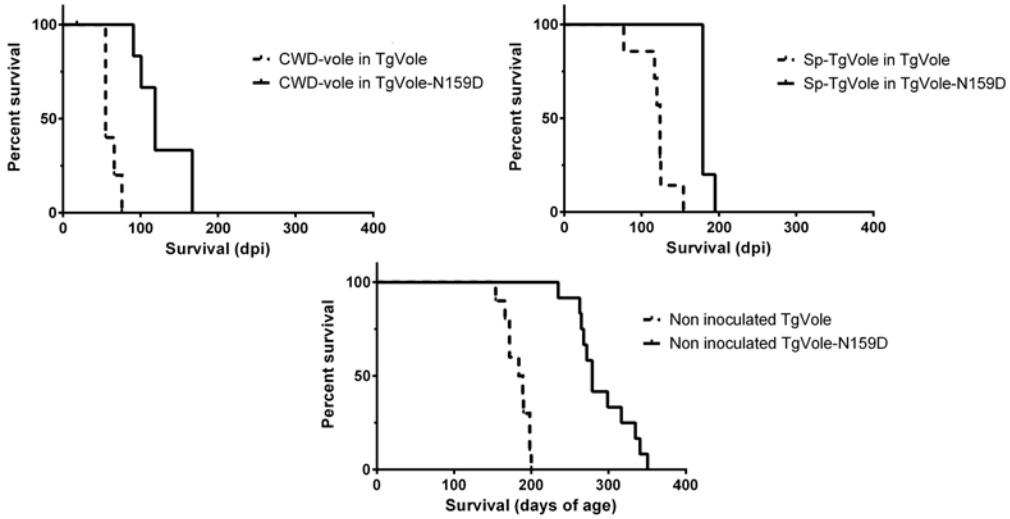
[b] Survival times were calculated as the number of days between inoculation and sacrifice of mice inoculated with CWD-vole or Sp-TgVole isolates, provided that the mouse developed clinical signs consistent with a TSE. Survival times are expressed as the mean (± SEM) number of dpi.

[c] For non-inoculated mice survival times were considered as the mean age at which the animals sporadically developed clinical signs consistent with a TSE and were euthanized. The age at which animals succumbed to disease is expressed as mean (± SEM) days of age.

SEM, standard error of the mean; dpi, days post-inoculation; NA, not applicable.

[d] Prolongation of survival time in TgVole-N159D mice is expressed as the percentage increase in mean survival time relative to TgVole mice.

[e] One animal from the group inoculated with CWD-vole isolate group died during the initial stages of the study due to a concomitant disease. This animal exhibited no spongiform lesions or PrP^{Sc} deposits and was excluded from all analyses.



232

233 **Figure 1. Survival curves for TgVole and TgVole-N159D mice (non-inoculated or after**
234 **challenge with CWD-vole or Sp-TgVole isolates)**. Analysis of survival curves using the log
235 rank test (α=0.050) revealed significant differences between TgVole and TgVole-N159D mice
236 after inoculation with either CWD-vole (p=0.0007) or Sp-TgVole (p=0.0011) isolates, and
237 between non-inoculated TgVole and TgVole-N159D mice (p<0.0001) that developed
238 spontaneous forms of the disease. Survival periods are expressed as days post-inoculation (dpi)
239 for inoculated groups, and as days of age at which mice succumbed to the spontaneous TSE for
240 non-inoculated groups.

241

242 **Expression of the N159D substitution does not alter the neuropathological features**
243 **of the disease**

244 Expression of the PrP^C N159D substitution, a key amino acid substitution
245 associated with prion disease resistance in dogs [6], considerably delayed the onset of
246 clinical signs, but did not significantly alter the neuropathological features exhibited by
247 TgVole-N159D mice. Similar results were observed in our previous study, in which the
248 effects of this dog-specific substitution were studied in the mouse PrP backbone [20].

Semi-quantitative analysis of spongiosis and prion protein deposition patterns in 9 brain areas revealed no significant differences between TgVole-N159D and TgVole mice inoculated with the same isolate (Fig. 2). All mice inoculated with the CWD-vole strain showed moderate spongiform changes and discretely distributed PrP^{Sc} deposits, which were particularly conspicuous in the thalamus. In other brain regions, such as the hippocampus, spongiosis was minimal and PrP^{Sc} deposition scores were very low (Figs. 2 and 3). These neuropathological features coincide with those described in I109 bank voles infected with the same strain, in which prominent involvement of the thalamus has been reported [16]. Moreover, both TgVole-N159D and TgVole mice inoculated with the atypical Sp-TgVole isolate exhibited severe vacuolar changes in the cortex (Fc and Tc) and in the hippocampus, in which we observed the most intense PrP^{Sc} deposition for this isolate (Figs. 2 and 3). To verify that the Sp-TgVole strain maintained its neuropathological characteristics after experimental transmission to TgVole and TgVole-N159D mice, we performed a histopathological assessment of brain samples from TgVole and TgVole-N159D animals that spontaneously developed the disease at ~182 and ~292 days of age, respectively. The lesion profiles and PrP^{Sc} deposition patterns in these animals were almost identical to one another and to those of mice inoculated with the Sp-TgVole isolate (Fig. 2).

The fact that similar neuropathological features were observed in TgVole and TgVole-N159D mice inoculated with the Sp-TgVole strain, and in non-inoculated TgVole mice, suggests the development of the same spontaneous disease in these inoculated mice in an accelerated or induced manner.

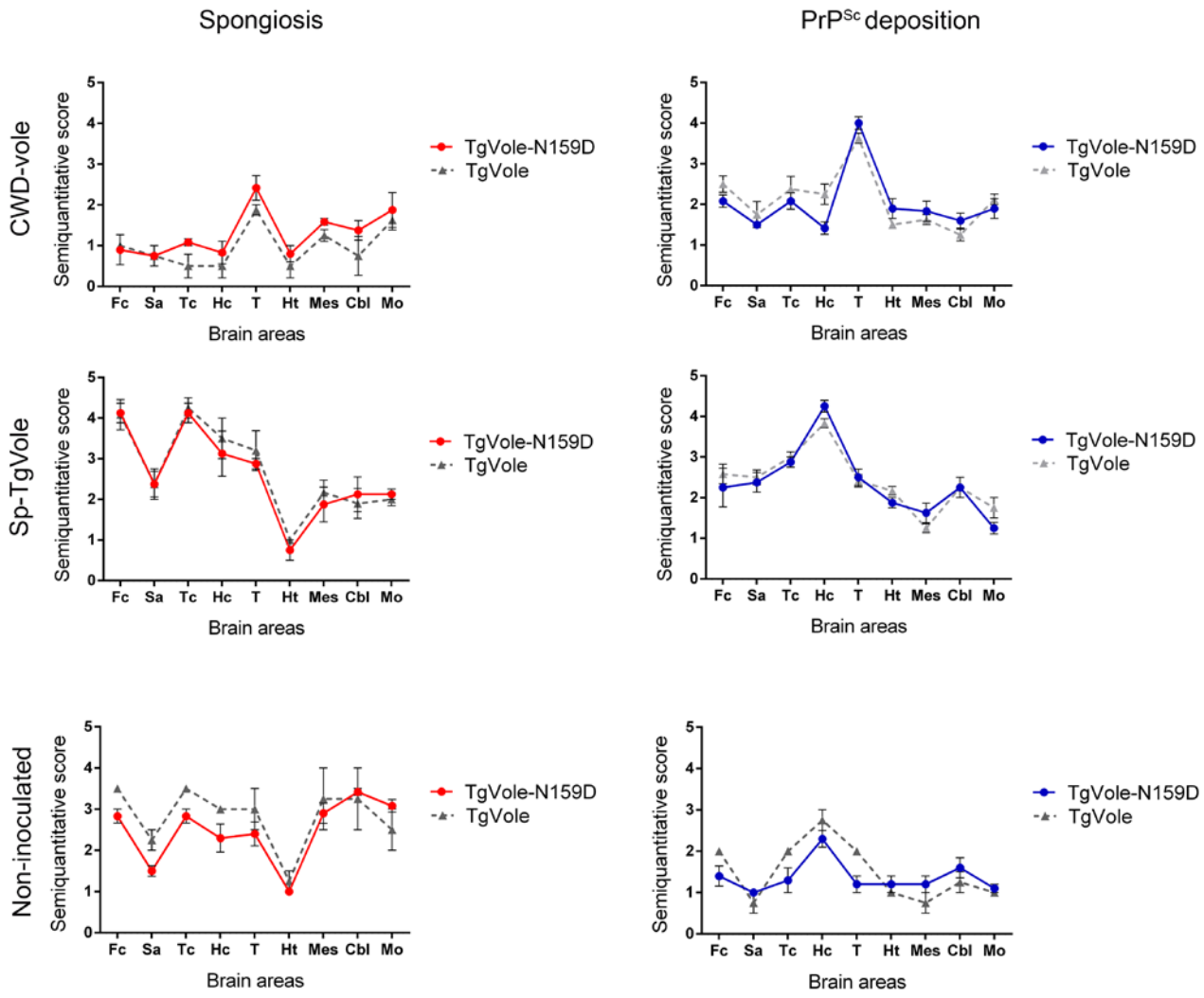


Figure 2. Spongiosis and PrP^{Sc} deposition profiles in the brains of TgVole and TgVole-N159D mice inoculated with CWD-vole or Sp-TgVole prion isolates, or non inoculated. Spongiosis and PrP^{Sc} deposition were evaluated semiquantitatively on a scale of 0 (absence of lesions/deposits) to 5 (high intensity lesions/deposition) in the following nine brain areas: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellum (Cbl), and medulla oblongata (Mo). Graphs represent the mean with SEM of at least 5 mice per group. Comparison of the lesion and PrP^{Sc} deposition profiles of TgVole and TgVole-

283 N159D mice revealed no significant differences between groups for any parameter
284 (α=0.05, Mann-Whitney *U* test).

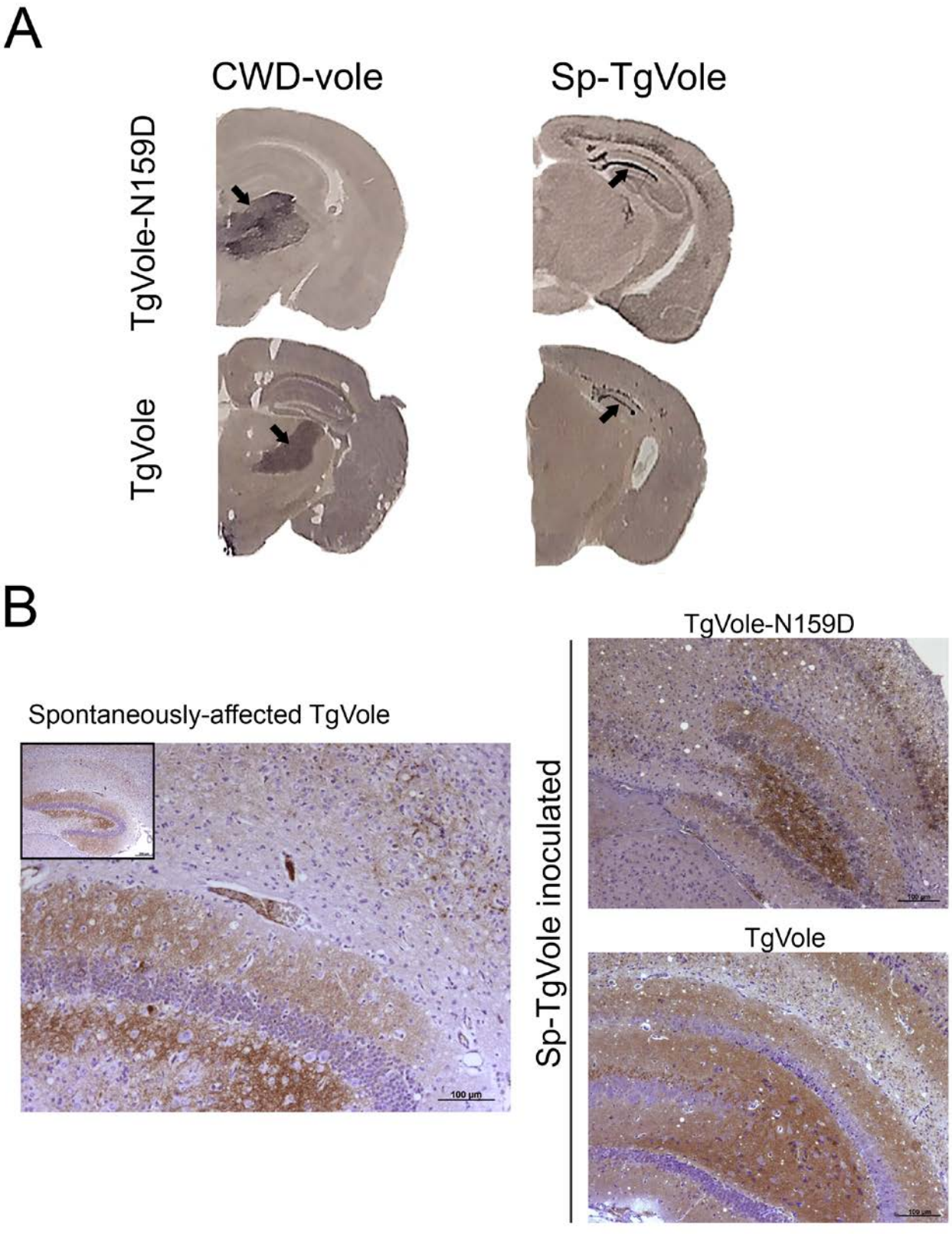


Figure 3. a PET blot images of coronal brain sections from TgVole and TgVole-N159D mice inoculated with CWD-vole or Sp-TgVole isolates. The distribution pattern of PrP^{Sc} deposition (dark purple) in mice expressing the N159D substitution is very similar to that of non-inoculated TgVole controls. Animals inoculated with the Sp-TgVole strain show marked PrP^{Sc} deposition in the hippocampus. By contrast, in CWD-vole-inoculated animals immunolabelling with the Sha31 antibody is much weaker in the hippocampus, and strongest in the thalamus. b Immunohistochemical analysis of a TgVole mouse with the spontaneous form of the disease and of TgVole-N159D and TgVole mice inoculated with the Sp-TgVole strain. Note that the morphology and distribution of PrP^{Sc} deposits is almost identical in the three mice, all of which show abundant granular PrP^{Sc} deposition in the dentate gyrus and in Ammon's horn of the hippocampus. Immunodetection was performed using the 6H4 monoclonal antibody (1:100).

Discussion

The role in prion disease resistance of certain naturally occurring variants in the amino acid sequence of PrP^{C} has been intensively studied. The presence of at least one arginine at codon 171 in sheep PrP^{C} appears to confer low susceptibility to classical scrapie infection [29,30]. Studies have demonstrated that this single amino acid substitution exerts a dominant-negative inhibitory effect on prion replication both *in vitro* and *in vivo* [31-34]. Heterozygosity for specific human PrP^{C} polymorphisms also protects against acquired, sporadic, and some familial prion diseases [35,36]. The resistance-associated human polymorphisms E219K and G127V not only confer strong protection against human prion diseases, but also exert a dominant-negative inhibitory effect on prion propagation when coexpressed with wild-type prion protein [36,33,37]. Introduction of these naturally occurring single mutations into an exogenous PrP^{C}

311 therefore represents a potential therapeutic strategy [33,37]. However, when searching
312 for dominant-negative PrPs it is important to bear in mind that susceptibility to TSE
313 depends on both the *PRNP* genotype and the infectious strain [38,39]. Although strong,
314 the resistance to prion propagation conferred by most of these polymorphisms is strain-
315 specific [40-42]. In this study, we investigated the potential protective effect of the D163
316 (D159 in bank vole numbering) PrP^C residue. This amino acid is almost exclusive to canid
317 species [5,6], in which no naturally occurring TSE have been described. Moreover, the
318 PrP^C form expressed by these species is highly resistant to misfolding *in vitro* [7] and
319 appears not to undergo misfolding *in vivo* [6,8].

320 We previously demonstrated that mice overexpressing a mutated prion protein
321 carrying the N158D amino acid substitution are completely resistant to prion infection
322 when inoculated with a variety of mouse-adapted prion strains [6]. Moreover, we have
323 shown that coexpression of wild-type mouse PrP^C and a mutant PrP^C variant carrying this
324 specific dog amino acid substitution has a dominant-negative effect on the *in vivo*
325 propagation of mouse-adapted prion strains of scrapie and BSE origins [20]. However,
326 could this amino acid substitution, characteristic of the most resistant mammalian species,
327 prevent the misfolding of a PrP^C characterized by an extraordinary promiscuity to
328 propagate numerous prion strains [15]?

329 Here, we show that in mice overexpressing bank vole PrP^C I109, whose
330 misfolding ability is such that its single overexpression leads to the development of a
331 spontaneous prion disease [18], the presence of the N159D substitution in PrP^C
332 significantly delays the onset of clinical signs. These findings suggest that the protective
333 effect of this amino acid change characteristic of canids is stronger when expressed in
334 mouse PrP^C [6]. Although non-inoculated TgVole-N159D mice present a delay in the
335 onset of clinical signs, we have observed that the expression of this resistance-associated

substitution does not prevent spontaneous prion formation in these animals. However, it is important to remember that bank vole PrP^C is greatly prone to conversion and allows the propagation of prion strains which are refractory to be transmitted in wild-type and transgenic mice. [12,14,43]. Bank voles expressing the I109 polymorphic variant of PrP^C are more susceptible to certain familial prion disorders than transgenic mice overexpressing homologous PrP carrying the corresponding mutation that causes the disease in humans [14,44]. However, we show that the N159D substitution can increase survival time in a model that overexpresses a form of PrP^C that is highly susceptible to misfolding induced by almost all prion strains. The prolongation of survival time was especially striking in TgVole-N159D mice after inoculation with the classical CWD-vole prion strain, which is the prion strain causing the shortest survival times described to date [16]: survival time in these mice was 108% longer than that of TgVole mice. While a significant increase in survival time was also observed in TgVole-N159D mice inoculated with Sp-TgVole isolate, an atypical strain of spontaneous origin, this effect was less marked than that observed for the CWD-vole strain (Table 1). Thus, the protective effect of the N159D substitution, although considerable in all cases, appears not to be homogeneous across strains. This observation is in good agreement with our previous findings in mice coexpressing the protein carrying the dog-specific substitution, in which the mutated protein inhibited prion propagation in a strain-specific manner [20]. It could be discussed, however, that comparing a single transgenic line carrying the mutation (TgVole-N159D mice) with a single control line (TgVole mice) is a too straightforward approach to evaluate the protective effect of the substitution. We generated other mutated lines, but their PrP expression levels were not comparable with those of their corresponding controls. Nevertheless, the goal of the present study is to analyze the transmission barrier to TgVole-N159D mice, and we consider that the most important

parameters determining that transmission are the PrP expression level and the distribution of PrP. TgVole-N159D and TgVole transgenic lines have a normal cellular distribution of PrP (Supplementary figure 2) and they show no differences between PrP expression levels or electrophoretic migration patterns (Figure 3). Therefore, we believe that the comparison between these lines is a suitable way to evaluate the effect of the N-to-D substitution.

Analysis of neuropathological features in TgVole-N159D and TgVole mice revealed no significant differences in lesional or PrP^{Sc} deposition profiles (Fig. 2). All mice inoculated with the Sp-TgVole isolate, generated by spontaneous misfolding of I109 PrP^{C}, showed near identical neuropathological profiles. This profile was clearly distinguishable from that of mice inoculated with the CWD-vole strain (Figs. 2 and 3). The distribution and morphology of PrP^{Sc} deposition in mice inoculated with the Sp-TgVole isolate was comparable to that of TgVole-N159D and TgVole mice that developed spontaneous forms of the disease (Fig. 2 and 3b). However, it should be borne in mind that non-inoculated TgVole-N159D and TgVole mice develop spontaneous TSE. Therefore, once they have exceeded the age at which this spontaneous disorder develops, it cannot be determined with certainty whether the observed neuropathology is a result of this phenomenon or a consequence of inoculation. Comparison of age at disease onset revealed that mice inoculated with the Sp-TgVole isolate succumbed to disease at a younger age than non-inoculated TgVole-N159D and TgVole mice (Table 1). Inoculation of the Sp-TgVole isolate thus appears to cause a seeding acceleration phenomenon, *i.e.*, the spontaneous pathological process characteristic of the model is accelerated by exogenous inoculation of the isolate [45]. Similar findings were reported in a study using transgenic mice overexpressing bank vole I109 prion protein [18]; inoculation of brain extracts from spontaneously diseased mice accelerated disease onset, reproducing the

neuropathological hallmarks seen in transgenic mice expressing the same transgene. Moreover, as mentioned, expression of the substitution did not impede the spontaneous generation of the prion, and TgVole-N159D mice experimented an increase in survival similar to that of Sp-TgVole inoculated animals. These results agree with the suggestion that the effects of the N159D substitution are strain-dependent, and that both non-inoculated and Sp-TgVole inoculated mice propagate the same strain. In addition, it could be discussed that the delay in the onset of clinical signs observed in Sp-TgVole inoculated TgVole-N159D mice could be associated with the fact that this isolate was obtained from spontaneously sick TgVole animals. Thus, it could be possible that Sp-TgVole isolate is more adapted to the TgVole model. However, we should consider that non-inoculated TgVole-N159D mice show an almost identical delay in the onset of the disease. Moreover, in a parallel experiment, we inoculated brain homogenates from spontaneously sick TgVole-N159D mice in TgVole and TgVole-N159D mice. We observed again that TgVole-N159D inoculated mice presented a survival period ~50% longer than TgVole animals (data not shown); although the isolate was obtained from TgVole-N159D mice and could therefore be better adapted to this model. We can conclude that while expression of the resistance-associated amino acid change significantly increased survival times, it did not alter the pathological features of the inoculated strains. These results are in agreement with our previous findings [20], and suggest that the delayed appearance of clinical signs in TgVole-N159D mice is not caused by strain modifications resulting from the N159D substitution.

The precise molecular mechanisms by which the N159D substitution prolongs survival time in TgVole-N159D mice remain unclear. However, this residue can extend the survival period in both inoculated mice and those that develop spontaneous disease, as evidenced by the significant differences in survival time between TgVole (182±5 days)

411 and TgVole-N159D mice (292±10 days) with spontaneous disease (Table 1 and Fig. 1).
412 Several theories have been proposed to explain the molecular basis of the anti-prion
413 propagation effect of certain single amino acid changes in PrP^{C}. We previously described
414 the fully protective effect of the N-to-D substitution in mouse PrP against prion
415 inoculation [6], and the dominant negative effect of this protective mutation [20]. It has
416 been suggested that coexpression with wild-type PrP of certain heterologous PrPs
417 carrying putative protective mutations may interfere with the interaction between similar
418 PrP monomers [46-48]. This interference could potentially disrupt the mechanism by
419 which PrP^{C} is converted into PrP^{Sc} [48], since allelic variants can be structurally
420 incompatible [49]. In fact, this disruptive effect has been tested as a potential anti-prion
421 therapy both *in vitro* [50] and *in vivo* [51], with successful results. Furthermore, the
422 introduction of single point mutations, and the heterologous interference they cause, has
423 also been proposed to account for the long survival periods observed when a prion strain
424 is transmitted to a new host [48]. The N159D substitution may exert a protective effect
425 by inducing protein alterations that attenuate the rate of fibril formation and the stability
426 of newly formed fibrils [6]. This molecular mechanism has been previously proposed for
427 other PrP^{C} amino acid substitutions [52,37]. In fact, it has been shown that the protective
428 human PrP^{C} variant G127V, which also acts as a dominant-negative protein [37], hinders
429 the formation of dimers and stable fibrils, thereby protecting against the development of
430 prion diseases [53]. Given that the N-to-D substitution at this specific position
431 significantly alters the surface charge of PrP [6], the N159D substitution may have a
432 similar effect in the context of bank vole PrP^{C}.

433 The results presented here indicate that the introduction of a specific canid N-to-
434 D amino acid substitution into exogenously administered PrP^{C} not only confers complete
435 resistance to TSE in certain mouse models but also significantly increases survival times

in models overexpressing PrPs that are highly susceptible to misfolding. Moreover, the N159D substitution delays the onset of clinical signs of both infectious and spontaneous forms of prion diseases. Together with our previous findings demonstrating a dominant-negative effect of prion protein carrying the N-to-D substitution against a variety of prion strains of different origins [20], we can conclude that this mutation could represent a useful tool to control the propagation of different prion strains when present in the correct PrP background.

Acknowledgments

This work was supported financially by the Spanish (AGL2015-65046-C2-1-R, AGL2015-65560-R) (MINECO/FEDER), and Interreg (POCTEFA EFA148/16) grants. We thank MINECO for the Severo Ochoa Excellence Accreditation (SEV-2016-0644). The authors would like to thank the following for their support: the IKERBasque Foundation, the staff at the CIC bioGUNE animal facility and Patricia Piñeiro and Dr. Jan Langeveld from Central Veterinary Institute, Wageningen for kindly providing the 12B2 antibody. Alicia Otero was supported by a research grant from the Government of Aragón (C020/2014) co-financed by the European Social Fund.

Author contribution statement

JC and RB conceived the study; AO, CH, NFB, HE, and BM performed most of the experiments; MASM and RN collaborated in the creation of the transgenic lines used; AO, HE, JJB, RB and JC evaluated the results; AO, RB, JJB, HE, and JC wrote and reviewed the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

460 1. Colby DW, Prusiner SB (2011) Prions. Cold Spring Harb Perspect Biol 3 (1):a006833.
461 doi:10.1101/cshperspect.a006833
462 2. Collins SJ, Lawson VA, Masters CL (2004) Transmissible spongiform
463 encephalopathies. Lancet 363 (9402):51-61. doi:10.1016/S0140-6736(03)15171-9
464 3. Prusiner SB (1998) The prion diseases. Brain Pathol 8 (3):499-513
465 4. Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. Science 216
466 (4542):136-144
467 5. Stewart P, Campbell L, Skogtvedt S, Griffin KA, Arnemo JM, Tryland M, Girling S,
468 Miller MW, Tranulis MA, Goldmann W (2012) Genetic predictions of prion disease
469 susceptibility in carnivore species based on variability of the prion gene coding region.
470 PLoS One 7 (12):e50623. doi:10.1371/journal.pone.0050623
471 6. Fernandez-Borges N, Parra B, Vidal E, Erana H, Sanchez-Martin MA, de Castro J,
472 Elezgarai SR, Pumarola M, Mayoral T, Castilla J (2017) Unraveling the key to the
473 resistance of canids to prion diseases. PLoS Pathog 13 (11):e1006716.
474 doi:10.1371/journal.ppat.1006716
475 7. Vidal E, Fernandez-Borges N, Pintado B, Ordonez M, Marquez M, Fondevila D, Torres
476 JM, Pumarola M, Castilla J (2013) Bovine spongiform encephalopathy induces
477 misfolding of alleged prion-resistant species cellular prion protein without altering its
478 pathobiological features. J Neurosci 33 (18):7778-7786. doi:10.1523/JNEUROSCI.0244-
479 13.2013
480 8. Polymenidou M, Trusheim H, Stallmach L, Moos R, Julius C, Miele G, Lenz-Bauer C,
481 Aguzzi A (2008) Canine MDCK cell lines are refractory to infection with human and
482 mouse prions. Vaccine 26 (21):2601-2614. doi:10.1016/j.vaccine.2008.03.035
483 9. Khan MQ, Sweeting B, Mulligan VK, Arslan PE, Cashman NR, Pai EF, Chakrabartty
484 A (2010) Prion disease susceptibility is affected by beta-structure folding propensity and

485 local side-chain interactions in PrP. Proc Natl Acad Sci U S A 107 (46):19808-19813.
486 doi:10.1073/pnas.1005267107
487 10. Chianini F, Fernandez-Borges N, Vidal E, Gibbard L, Pintado B, de Castro J, Priola
488 SA, Hamilton S, Eaton SL, Finlayson J, Pang Y, Steele P, Reid HW, Dagleish MP,
489 Castilla J (2012) Rabbits are not resistant to prion infection. Proc Natl Acad Sci U S A
490 109 (13):5080-5085. doi:10.1073/pnas.1120076109
491 11. Bian J, Khaychuk V, Angers RC, Fernandez-Borges N, Vidal E, Meyerett-Reid C,
492 Kim S, Calvi CL, Bartz JC, Hoover EA, Agrimi U, Richt JA, Castilla J, Telling GC (2017)
493 Prion replication without host adaptation during interspecies transmissions. Proc Natl
494 Acad Sci U S A 114 (5):1141-1146. doi:10.1073/pnas.1611891114
495 12. Nonno R, Di Bari MA, Cardone F, Vaccari G, Fazzi P, Dell'Omo G, Cartoni C,
496 Ingrosso L, Boyle A, Galeno R, Sbriccoli M, Lipp HP, Bruce M, Pocchiari M, Agrimi U
497 (2006) Efficient transmission and characterization of Creutzfeldt-Jakob disease strains in
498 bank voles. PLoS Pathog 2 (2):e12. doi:10.1371/journal.ppat.0020012
499 13. Agrimi U, Nonno R, Dell'Omo G, Di Bari MA, Conte M, Chiappini B, Esposito E,
500 Di Guardo G, Windl O, Vaccari G, Lipp HP (2008) Prion protein amino acid determinants
501 of differential susceptibility and molecular feature of prion strains in mice and voles.
502 PLoS Pathog 4 (7):e1000113. doi:10.1371/journal.ppat.1000113
503 14. Pirisinu L, Di Bari MA, D'Agostino C, Marcon S, Riccardi G, Poleggi A, Cohen ML,
504 Appleby BS, Gambetti P, Ghetti B, Agrimi U, Nonno R (2016) Gerstmann-Straussler-
505 Scheinker disease subtypes efficiently transmit in bank voles as genuine prion diseases.
506 Sci Rep 6:20443. doi:10.1038/srep20443
507 15. Watts JC, Giles K, Patel S, Oehler A, DeArmond SJ, Prusiner SB (2014) Evidence
508 that bank vole PrP is a universal acceptor for prions. PLoS Pathog 10 (4):e1003990.
509 doi:10.1371/journal.ppat.1003990

16. Di Bari MA, Nonno R, Castilla J, D'Agostino C, Pirisinu L, Riccardi G, Conte M, Richt J, Kunkle R, Langeveld J, Vaccari G, Agrimi U (2013) Chronic wasting disease in bank voles: characterisation of the shortest incubation time model for prion diseases. PLoS Pathog 9 (3):e1003219. doi:10.1371/journal.ppat.1003219

17. Cartoni C, Schinina ME, Maras B, Nonno R, Vaccari G, Di Baria MA, Conte M, Liu QG, Lu M, Cardone F, Windl O, Pocchiari M, Agrimi U (2005) Identification of the pathological prion protein allotypes in scrapie-infected heterozygous bank voles (Clethrionomys glareolus) by high-performance liquid chromatography-mass spectrometry. J Chromatogr A 1081 (1):122-126

18. Watts JC, Giles K, Stohr J, Oehler A, Bhardwaj S, Grillo SK, Patel S, DeArmond SJ, Prusiner SB (2012) Spontaneous generation of rapidly transmissible prions in transgenic mice expressing wild-type bank vole prion protein. Proc Natl Acad Sci U S A 109 (9):3498-3503. doi:10.1073/pnas.1121556109

19. Will RG, Ironside JW (2017) Sporadic and Infectious Human Prion Diseases. Cold Spring Harb Perspect Med 7 (1). doi:10.1101/cshperspect.a024364

20. Otero A, Bolea R, Hedman C, Fernandez-Borges N, Marin B, Lopez-Perez O, Barrio T, Erana H, Sanchez-Martin MA, Monzon M, Badiola JJ, Castilla J (2017) An Amino Acid Substitution Found in Animals with Low Susceptibility to Prion Diseases Confers a Protective Dominant-Negative Effect in Prion-Infected Transgenic Mice. Mol Neurobiol. doi:10.1007/s12035-017-0832-8

21. Castilla J, Gutierrez-Adan A, Brun A, Doyle D, Pintado B, Ramirez MA, Salguero FJ, Parra B, San Segundo FD, Sanchez-Vizcaino JM, Rogers M, Torres JM (2004) Subclinical bovine spongiform encephalopathy infection in transgenic mice expressing porcine prion protein. J Neurosci 24 (21):5063-5069. doi:Doi 10.1523/Jneurosci.5400-03.2004

535 22. Fernandez-Borges N, Di Bari MA, Erana H, Sanchez-Martin M, Pirisinu L, Parra B,
536 Elezgarai SR, Vanni I, Lopez-Moreno R, Vaccari G, Venegas V, Charco JM, Gil D,
537 Harrathi C, D'Agostino C, Agrimi U, Mayoral T, Requena JR, Nonno R, Castilla J (2018)
538 Cofactors influence the biological properties of infectious recombinant prions. Acta
539 Neuropathol 135 (2):179-199. doi:10.1007/s00401-017-1782-y
540 23. Fraser H, Dickinson AG (1968) The sequential development of the brain lesion of
541 scrapie in three strains of mice. J Comp Pathol 78 (3):301-311
542 24. Schulz-Schaeffer WJ, Tschoke S, Kranefuss N, Drose W, Hause-Reitner D, Giese A,
543 Groschup MH, Kretzschmar HA (2000) The paraffin-embedded tissue blot detects
544 PrP(Sc) early in the incubation time in prion diseases. Am J Pathol 156 (1):51-56.
545 doi:10.1016/S0002-9440(10)64705-0
546 25. Andreoletti O, Simon S, Lacroux C, Morel N, Tabouret G, Chabert A, Lugan S,
547 Corbiere F, Ferre P, Foucras G, Laude H, Eychenne F, Grassi J, Schelcher F (2004) PrPSc
548 accumulation in myocytes from sheep incubating natural scrapie. Nat Med 10 (6):591-
549 593. doi:10.1038/nm1055
550 26. Monleon E, Monzon M, Hortells P, Bolea R, Acin C, Vargas F, Badiola JJ (2005)
551 Approaches to Scrapie diagnosis by applying immunohistochemistry and rapid tests on
552 central nervous and lymphoreticular systems. J Virol Methods 125 (2):165-171.
553 doi:10.1016/j.jviromet.2005.01.013
554 27. Castilla J, Saa P, Hetz C, Soto C (2005) In vitro generation of infectious scrapie
555 prions. Cell 121 (2):195-206. doi:10.1016/j.cell.2005.02.011
556 28. Pirisinu L, Marcon S, Di Bari MA, D'Agostino C, Agrimi U, Nonno R (2013)
557 Biochemical characterization of prion strains in bank voles. Pathogens 2 (3):446-456.
558 doi:10.3390/pathogens2030446

559 29. Westaway D, Zuliani V, Cooper CM, Da Costa M, Neuman S, Jenny AL, Detwiler L,
560 Prusiner SB (1994) Homozygosity for prion protein alleles encoding glutamine-171
561 renders sheep susceptible to natural scrapie. Genes Dev 8 (8):959-969
562 30. Clouscard C, Beaudry P, Elsen JM, Milan D, Dussaucy M, Bounneau C, Schelcher F,
563 Chatelain J, Launay JM, Laplanche JL (1995) Different allelic effects of the codons 136
564 and 171 of the prion protein gene in sheep with natural scrapie. J Gen Virol 76 (Pt
565 8):2097-2101. doi:10.1099/0022-1317-76-8-2097
566 31. Kaneko K, Zulianello L, Scott M, Cooper CM, Wallace AC, James TL, Cohen FE,
567 Prusiner SB (1997) Evidence for protein X binding to a discontinuous epitope on the
568 cellular prion protein during scrapie prion propagation. Proc Natl Acad Sci U S A 94
569 (19):10069-10074
570 32. Zulianello L, Kaneko K, Scott M, Erpel S, Han D, Cohen FE, Prusiner SB (2000)
571 Dominant-negative inhibition of prion formation diminished by deletion mutagenesis of
572 the prion protein. J Virol 74 (9):4351-4360
573 33. Perrier V, Kaneko K, Safar J, Vergara J, Tremblay P, DeArmond SJ, Cohen FE,
574 Prusiner SB, Wallace AC (2002) Dominant-negative inhibition of prion replication in
575 transgenic mice. Proc Natl Acad Sci U S A 99 (20):13079-13084.
576 doi:10.1073/pnas.182425299
577 34. Geoghegan JC, Miller MB, Kwak AH, Harris BT, Supattapone S (2009) Trans-
578 dominant inhibition of prion propagation in vitro is not mediated by an accessory
579 cofactor. PLoS Pathog 5 (7):e1000535. doi:10.1371/journal.ppat.1000535
580 35. Palmer MS, Dryden AJ, Hughes JT, Collinge J (1991) Homozygous prion protein
581 genotype predisposes to sporadic Creutzfeldt-Jakob disease. Nature 352 (6333):340-342.
582 doi:10.1038/352340a0

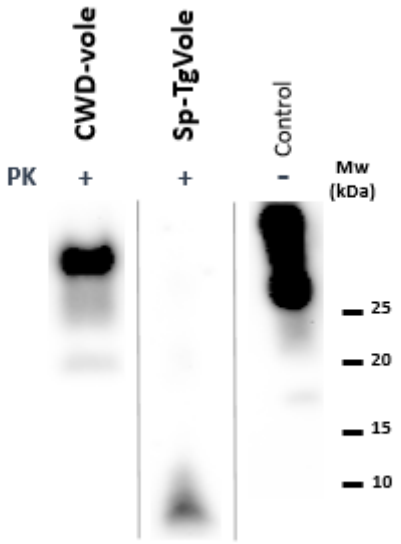
583 36. Shibuya S, Higuchi J, Shin RW, Tateishi J, Kitamoto T (1998) Codon 219 Lys allele
584 of PRNP is not found in sporadic Creutzfeldt-Jakob disease. Ann Neurol 43 (6):826-828.
585 doi:10.1002/ana.410430618
586 37. Asante EA, Smidak M, Grimshaw A, Houghton R, Tomlinson A, Jeelani A,
587 Jakubcova T, Hamdan S, Richard-Londt A, Linehan JM, Brandner S, Alpers M, Whitfield
588 J, Mead S, Wadsworth JD, Collinge J (2015) A naturally occurring variant of the human
589 prion protein completely prevents prion disease. Nature 522 (7557):478-481.
590 doi:10.1038/nature14510
591 38. Hill AF, Joiner S, Linehan J, Desbruslais M, Lantos PL, Collinge J (2000) Species-
592 barrier-independent prion replication in apparently resistant species. Proc Natl Acad Sci
593 U S A 97 (18):10248-10253
594 39. Collinge J (2001) Prion diseases of humans and animals: their causes and molecular
595 basis. Annu Rev Neurosci 24:519-550. doi:10.1146/annurev.neuro.24.1.519
596 40. Atarashi R, Sim VL, Nishida N, Caughey B, Katamine S (2006) Prion strain-
597 dependent differences in conversion of mutant prion proteins in cell culture. J Virol 80
598 (16):7854-7862. doi:10.1128/JVI.00424-06
599 41. Striebel JF, Race B, Meade-White KD, LaCasse R, Chesebro B (2011) Strain specific
600 resistance to murine scrapie associated with a naturally occurring human prion protein
601 polymorphism at residue 171. PLoS Pathog 7 (9):e1002275.
602 doi:10.1371/journal.ppat.1002275
603 42. Houston F, Goldmann W, Chong A, Jeffrey M, Gonzalez L, Foster J, Parnham D,
604 Hunter N (2003) Prion diseases: BSE in sheep bred for resistance to infection. Nature 423
605 (6939):498. doi:10.1038/423498a
606 43. Di Bari MA, Chianini F, Vaccari G, Esposito E, Conte M, Eaton SL, Hamilton S,
607 Finlayson J, Steele PJ, Dagleish MP, Reid HW, Bruce M, Jeffrey M, Agrimi U, Nonno R

608 (2008) The bank vole (Myodes glareolus) as a sensitive bioassay for sheep scrapie. J Gen
609 Virol 89 (Pt 12):2975-2985. doi:10.1099/vir.0.2008/005520-0
610 44. Asante EA, Linehan JM, Smidak M, Tomlinson A, Grimshaw A, Jeelani A,
611 Jakubcova T, Hamdan S, Powell C, Brandner S, Wadsworth JD, Collinge J (2013)
612 Inherited prion disease A117V is not simply a proteinopathy but produces prions
613 transmissible to transgenic mice expressing homologous prion protein. PLoS Pathog 9
614 (9):e1003643. doi:10.1371/journal.ppat.1003643
615 45. Fernandez-Borges N, Erana H, Elezgarai SR, Harrathi C, Gayosso M, Castilla J
616 (2013) Infectivity versus Seeding in Neurodegenerative Diseases Sharing a Prion-Like
617 Mechanism. Int J Cell Biol 2013:583498. doi:10.1155/2013/583498
618 46. Prusiner SB, Scott M, Foster D, Pan KM, Groth D, Mirenda C, Torchia M, Yang SL,
619 Serban D, Carlson GA, et al. (1990) Transgenetic studies implicate interactions between
620 homologous PrP isoforms in scrapie prion replication. Cell 63 (4):673-686
621 47. Hope J, Morton LJ, Farquhar CF, Multhaup G, Beyreuther K, Kimberlin RH (1986)
622 The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge
623 distribution and N-terminal protein sequence as predicted for the normal brain protein
624 (PrP). EMBO J 5 (10):2591-2597
625 48. Priola SA, Caughey B, Race RE, Chesebro B (1994) Heterologous PrP molecules
626 interfere with accumulation of protease-resistant PrP in scrapie-infected murine
627 neuroblastoma cells. J Virol 68 (8):4873-4878
628 49. Jahandideh S, Jamalan M, Faridounnia M (2015) Molecular dynamics study of the
629 dominant-negative E219K polymorphism in human prion protein. J Biomol Struct Dyn
630 33 (6):1315-1325. doi:10.1080/07391102.2014.945486

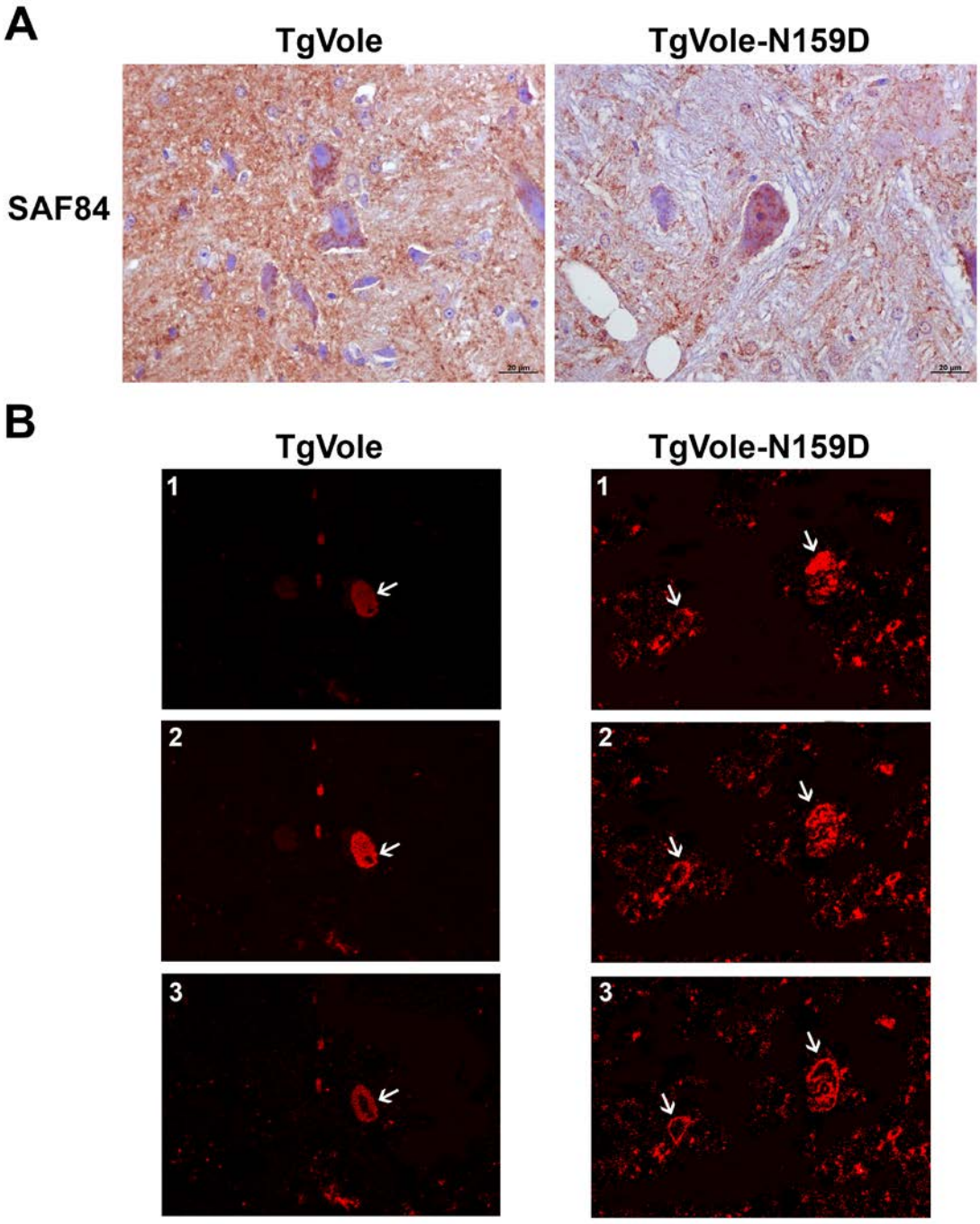
631 50. Horiuchi M, Priola SA, Chabry J, Caughey B (2000) Interactions between
632 heterologous forms of prion protein: binding, inhibition of conversion, and species
633 barriers. Proc Natl Acad Sci U S A 97 (11):5836-5841. doi:10.1073/pnas.110523897
634 51. Seelig DM, Goodman PA, Skinner PJ (2016) Potential approaches for heterologous
635 prion protein treatment of prion diseases. Prion 10 (1):18-24.
636 doi:10.1080/19336896.2015.1123372
637 52. Lee CI, Yang Q, Perrier V, Baskakov IV (2007) The dominant-negative effect of the
638 Q218K variant of the prion protein does not require protein X. Protein Sci 16 (10):2166-
639 2173. doi:10.1110/ps.072954607
640 53. Zhou S, Shi D, Liu X, Liu H, Yao X (2016) Protective V127 prion variant prevents
641 prion disease by interrupting the formation of dimer and fibril from molecular dynamics
642 simulations. Sci Rep 6:21804. doi:10.1038/srep21804

643

Supplementary material



Supplementary Figure 1. Biochemical analyses of the two inoculated prion strains. Biochemical analysis of proteinase-K (PK)-resistant PrP^{Sc} in brain homogenates from I109 TgVole mice inoculated with the CWD-vole strain and from spontaneously generated TgVole I109 PrP^{Sc} (Sp-TgVole strain). Representative brain homogenates were digested with 80 µg/ml and 200 µg/ml of PK, respectively. Samples were run in the different gels, as indicated by the grey line. CWD-vole strain shows a classical electrophoretic pattern. By contrast, the spontaneously generated TgVole I109 prion strain results in accumulation of atypical prions, characterized by an electrophoretic migration pattern similar to that observed in human GSS, with a predominant 7–10-kDa PK-resistant band. 12B2 monoclonal antibody (1:2,500). Control, undigested TgVole whole brain homogenate. MW, molecular weight.



Supplementary Figure 2: Histological localization of PrP in TgVole and TgVole-N159D mouse brains. **a** Immunohistochemical detection of PrP in medulla oblongata from a TgVole and a TgVole-N159D mouse using SAF84 monoclonal antibody. Both animals show a normal neuroanatomic distribution of PrP presenting intense immunoreactivity in the neuronal bodies.

b TgVole and TgVole-N159D brain serial optical z-sections by confocal microscopy (x20). To determine the cellular localization of PrP in the transgenic lines used, the fluorescence emission from a TgVole and a TgVole-N159D mouse brain was analyzed by confocal microscopy. The fluorescence emission resulted from excitation with 594-nm laser and was detected using long-pass 615-nm filter. 0,5 µm z-stacks of digital images were captured using Zen 2008 software (Carl Zeiss Microimaging) with 20x (NA 1.3) objective. Both animals present a very intense neuronal staining for PrP, which was detected in the neuronal membrane (arrows). (SAF84 antibody, 1:1000).