1 **A single amino acid substitution, found in mammals with low susceptibility to prion**

2 **diseases, delays propagation of two prion strains in highly susceptible transgenic**

3 **mouse models**

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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.

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

62 prion diseases in humans, and their origin remains unknown, the generation of these

63 models is essential for research. It has been suggested that sporadic prion diseases may

64 be caused by random and stochastic misfolding of PrP^C, resulting in the accumulation of

65 PrP^{Sc} and consequent clinical and neuropathological features associated with prion

66 disorders [19].

67   The canid D163 PrP^C polymorphism is likely the main determinant of Canidae

68 resistance to prion diseases. Indeed, the presence of this single substitution in mouse PrP^C

69 (N158D substitution in mouse PrP^C numbering) prevents prion propagation both *in vitro*

70 and *in vivo* [6]. We previously demonstrated that *in vivo* coexpression of the N158D PrP^C

71 variant and wild-type mouse PrP^C significantly delays disease onset in transgenic mice

72 inoculated with several prion strains of different origins and characteristics [20],

73 suggesting that N158D PrP^C is a promising candidate in the search for proteins with

74 dominant-negative effects against a broad spectrum of prion strains. In the present study,

75 we investigated whether this substitution could also prevent or delay the onset of prion

76 disease in a highly susceptible model. Transgenic mice overexpressing bank vole I109

77 PrP and carrying this specific residue (TgVole-N159D mice), were inoculated with two

78 prion isolates, and the resulting survival times compared with those of transgenic mice

79 expressing comparable levels of bank vole PrP^C (TgVole mice) but lacking this PrP^C

80 residue. To corroborate the ability of this amino acid substitution to confer protection

81 against prion propagation, we used two distinct strains with very different

82 neuropathological and biochemical features. For both inoculated prion strains, survival

83 periods in TgVole-N159D mice were 52–108% longer than those of TgVole mice. These

84 results are in good agreement with our previous findings demonstrating that expression

85 of this specific amino acid substitution, even when expressed in a PrP^C highly susceptible

86 to misfolding, interferes with prion propagation and delays the onset of disease caused by

87 multiple, distinct prion strains.

Materials and Methods

Ethics statement

90   All procedures involving animals were approved by the University of Zaragoza's

91 Ethics Committee for Animal Experiments (permit number PI32/13) and were performed

92 in accordance with recommendations for the care and use of experimental animals and

93 with Spanish law (R.D. 1201/05).

Inoculation of transgenic mice and sample processing

95   Two different transgenic mouse models were used in the present study: transgenic

96 mice expressing ∼3-4x the I109 polymorphic variant of bank vole PrP and carrying the

97 critical dog amino acid substitution (I109-N159D PrP^C), hereafter referred to as TgVole-

98 N159D mice, and mice overexpressing ∼3-4x bank vole I109 PrP^C, hereafter referred to

99 as TgVole mice, which were used as controls. The murine *PRNP* promoter was used for

100 both I109-N159D and I109 PrP^C expression, in a murine *Prnp$^{0/0}$* background. Mice were

101 generated and characterized as previously described [21].

102   Mice were anesthetized with isoflurane and intracerebrally inoculated (right

103 cerebral hemisphere) with 20 µl of a 1 % brain homogenate. Both TgVole-N159D and

104 TgVole mice were inoculated with one of two different isolates: CWD-vole, a CWD

105 strain adapted to bank voles that contains I109 PrP and is characterized by very short

106 survival times [16]; and an atypical prion isolate (Sp-TgVole isolate) of spontaneous

107 origin. Sp-TgVole inoculum was obtained from brain homogenates from TgVole mice

108 [22] that were sacrificed at 182±5 days of age after developing a spontaneous

109 neurodegenerative disorder linked to the overexpression of the I109 variant of bank vole

110 PrP [18] (Supplementary Fig. 1). Intracerebral injections were performed using a 50-µl

111 precision syringe and a 25-G needle. After inoculation, mice received a subcutaneous

112 injection of buprenorphine (0.3 mg/kg) to induce analgesia.

113 Mice were monitored for the onset of neurologic signs after inoculation, sacrificed

114 by cervical dislocation upon detection of clinical signs of terminal disease (*i.e.,* severe

115 locomotor disorders, poor body condition, and any signs of impaired feeding ability), and

116 their brains collected. Coronal sections were cut at the level of the frontal cortex and

117 medulla oblongata and immediately stored at -80 ºC for biochemical analyses. The

118 remaining brain tissue was stored in 10 % formalin fixative for histological analyses.

119 **Histopathological evaluation**

120 Formalin-fixed brains were sectioned transversely at four standard levels for

121 neuropathological analyses of the following brain areas: frontal cortex (Fc), septal area

122 (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht),

123 mesencephalon (Mes), cerebellar cortex (Cbl), and medulla oblongata (Mo) [23].

124 Formalin-fixed brain tissues were embedded in paraffin wax, and 4-µm-thick tissue

125 sections were mounted on microscope slides for hematoxylin-eosin staining. The

126 intensity and distribution of spongiform changes were blindly evaluated using an optical

127 microscope (Zeiss Axioskop 40) and semiquantitatively scored on a scale of 0 (absence

128 of lesions) to 5 (high intensity lesions) in each of the aforementioned brain regions.

129 **Analysis of PrP^{Sc} deposition**

130 The detection of PrP^{Sc} deposition in paraffin-embedded brains was performed

131 using the paraffin-embedded tissue (PET) blot technique, as previously described [24,25].

132 PrP^{Sc} was detected by incubation with the Sha31 primary monoclonal antibody (1:8,000;

133   SPI-Bio), followed by an alkaline phosphatase-coupled goat anti-mouse antibody (1:500;

134   Dako). Immunolabeling was visualized using the NBT/BCIP substrate chromogen (nitro

135   blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate; Sigma-Aldrich). The presence,

136   intensity, and distribution of PrP^{Sc} aggregates were evaluated using a Zeiss Stemi DV4

137   stereomicroscope and semiquantitatively scored as described for spongiform lesions.

138   The distribution of PrP^{Sc} deposition was also analyzed by immunohistochemistry

139   using a previously described protocol [26], with some modifications. Paraffin-embedded

140   sections were pre-incubated with 98 % formic acid for 5 min and underwent hydrated

141   autoclaving in citrate buffer for 20 min at 96 ºC. Peroxidase activity was blocked for 5

142   min using a peroxidase blocking reagent (Dako). Immunodetection of PrP^{Sc} was achieved

143   by incubation with 6H4 monoclonal antibody (1:100, Prionics) followed by an anti-mouse

144   Envision polymer (Dako). Sections were subsequently incubated with DAB

145   (diaminobenzidine, Dako) and counterstained with hematoxylin.

Biochemical analysis of inoculated strains

147   Proteinase K (PK) resistant PrP^{Sc} was detected and characterized in both the

148   CWD-vole and Sp-TgVole inocula before inoculation. To this end, 10 % brain

149   homogenates from CWD-vole I109 inoculated animals (CWD-vole strain) were

150   incubated with 80 µg/ml PK for 1 h at 42 ºC with constant agitation (450 rpm), as

151   previously described [27]. Biochemical characterization of spontaneously generated

152   TgVole I109 prions (Sp-TgVole strain) was also carried out as reported previously [28].

153   Briefly, brain homogenates (20% w/v) from clinically diseased animals were mixed with

154   an equal volume of 100 mM Tris-HCl + 4 % sarkosyl and incubated for 30 min at 37 ºC.

155   Homogenates were then digested with 200 µg/ml PK (Sigma-Aldrich) for 1 h at 55 ºC

156   with gentle agitation. Aliquots of samples were mixed with an equal volume of

157 isopropanol/butanol (1:1 v/v) and centrifuged for 5 min at 20,000 × g. Supernatants were

158 discarded and pellets were re-suspended in denaturing sample buffer (NuPage). Both

159 CWD-vole and Sp-TgVole inocula were analyzed by Western blotting using the 12B2

160 antibody (1:2,500), which recognizes the 89–93 epitopes of bank vole PrP.

161 **Histological analysis of PrP distribution**

162 The histological localization and distribution of cellular PrP in the brains of both

163 TgVole-N159D and TgVole mice was analyzed by immunohistochemistry as previously

164 described [20]. Briefly, paraffin-embedded brain sections were incubated with a 1%

165 peroxidase solution for 20 min followed by hydrated autoclaving at 100°C in citrate

166 buffer for 30 min. PrP immunodetection was performed overnight at 4°C using SAF84

167 (1:1,000; Cayman Chemical) antibody. The anti-mouse Envision polymer (Dako) was

168 used as the visualization system and DAB (diaminobenzidine, Dako) as the chromogen.

169 The cellular localization of PrP was further analyzed in the brains of TgVole-

170 N159D and TgVole mice using immunofluorescence and confocal imaging. The

171 immunofluorescence staining was performed as described previously [20]. Paraffin-

172 embedded tissue sections from TgVole-N159D and TgVole mice were pre-treated with

173 1% peroxidase for 30 min. Sections were subsequently permeabilized with 0,1% Triton

174 X-100 for 3 h at room temperature and subjected to hydrated autoclaving.

175 Immunodetection was carried out with SAF84 antibody (1:1000) followed by a goat anti-

176 mouse IgG biotin conjugate (1:100; Invitrogen) and an Alexa fluor 594 streptavidin

177 conjugate (1:1000; Invitrogen). Sections were observed under a Zeiss laser-scanning

178 confocal microscope LSM 510 (Carl Zeiss MicroImaging).

179 **Data analysis**

180   Survival times were analyzed using the Kaplan-Meier method and the survival

181   curves for mice carrying the N159D substitution were compared with those of controls

182   using the log rank test ($\alpha = 0.05$). Differences in histopathological and PrP^{Sc} deposition

183   profiles between transgenic mouse models were analyzed using the nonparametric Mann-

184   Whitney U-test, with p-values <0.05 considered significant. GraphPad Prism version 6.0

185   (GraphPad Software, La Jolla, CA, USA) was used for data analysis and to generate

186   Kaplan Meier curves and histopathology graphs.

187   **Results**

188   **Expression of the N159D PrP^C substitution markedly increases survival time in**

189   **mice challenged with the CWD-vole and Sp-TgVole strains**

190   Transgenic mice expressing bank vole I109 PrP^C and carrying the prion

191   resistance-associated N159D amino acid substitution (TgVole-N159D mice) were

192   intracerebrally inoculated with either the classical CWD-vole strain or the atypical Sp-

193   TgVole strain (Supplementary Fig. 1). As controls, TgVole mice expressing comparable

194   levels of bank vole I109 PrP^C (∼3-4×) were challenged with the same isolates. Both

195   transgenic lines present a normal cellular distribution of PrP through the brain and show

196   a good expression of PrP in the cellular membrane (Supplementary Fig. 2). After

197   challenge with the CWD-vole strain, survival time in TgVole-N159D mice was 108 %

198   longer than that of control TgVole mice, in which the mean survival time was 61±4 days

199   post-inoculation (dpi). In TgVole-N159D mice inoculated with the Sp-TgVole isolate,

200   survival time was 52 % longer than that of control TgVole mice (Table 1).

201   In both the TgVole and TgVole-N159D transgenic mice, overexpression of bank

202   vole I109 PrP^C (∼3-4x) leads to the development of a spontaneous neurodegenerative

203   disorder. However, TgVole-N159D mice develop the spontaneous form of the disease

204 showing an increase in survival time of 60 % relative to non-inoculated TgVole animals

205 (Table 1). This relative increase in survival is comparable to that observed in TgVole-

206 N159D mice inoculated with the Sp-TgVole strain. For both inoculated strains, survival

207 time was significantly longer in TgVole-N159D versus TgVole mice (Fig. 1). Significant

208 differences in survival were obtained between TgVole-N159D and TgVole mice for both

209 strains inoculated and between non-inoculated, spontaneously sick TgVole-N159D and

210 TgVole mice (Fig. 1).

211 Despite the significant delay in disease onset, TgVole-N159D mice exhibited

212 clinical signs of neurodegeneration identical to those seen in TgVole mice. Animals

213 inoculated with the CWD-vole strain exhibited dorsal kyphosis, circling behavior,

214 cachexia, and tremor. By contrast, those inoculated with the Sp-TgVole isolate showed

215 mild kyphosis and rapidly progressing ataxia.

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

217

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%

218

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

220   [b] Survival times were calculated as the number of days between inoculation and sacrifice of mice

221   inoculated with CWD-vole or Sp-TgVole isolates, provided that the mouse developed clinical

222   signs consistent with a TSE. Survival times are expressed as the mean (± SEM) number of dpi.

223   [c] For non-inoculated mice survival times were considered as the mean age at which the animals

224   sporadically developed clinical signs consistent with a TSE and were euthanized. The age at

225   which animals succumbed to disease is expressed as mean (± SEM) days of age.

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

227   [d] Prolongation of survival time in TgVole-N159D mice is expressed as the percentage increase in

228   mean survival time relative to TgVole mice.

229   [e] One animal from the group inoculated with CWD-vole isolate group died during the initial stages

230   of the study due to a concomitant disease. This animal exhibited no spongiform lesions or PrP^{Sc}

231   deposits and was excluded from all analyses.

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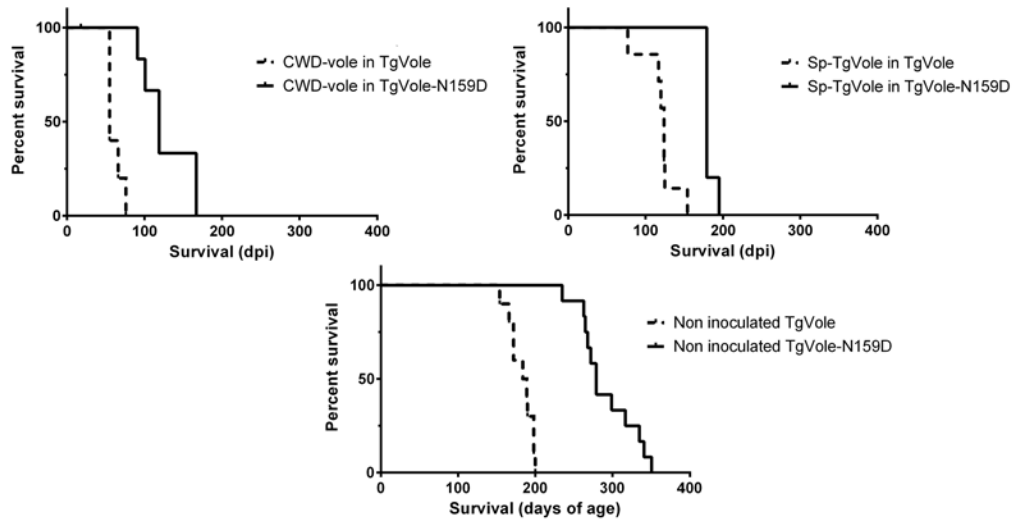
Figure 1. Survival curves for TgVole and TgVole-N159D mice (non-inoculated or after challenge with CWD-vole or Sp-TgVole isolates). Analysis of survival curves using the log rank test (α=0.050) revealed significant differences between TgVole and TgVole-N159D mice after inoculation with either CWD-vole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

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

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

249   Semi-quantitative analysis of spongiosis and prion protein deposition patterns in

250   9 brain areas revealed no significant differences between TgVole-N159D and TgVole

251   mice inoculated with the same isolate (Fig. 2). All mice inoculated with the CWD-vole

252   strain showed moderate spongiform changes and discretely distributed PrP^{Sc} deposits,

253   which were particularly conspicuous in the thalamus. In other brain regions, such as the

254   hippocampus, spongiosis was minimal and PrP^{Sc} deposition scores were very low (Figs.

255   2 and 3). These neuropathological features coincide with those described in I109 bank

256   voles infected with the same strain, in which prominent involvement of the thalamus has

257   been reported [16]. Moreover, both TgVole-N159D and TgVole mice inoculated with the

258   atypical Sp-TgVole isolate exhibited severe vacuolar changes in the cortex (Fc and Tc)

259   and in the hippocampus, in which we observed the most intense PrP^{Sc} deposition for this

260   isolate (Figs. 2 and 3). To verify that the Sp-TgVole strain maintained its

261   neuropathological characteristics after experimental transmission to TgVole and TgVole-

262   N159D mice, we performed a histopathological assessment of brain samples from TgVole

263   and TgVole-N159D animals that spontaneously developed the disease at ∼182 and ∼292

264   days of age, respectively. The lesion profiles and PrP^{Sc} deposition patterns in these

265   animals were almost identical to one another and to those of mice inoculated with the Sp-

266   TgVole isolate (Fig. 2).

267   The fact that similar neuropathological features were observed in TgVole and

268   TgVole-N159D mice inoculated with the Sp-TgVole strain, and in non-inoculated

269   TgVole mice, suggests the development of the same spontaneous disease in these

270   inoculated mice in an accelerated or induced manner.
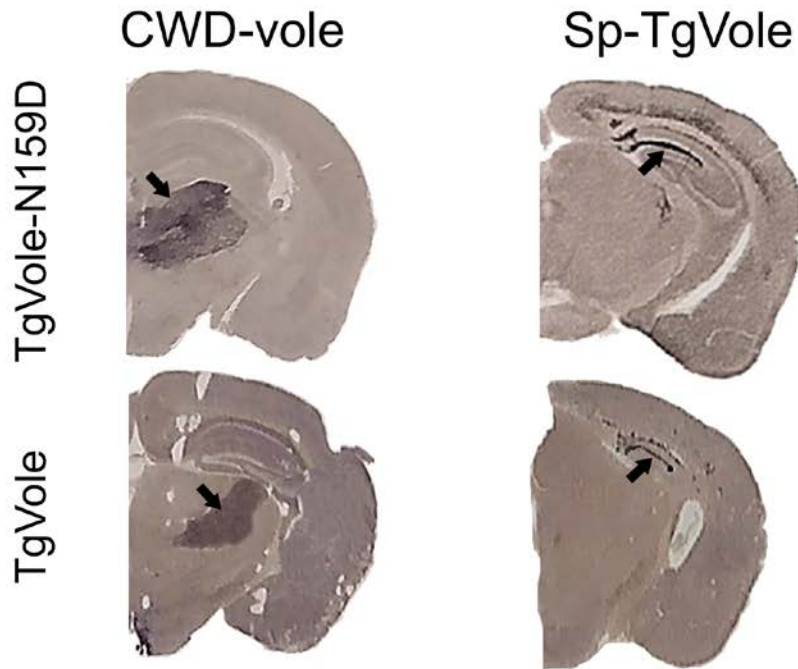
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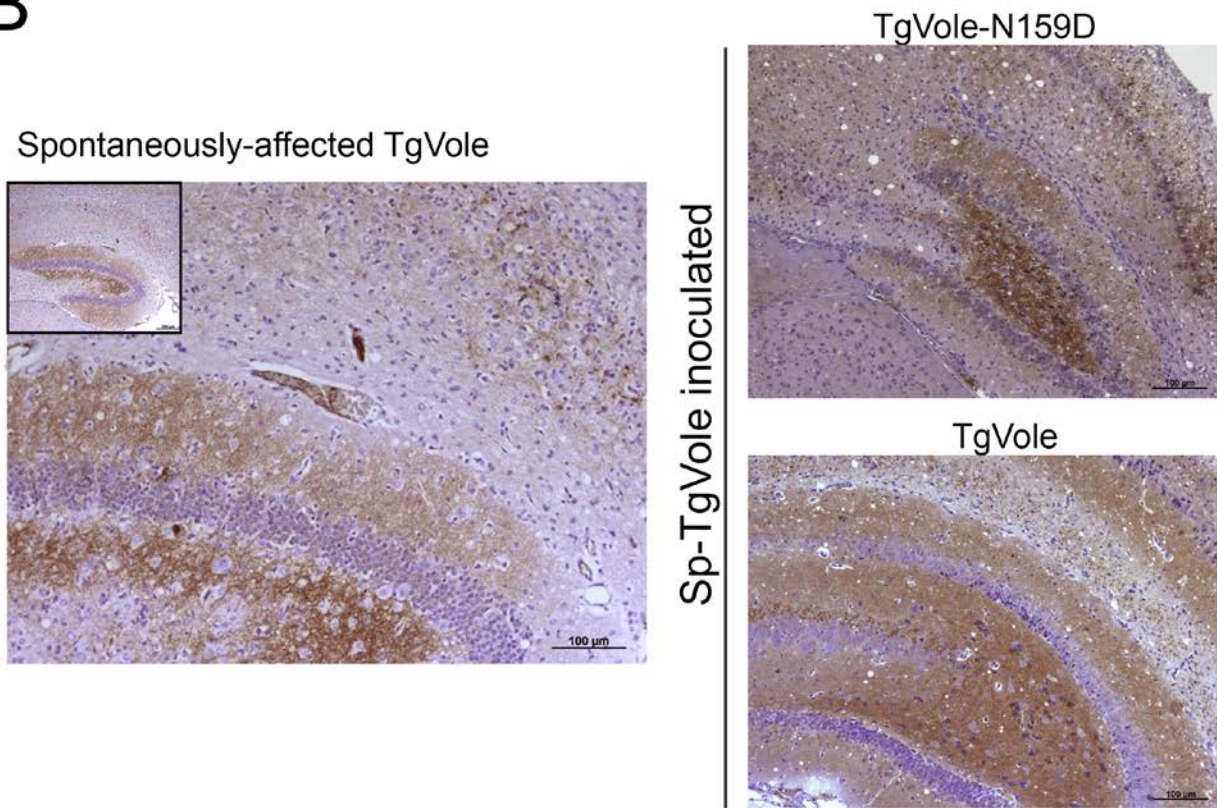
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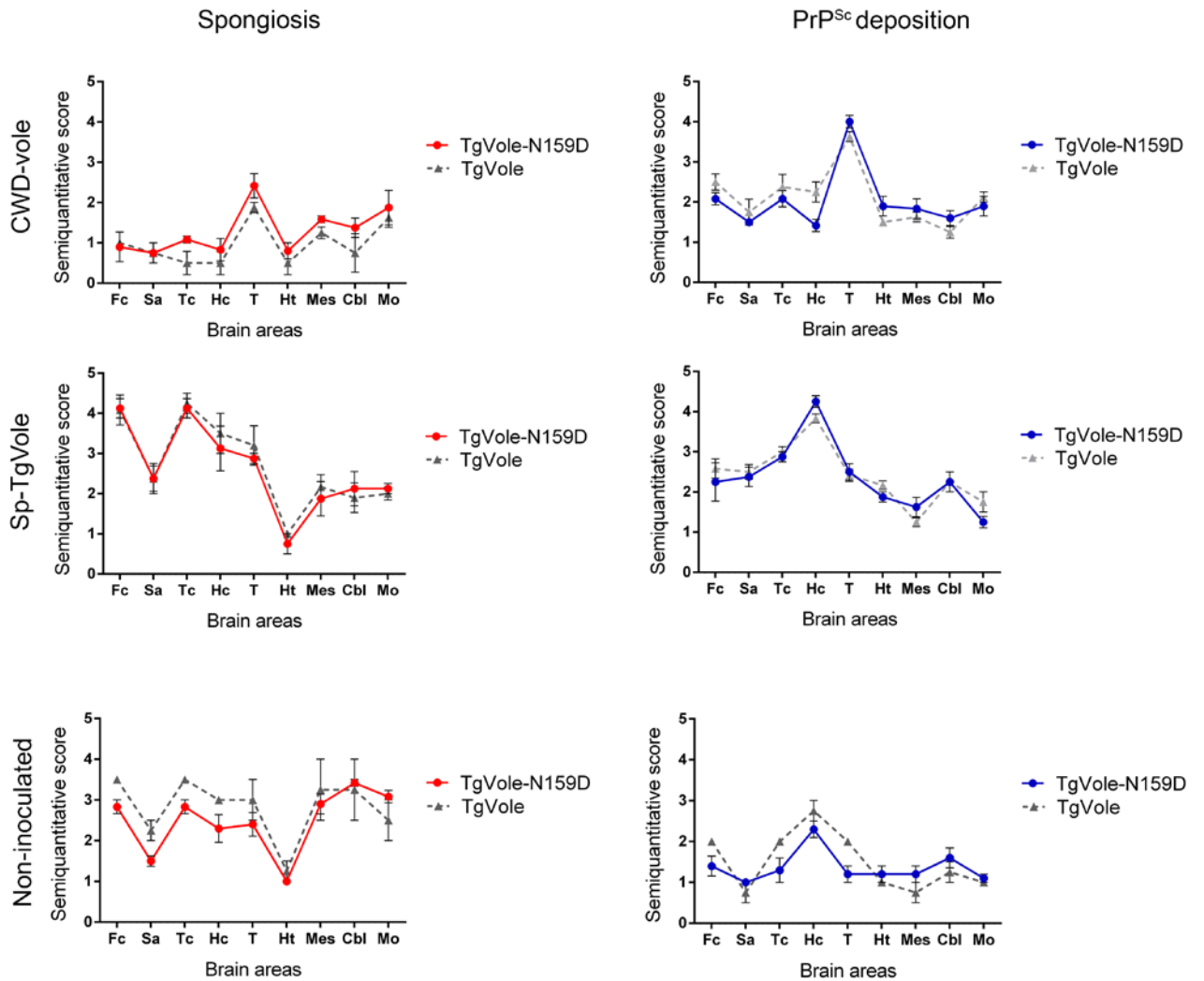
Figure 2. Spongiosis and PrP^Sc deposition profiles in the brains of TgVole and TgVole-N159D mice inoculated with CWD-vole or Sp-TgVole prion isolates, or non inoculated. Spongiosis and PrP^Sc deposition were evaluated semiquantitatively on a scale of 0 (absence of lesions/deposits) to 5 (high intensity 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).

A

CWD-vole   Sp-TgVole

TgVole-N159D

TgVole

B

Spontaneously-affected TgVole

Sp-TgVole inoculated

TgVole-N159D

TgVole

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}

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

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

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

386 neuropathological hallmarks seen in transgenic mice expressing the same transgene.

387 Moreover, as mentioned, expression of the substitution did not impede the spontaneous

388 generation of the prion, and TgVole-N159D mice experimented an increase in survival

389 similar to that of Sp-TgVole inoculated animals. These results agree with the suggestion

390 that the effects of the N159D substitution are strain-dependent, and that both non-

391 inoculated and Sp-TgVole inoculated mice propagate the same strain. In addition, it could

392 be discussed that the delay in the onset of clinical signs observed in Sp-TgVole inoculated

393 TgVole-N159D mice could be associated with the fact that this isolate was obtained from

394 spontaneously sick TgVole animals. Thus, it could be possible that Sp-TgVole isolate is

395 more adapted to the TgVole model. However, we should consider that non-inoculated

396 TgVole-N159D mice show an almost identical delay in the onset of the disease.

397 Moreover, in a parallel experiment, we inoculated brain homogenates from spontaneously

398 sick TgVole-N159D mice in TgVole and TgVole-N159D mice. We observed again that

399 TgVole-N159D inoculated mice presented a survival period ~50% longer than TgVole

400 animals (data not shown); although the isolate was obtained from TgVole-N159D mice

401 and could therefore be better adapted to this model.   We can conclude that while

402 expression of the resistance-associated amino acid change significantly increased survival

403 times, it did not alter the pathological features of the inoculated strains. These results are

404 in agreement with our previous findings [20], and suggest that the delayed appearance of

405 clinical signs in TgVole-N159D mice is not caused by strain modifications resulting from

406 the N159D substitution.

407   The precise molecular mechanisms by which the N159D substitution prolongs

408 survival time in TgVole-N159D mice remain unclear. However, this residue can extend

409 the survival period in both inoculated mice and those that develop spontaneous disease,

410 as evidenced by the significant differences in survival time between TgVole (182±5 days)

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

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

436   in models overexpressing PrPs that are highly susceptible to misfolding. Moreover, the

437   N159D substitution delays the onset of clinical signs of both infectious and spontaneous

438   forms of prion diseases. Together with our previous findings demonstrating a dominant-

439   negative effect of prion protein carrying the N-to-D substitution against a variety of prion

440   strains of different origins [20], we can conclude that this mutation could represent a

441   useful tool to control the propagation of different prion strains when present in the correct

442   PrP background.

452   **Author contribution statement**

453   JC and RB conceived the study; AO, CH, NFB, HE, and BM performed most of the

454   experiments; MASM and RN collaborated in the creation of the transgenic lines used;

455   AO, HE, JJB, RB and JC evaluated the results; AO, RB, JJB, HE, and JC wrote and

456   reviewed the manuscript.

457   **Conflict of interest**

458   The authors declare that they have no conflict of interest.

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644 **Supplementary material**

645
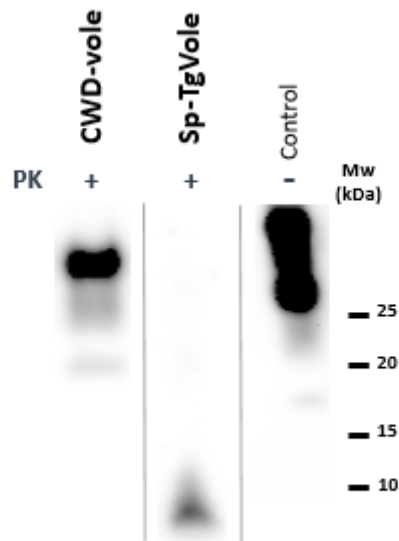
Supplementary Figure 1. Biochemical analyses of the two inoculated prion strains.
Biochemical analysis of proteinase-K (PK)-resistant PrP^{Sc} in brain homogenates from I109 TgVole mice inoculated with the CWD-vole strain and from spontaneously generated TgVole I109 PrP^{Sc} (Sp-TgVole strain). Representative 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.

A SAF84 | TgVole | TgVole-N159D

B | TgVole | TgVole-N159D

657
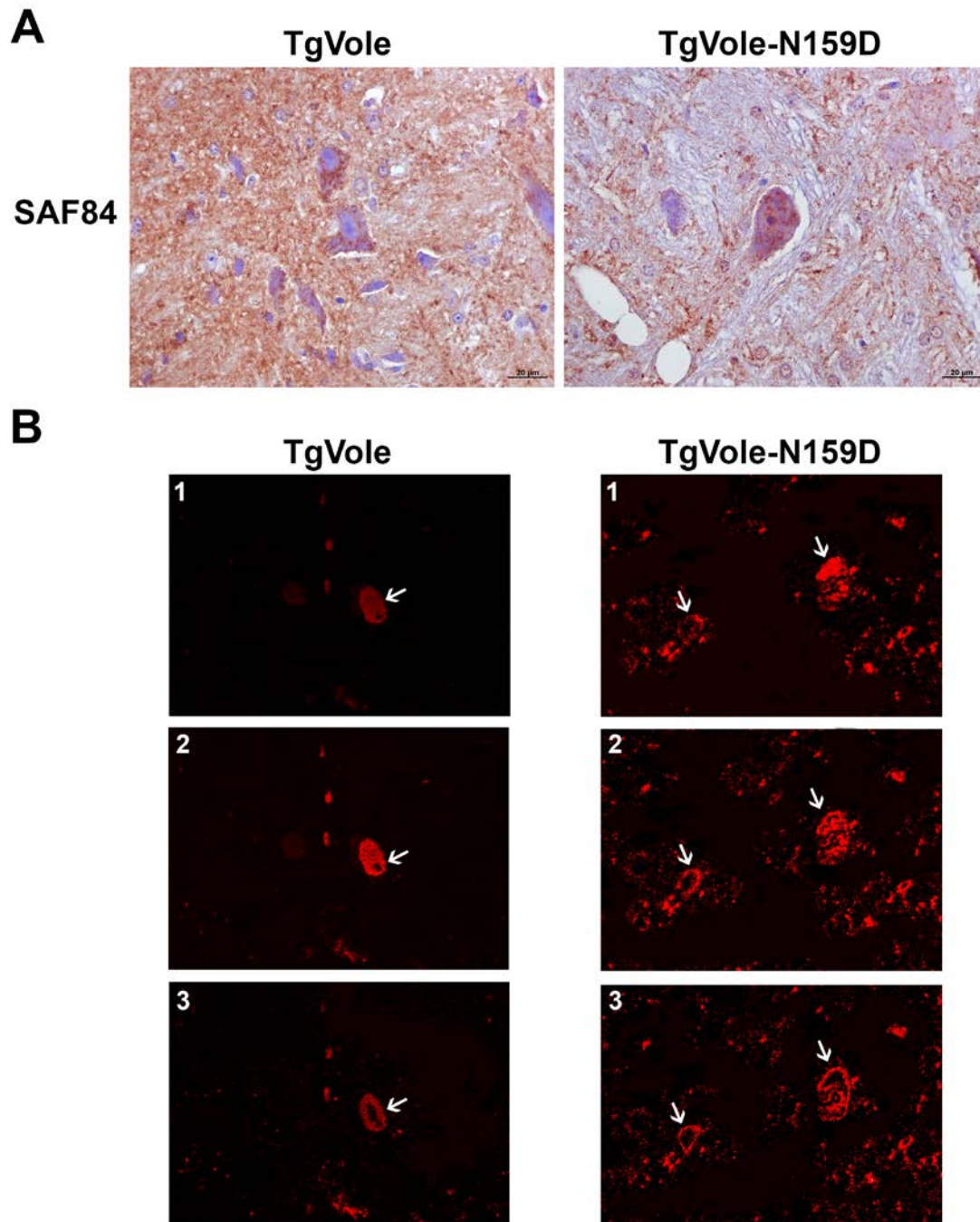
Supplementary Figure 2: Histological localization of PrP in TgVole and TgVole-N159D mouse brains. **a** Immunohistochemical detection of PrP in medulla oblongata from a TgVole and a TgVole-N159D mouse using SAF84 monoclonal antibody. Both animals show a normal neuroanatomic distribution of PrP presenting intense immunoreactivity in the neuronal bodies.

663   b TgVole and TgVole-N159D brain serial optical z-sections by confocal microscopy

664   (x20). To determine the cellular localization of PrP in the transgenic lines used, the

665   fluorescence emission from a TgVole and a TgVole-N159D mouse brain was analyzed

666   by confocal microscopy. The fluorescence emission resulted from excitation with 594-

667   nm laser and was detected using long-pass 615-nm filter. 0,5 µm z-stacks of digital

668   images were captured using Zen 2008 software (Carl Zeiss Microimaging) with 20x (NA

669   1.3) objective. Both animals present a very intense neuronal staining for PrP, which was

670   detected in the neuronal membrane (arrows). (SAF84 antibody, 1:1000).

671