Homozygous R136S mutation in PRNP gene causes recessive inherited early onset prion disease

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Homozygous R136S mutation in *PRNP* gene causes recessive inherited early onset prion disease

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Abstract

Background: More than 40 pathogenic heterozygous *PRNP* mutations causing inherited prion diseases have been identified to date. Recessive inherited prion disease has not been described to date.

Methods: We describe the clinical and neuropathological data of inherited early-onset prion disease caused by the rare *PRNP* homozygous mutation R136S. *In vitro* PrP^{Sc} propagation studies were performed using recombinant-adapted Protein Mysfolding Cyclic Amplification technique. Brain material from two R136S homozygous patients were intracranially inoculated in TgMet129 and TgVal129 transgenic mice to assess the transmissibility of this rare inherited form of prion disease.

Results: The index case presented symptoms of early-onset dementia beginning at the age of 49 and died at the age of 53. Neuropathological evaluation of the proband revealed abundant multicentric PrP plaques and Western blotting revealed a 6-8 kDa protease-resistant, unglycosylated PrP fragment, consistent with a Gerstmann-Sträussler-Scheinker phenotype. Her youngest sibling suffered from progressive cognitive decline, motor impairment and myoclonus with onset in her late 30s and died at the age of 48. Genetic analysis revealed the presence of the R136S mutation in homozygosis in the two affected subjects linked to homozygous methionine at codon 129. One sibling carrying the heterozygous R136S mutation, linked to homozygous methionine at codon 129, is still asymptomatic at the age of 74. The inoculation of human brain homogenates from our index case and an independent case from a Portuguese family with the same mutation in transgenic mice expressing human PrP and in vitro propagation of PrP^{Sc} studies failed to show disease transmissibility.

Conclusion: In conclusion, biallelic R136S substitution is a rare variant that produces inherited early-onset human prion disease with a Gerstmann-Sträussler-Scheinker

neuropathological and molecular signature. Even if the R136S variant is predicted to be "probably damaging", heterozygous carriers are protected, at least from an early onset providing the first evidence for a recessive pattern of inheritance in human prion diseases.

Keywords: gene *PRNP*; GSS; homozygous; neuropathology; prion

Background

Genetic prion diseases represent ~15% of human prion diseases [1] and are caused by genetic variants in the *PRNP* gene, located on the chromosome 20. More than 40 rare variants have been described to cause genetic prion disease. Importantly, these mutations are linked to the polymorphism at codon 129, coding either for methionine or valine, which frequently determines the phenotypic presentation. Genetic prion diseases usually present family history of disease. However in some genetic cases, the familial tree [2] could not identify other cases in the family or suggests incomplete penetrance, which has been attributed to underdiagnosis or low clinical expression during the lifespan [3,4]. Mutations induce misfolding of the cellular prion protein (PrP^{C}), and the accumulation of the disease associated isoform PrP^{Sc} [5,6]. According to the clinic-neuropathological features, inherited prion diseases can be classified as genetic Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker (GSS) syndrome, Fatal Familial Insomnia and Prion diseases associated to octapeptide insertions although there are also some variants presenting an intermediate clinical phenotype or not fitting into these categories.[7] The clinical diagnosis of genetic prion disorders might be challenging, especially in patients with non-prototypical phenotypes. Analysis of the human *PRNP* gene is recommended whenever such as disease form is suspected [8,9].

Up to date, all described pathogenic variants cause the disease in heterozygous carriers, being the disease inherited with an autosomal dominant pattern. Very few individuals have been reported to be homozygous for *PRNP* variants [10-13]. Most of them are homozygous carriers of well-known pathogenic mutations which cause also the disease in heterozygous carriers from areas of high prevalence of the mutation. In 2006, Pacheco et al. [14] reported at the Prion 2006 meeting a Portuguese patient carrying the homozygous R136S variant with family history of disease in two siblings, but not additional information is available in the literature.

In this report, we describe the clinical, neuropathological and biochemical phenotype of genetic prion disease associated with a biallelic R136S substitution in the *PRNP* gene in a Spanish family. We also present the results of *in vivo* and *in vitro* transmissibility and propagation studies of this rare variant.

Materials and methods

Clinical data

The index case and two asymptomatic siblings were studied at the Alzheimer's disease and other cognitive disorders unit at Hospital Clínic de Barcelona, Barcelona, Spain. Clinical information also included data from other centres specialized in cognitive disorders were the patient was evaluated and followed after diagnosis. The youngest sister and affected patient was examined at Hospital of Leon, Leon, Spain. The clinical information associated with other members of the family was obtained from medical records or was provided by the family members themselves.

Genetic studies

PRNP exon 2 was amplified by PCR of genomic DNA extracted from whole peripheral blood as previously described [15] at Hospital Clínic de Barcelona. We used two pathogenic prediction softwares: SIFT [16] and Polymorphism Phenotyping v2 (Polyphen-2 (score 1.0)) [17] to predict the potential pathogenic effect of the observed variant. Copy number variation analysis was performed using Affymetrix SNP6.0 arrays.

Neuropathology and immunoblotting

Neuropathologic examination was performed according to standardized protocols at Neurological Tissue Bank of Hospital Clínic-IDIBAPS in Barcelona, Spain. Right brain

hemisphere was dissected in coronal sections and frozen while left brain hemisphere was fixed by immersion in 4% formalin for three weeks and after fixation and cutting were treated with 98% formic acid for one hour. Upon several water washes, brain material covering at least 30 different brain areas was postfixed in 10% formalin for at least 48 hours and embedded in paraffin. For histologic evaluation, 5-um-thick sections were stained with hematoxylin & eosin and Periodic Acid-Schiff (PAS). Immunohistochemistry (IHC) was performed using the Autostainer System method (Dako) with various antibodies including anti-PrP (12F10; Bertin-bioreagent, France), anti-βA4 (6F/3D, Dako, Glostrup, Denmark), anti-tau (AT8; Thermo Scientific, USA), anti-α-synuclein (5G4; Analytik Jena, Germany), anti-ubiquitin (P4D1; Cell signalling, USA), anti-α-internexin (2E3, Invitrogen, USA) and anti-TDP-43 (2E2-D3, Abnova, Taiwan). Disease evaluation was performed according to international consensus criteria [18].

Samples of frontal, temporal, parietal and occipital cortices, hypothalamus, thalamus and cerebellum were processed for Western Blotting. Human brain tissue was homogenized in 10% (w/v) in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM Tris) at pH 6.9. To detect brain PrP^{res} brain homogenate were subjected to digestion with 2 U/ml of proteinase K (PK) (Roche Diagnostics) at 37°C for 1 hour. After blocking PK activity with phenylmethylsulfonyl fluoride (PMSF, final concentration 3.6 mM), samples were boiled in sample buffer (final concentration: 3% SDS, 4% β-mercaptoethanol, 10% glycerol, 2 mM EDTA, 62.5 mM Tris) for 6 min at 100 °C. Digested samples were loaded into 12% Tris-Glycine gel (Criterion, Bio-Rad). After electrophoretic transference of proteins onto polyvinydene fluoride membranes (Bio-Rad) and after blocking the membrane 1 hour at 37ªC. After electrophoretic transference of proteins onto polyvinylidene fluoride membranes (Bio-Rad) membranes were incubated with 3F4 (1:30.000; Millipore). 3F4 recognizes the 109-112 epitope of the human-PrP^{C} sequence.

Immunocomplexes were detected by 1 hour membrane incubation with horseradish peroxidase conjugated antimouse IgG (GE Healthcare Amersham Biosciences) and development with enhanced chemiluminescence in ECL Select (GE Healthcare Amersham Biosciences). For PrP^{Sc} deglycosylation, N-Linked glycans were removed by using a peptide-N-glycosidase (PNGaseF+) F kit (New England Biolabs) according to the manufacturer's instructions.

In vitro propagation of PrP by PMCA

In vitro PrP^{Sc} propagation studies were performed at the Prion Research Lab at CICbioGune, in Bizkaia, Spain.Two different type of experiments were performed using recombinant-adapted Protein Mysfolding Cyclic Amplification (recPMCA) technique as previously described [19]. In the experiment focused on the in vitro propagation of human rec-PrP^{Sc} using R136 vs. S136 human rec-PrP as substrates, samples were complemented with chicken brain homogenate and seeded with different dilutions of recPMCA-adapted CJD MM1 misfolded rec-PrP^{Sc} and subjected to a unique 24 h round of standard recPMCA. The experiment focused on the evaluation of the spontaneous propensity to misfold of the R136S variant, was performed twice in duplicates of four and compared with other human mutated rec-PrP and wt rec-PrP. Samples were subjected to 15 rounds of recPMCA [20]. Amplified samples were digested with 50-80 µg/ml of PK and analyzed by western blot using monoclonal antibody D18 (1:5,000).

Transmission studies in human PrP transgenic mice

Inoculation of transgenic mice was performed at Centro de Investigación en Sanidad Animal (CISA-INIA), Madrid, Spain. Inocula were prepared from fresh frozen cerebellar tissue from the proband and from one independent case from the Portuguese family [14] carrying the homozygous R136S variant, as 10% (wt/vol) homogenates in 5% glucose distilled water.

Two different mouse models for human-PrP were inoculated: HuPrP-Tg340-Met129 (TgMet129) mouse line expressing the Met129-PrP^{C} variant [21] and HuPrP-Tg362-Val129 mouse line expressing the Val129-PrP^{C} variant (TgVal129) [22]. These transgenic mouse lines express 4-fold and 8-fold the level of PrP^{C} expression in human brain respectively. Six to nine individually identified mice 6–10-week-old were anesthetized and inoculated with 2 mg of 10% brain homogenate in the right parietal lobe by using a 25-gauge disposable hypodermic needle. After inoculation, mice were observed daily and their neurologic status was assessed weekly. At the established experimental endpoint (700 days postinoculation), animals were euthanized. After necropsy, part of the brain was fixed by using immersion in neutral-buffered 10% formalin (4% 2-formaldehyde) and used it for histopathology analysis while the rest of the tissue was frozen at -20°C and used for proteinase K–resistant PrP^{Sc} (PrP^{res}) detection by WB. For each inoculation experiment, mean survival times with standard deviation was calculated as well as the attack rate defined as the proportion of mice that scored positive for PrP^{res} divided by the number of total inoculated mice.

Western blotting in mouse bioassay

Western blotting analysis was done as previously described [23]. Briefly, 175 + 20 mg of frozen mouse brain tissue were homogenized in 5% glucose distilled water in grinding tubes (Bio-Rad) and adjusted to 10% (wt/vol) by using a TeSeE Precess 48TM homogenizer (Bio-Rad). To detect brain PrP^{res} 100 µL of 10% (wt/vol) brain homogenate were subjected to digestion with 40 µg/mL of PK in buffer 5% sarkosyl, 5% Triton X100, 1 M Urea and 16 mM Tris–HCl (pH 9.6) at 60 °C for 15 min. Digested samples were loaded samples into 12% Bis-Tris Gel (Criterion XT; Bio-Rad). After electrophoretic transference of proteins onto polyvinylidene fluoride membranes (Millipore) and blocking overnight with 2% bovine serum albumin blocking buffer, membranes were incubated with 12B2 [24] and Sha31 [25]

mAb at a concentration of 1 μg/mL. 12B2 recognizes the 89-WGQGG-93 epitope of the human-PrP^C sequence while Sha31 recognizes the 145-WEDRYYRE-152 epitope of the human-PrP^C sequence. Immunocomplexes were detected by 1 h membrane incubation with horseradish peroxidase conjugated antimouse IgG (GE Healthcare Amersham Biosciences) and development with enhanced chemiluminescence in ECL Select (GE Healthcare Amersham Biosciences). Images were captured using the ChemiDoc WRS+ System and processed them by using Image Lab 5.2.1 software (both Bio-Rad).

Histological analysis in mouse bioassay

Mouse brain samples were immediately fixed in neutral-buffered 10% formalin (4% 2-formaldehyde) during necropsy, coronally trimmed and processed routinely. After embedded in paraffin-wax, tissues were cut to 4μm thickness, de-waxed and rehydrated by standard procedures. Tissue slides were subjected to two different histological methods: hematoxylin&eosin staining for lesion profile evaluation by standard methods [26] and IHC. Lesion profile evaluation was done by semi-quantitative assessment of spongiform degeneration scoring vacuolization in the following brain areas: cerebral cortex (CCtx); striatum (Stri); hippocampus (Hpp); thalamus (Thal); hypothalamus (Hpth); midbrain (Midb); cerebellar cortex (CbCtx); pons/medulla oblongata (PoMe). For the IHC analysis, tissue slides were subjected to antigen retrieval and quenching of hydrogen peroxide, latter incubated with the Sha31 mAb [25] and subsequent steps of the IHC procedure were performed by a commercial immunoperoxidase technique (Vector-Elite ABC kit, Vector Laboratories) as per manufacturer's instructions finally counterstaining the sections with Harri's haematoxylin.

Results

Clinical characterization and family history

The index patient was a 51-year-old female who was referred to the Neurology department due to progressive memory decline, language problems and subtle changes in behaviour (apathy and childish behaviour) beginning at the age of 49 years (Table 1, Figure 1A, subject II.5). At first examination, her Minimental State examination Score was 28/30. The cognitive evaluation revealed moderate generalized cognitive impairment including mild verbal memory impairment and visuospatial, language and executive disturbances. The neurological examination was normal except for mild dysarthria. MRI examination was unremarkable (Figure 2). Cerebrospinal fluid showed elevated total tau levels (1262 pg/ml), Abeta42 (701pg/ml) within the normal range, slightly elevated phospho-tau (68.5 pg/ml), and reduced p-tau/t-tau ratio (0,054). The 14-3-3 Western-Blot test was negative. The patient deteriorated progressively over the next years with worsening of both cognitive and motor functions developing severe ataxia. She died at the age of 53 after disease duration of 4 years.

The youngest sibling of the index patient (Table 1, Figure 1A subject II-6) also suffered from cognitive decline, motor impairment and myoclonus with onset in her late 30s (38 years). Neurological examination, MRI scan and CSF 14-3-3 were normal at first examination, but the disease progressed and the patient was severely impaired after 10 years of disease. The patient died at the age of 48, but the postmortem neuropathological examination was not performed. The other four siblings were neurologically preserved (present ages from 63 to 74 years) (Figure 1A, II.1-II.4).

The proband's parents were born in a small village in the North-west of Spain, and were probably consanguineous but the degree of kinship is unknown. The mother (Figure 1A, subject I.2) died at the age of 89 years and was neurologically healthy according to their

family members. The father (Figure 1A, subject I.1) died at the age of 84 with dementia with a clinical onset at the age of 75 that had been classified as vascular cognitive impairment by the treating physician.

Genetic studies

The genetic study of the Spanish patient and her youngest sister showed the presence of a substitution of guanine for thymine (AGG to AGT) at codon 136 of the *PRNP* in the two alleles, causing the missense mutation R136S in homozygosis. This was linked to methionine homozygosis at codon 129. No deletions or duplications were detected in the 100kb region which includes the *PRNP* gene.

Of the four asymptomatic siblings, two underwent genetic testing. One asymptomatic 74-year-old sibling (Figure 1A, subject II.1) carried the R136S mutation in heterozygosis, which was also linked to homozygous methionine at codon 129. Another sibling showed a normal *PRNP* sequence and was also methionine homozygous at codon 129.

According to pathogenic prediction software, the R136S variant is considered to be damaging (SIFT) [16], or probably damaging (Polymorphism Phenotyping v2 (Polyphen-2 (score 1.0)) [17].

Neuropathological findings and immunoblotting

The brain of the index patient was removed after death at the Neurological Tissue Bank of the IDIBAPS Biobank in Barcelona. Unfixed brain weight was 1030g. Gross examination showed a moderate diffuse brain atrophy accentuated in the frontal lobes and mild nigral pallor. Histology revealed a severe involvement of grey matter that showed prominent neuronal loss, astrogliosis and microglial proliferation, especially in frontal cortex (Figure 3A). Despite observing a superficial laminar spongiosis, only mild bona fide spongiform

change was detected in grey matter which was characterized by a few small-sized vacuoles in cortical regions and basal ganglia. The most striking feature was the presence of abundant amyloid plaques consisting of multiple densely packed cores (multicentric plaques) that were variably PAS positive and strongly immunoreactive for PrP^{Sc} (12F10 antibody) after pretreating the tissue sections for removal of physiological PrP^{C}. These multicentric plaques were detected in all cortical areas - with less involvement of primary visual cortex, in basal ganglia, thalamic nuclei, and brainstem and in the molecular layer of cerebellar cortex. Moreover, multicentric PrP amyloid plaques induced a moderate neuritic component that was immunoreactive for phosphorylated tau (AT8).

PrP Western blotting displayed a multiple banding pattern with a prominent low molecular weight (mw) fragment of ~8kDa which is absent in the in comparison to the PrP Type 1 (sCJDMM1) control sample run in parallel, in which the lowest mw band detected was ~21kDa (Figure 4). The intensity of the bands was variable among different brain regions and after treatment with PNGaseF+. At visual inspection, the frontal cortex contained the highest amounts of PrP^{Sc}.

In vitro propagation of PrP^{Sc}

In order to evaluate the effect of the R136S mutation in the in vitro propagation of human rec-PrP^{Sc}, human 129M 136R (wt) rec-PrP, human 129M 136S rec-PrP and a mix of both rec-PrPs in ratio 1:1 were used as substrates for recPMCA and seeded with different dilutions of recPMCA-adapted CJD MM1 misfolded rec-PrP (10-1 – 10-8), and subjected to a unique 24 h round of standard recPMCA. No significant differences were found in the propagation ability of the R136 (wt) vs. the mutant R136S rec-PrP or the mix 1:1 of both proteins.

To evaluate the spontaneous propensity of the R136S recPrP to misfold compared to the wt recPrP and other GSS-related mutated recPrP (P105L and A117V), a serial rec-PMCA (15

rounds) was performed (Figure 6). All cases were associated with methionine at codon 129. The wt and the R136S PrP linked to the methionine polymorphic variant at codon 129 did not show spontaneous misfolding, in contrast to the other GSS-related recPrP.

Bioassay in human-PrP transgenic mice

Brain material from the two patients (the Spanish index case and the Portuguese index case) were intracranially inoculated in TgMet129 and TgVal129 transgenic mice (Table 2) to assess the transmissibility of this rare inherited form of prion disease. These mouse lines expressed in homozygosis the Met129-PrP^{C} variant or the Val129-PrP^{C} variant respectively. All mice were sacrificed at the end of their lifespan without showing any clinical sign suspicious of prion disease and remaining uninfected. No PrP^{res} was found in mice brains either by WB nor IHC (data not shown). Vacuolization was no different from the one associated with the natural aging process of non-inoculated control mice of the same transgenic lines by histology (data not shown).

Discussion

We report here that the biallelic R136S *PRNP* mutation causes inherited human prion disease with early onset of symptoms (30s-40s). The same mutation presented in heterozygous state in other family members did not induce the same phenotype. Clinically, the patients presented with early-onset dementia developing motor disturbances at follow-up. Neuropathological examination revealed the presence of abundant multicentric PrP amyloid plaques with mild neuritic component, along with severe neuronal loss, gliosis and microglial proliferation with only mild spongiform change, which is consistent with a GSS neuropathological phenotype. In contrast to previous description of homozygous mutations in *PRNP* [10-13,27] (Table 1), the disease in R136S families presented with an autosomal

315 recessive pattern of inheritance, keeping the heterozygous family members protected from
316 developing the disease, at least, at early ages. *In vitro* propagation and *in vivo* transmissibility
317 studies suggest that the PrP^{Sc} variant resulting from the R136S mutation failed to propagate
318 both *in vivo* and *in vitro* in the conditions analyzed, suggesting that this variant is biologically
319 less active than other mutations causing genetic prion diseases.

320 Previous described pathogenic mutations cause human prion disease in the heterozygous state
321 and show an autosomal dominant inheritance pattern. There are scarce examples in the
322 literature of patients affected by prion diseases carrying homozygous genetic variants.
323 Homozygous E200K carriers are associated with consanguinity in areas of high prevalence of
324 this variant. Homozygous carriers of the E200K mutation present an earlier age of onset than
325 heterozygous carriers but no other relevant differences in clinical features compared with
326 heterozygous carriers [10]. One case of prion disease linked to homozygous Q212P has been
327 described, but also another case has been reported in a heterozygous carrier [11]. Komatsu et
328 al. [12] reported the case of a homozygous V203I patient, in addition to three previously
329 reported heterozygous patients for this variant. However the lack of family history of disease
330 in all the V203I and the presence of this variant in normal controls suggest that this variant
331 could not be pathogenic [3] and more probably could represent benign variant or "risk"
332 variants that might increase the risk of developing a prion disease [11,27].

333 Minikel et al. [3] reported in 2016 the presence of the R136S variant in heterozygosis in two
334 alleles from 60.706 population control exomes but not in patients, suggesting that this
335 represents a very rare variant in the normal population even if the R136S variant is predicted
336 to be "probably damaging" in several pathogenicity prediction software. In our center, we
337 had not identified this genetic variant in more than 300 Spanish subjects (including both
338 patients and controls) analyzed so far. In 2006, Pacheco et al. [14] described one Portuguese
339 patient (Figure 1B, subject II.1) with similar clinical and neuropathological features of those

of our patients (early-onset dementia, motor symptoms in her early 50s and a GSS neuropathological phenotype) linked to the presence of the homozygous R136S variant. Two of her siblings presented history of similar disease in their early 50s, but not the parents who should be obligated carriers.

In both families (the present family and the Portuguese family) described heterozygous carriers were apparently protected from disease. As the genetic study in the Portuguese index case's mother (Figure 1B, subject I.2) and another family member neurologically preserved in their 80s and 90s revealed the presence of heterozygous R136S linked to 129MV, the authors suggested that the presence of valine in the trans-allele could prevent the expression of the disease. In contrast, in the Spanish family, one heterozygous carrier, homozygous for methionine at codon 129 is neurologically preserved in their 70s, suggesting that the expression of the disease does not depend on the codon 129 of the trans-allele but in the homozygous state or "dosage" of the mutant allele.

The expression of the disease only in homozygous subjects could be explained by a dose effect as it has been suggested for an earlier age of onset in homozygous E200K carriers [10]. A larger number of R136S PrP molecules (or the absence of wild-type protein) in the homozygous patient's brain may increase the chance of spontaneous conversion. Even if homozygous patients are at higher risk for the spontaneous conversion to PrP^{Sc} than heterozygous patients, on the other hand, the subsequent infective process based on PrP^{Sc}-PrP^{C} interaction might be less effective as there is only mutant PrP^{Sc} leading to longer disease duration.

Even if we cannot rule-out a late onset of the disease, the presence of known or obligated heterozygous carriers in neurological preserved family members that were already in their 70s, 80s or 90s make this possibility much less probable during the usual lifespan. In this

sense, we believe that both family trees (Figure 1), provide first evidence for a recessive pattern of inheritance in human prion diseases.

The clinical phenotype in the patients resembled that of insertion mutations (OPRI), which are characterized by early-onset progressive dementia with late motor dysfunction [6], whereas the neuropathological features were typical of GSS. The observed clinical phenotype is atypical for GSS, which more commonly present with motor signs followed by dementia, although it has also been linked to other point mutations (Y218N) [6] and OPRI [27,28]. Irrespectively of the clinical syndrome and the relative regional distribution of lesions, GSS is defined by the presence of multicentric PrP amyloid plaques in the cerebral and cerebellar cortices [7,18]. In contrast to other human prion diseases, the presence of spongiform change can be variable, from absent to severe, which could explain the lack of signal intensity changes in Diffusion Weighted-Imaging in MRI scans. In addition, western blot studies typically show PK-resistant non-glycosylated PrP^{Sc} fragments with a low molecular weight that oscillates between 6-8kDa depending on the PRNP pathogenic variant. Occasionally, however, the immunoblot profile can also show larger truncated PrP^{res} fragments with a molecular weight of 19-21 kDa, as observed in other sporadic or genetic prion diseases [29-31].

Animal modeling of human inherited prion diseases is not easy. Practically all attempts to generate transgenic mouse models for human inherited prion diseases using the human-PrP^{C} sequence had been unsuccessful apart from the recent reported exception of an A117V GSS model [32]. The rest of successful transgenic mouse models that develop spontaneously neurologic disorders when harboring mutations that have been reported in human inherited prion diseases have been made in mouse or bank vole PrP sequences or in chimeric mouse/human PrP proteins [33]. It seems that mouse and bank vole PrP proteins are more prone to spontaneous misfolding while human PrP is apparently more resistant. Alternatively,

389 interactions between mouse PrP and other than PrP mouse factors may be important for the
390 spontaneous generation of prions. In general terms for GSS modelling, models
391 overexpressing mouse, mouse/human chimeric and cow PrP harboring the respective
392 equivalents to human P102L mutation spontaneously developed a prion disease along with
393 neuropathological changes [33]. However, brain PrP^{res} detection and transmissibility to wild-
394 type mice was only achieved in the particular case of equivalent P113L mutation in cow-PrP^{C}
395 sequence producing a prion agent with features resembling those of classical bovine
396 spongiform encephalopathy [34]. When the A117V mutation was overexpressed in the mouse
397 sequence (A116V) at 4–6x levels, transgenic mice showed signs of prion disease including
398 neuropathology markers but no PrP^{res} were detected [35]. However, A117V GSS cases were
399 successfully transmitted into A117V human PrP transgenic mice Tg30 and Tg31 (2x and 3x
400 overexpression respectively) [32,36] from which Tg30 developed a spontaneous disease that
401 was later transmissible to both Tg30 and Tg31 mice as well as to wild-type V129 human PrP
402 transgenic mice [32]. It would be of interest to further investigate the effects of this
403 homozygous mutation in an animal model, as well as the production of heterozygous animals
404 to further investigate to which extent the presence of the wild-type allele dumpers the
405 spontaneous misfolding of the mutated one. The recessive inheritance pattern displayed by
406 this disease in humans points to a protective effect exerted by the wild-type variant and the *in*
407 *vitro* studies performed in this work also supports this hypothesis. However, a slower
408 misfolding rate of the wild-type allele that may finally contribute to disease development at
409 elder ages cannot be ruled out.

410 However, prion ability to cause direct cellular damage (toxicity) has often been dissociated
411 from the capacity of successfully transmit from an infected host to an uninfected recipient
412 (transmissibility) [37]. This fact, added to the difficulty of modelling inherited prion disease

that is further discussed below, may explain the lack of *in vivo* and *in vitro* propagation of the prion disease associated to R136S mutation.

This report has some limitations. First, the diagnosis of genetic prion disease is a hurdle task and some members of both extended families might have been underdiagnosed, lowering the possibilities of describing this mutation in other patients. Second, due to the lack of neuropathological evaluation in any of the asymptomatic heterozygous carriers, we could not rule-out subclinical prion disease at late ages, although this possibility seems less probable.

Conclusions

The biallelic R136S substitution is a rare *PRNP* mutation that induces inherited human prion disease presenting with early-onset dementia and motor impairment and neuropathological and molecular features consistent with GSS. Even if the R136S variant is predicted to be "probably damaging", heterozygous carriers are protected, at least, from an early-onset, providing the first pieces of evidence for a recessive pattern of inheritance in human prion diseases.

Abbreviations

Cellular prion protein: PrP^{C} ; cerebellar cortex: CbCtx; cerebral cortex: CCtx; Gerstmann-Sträussler-Scheinker: GSS; hippocampus:Hpp; hypothalamus: Hpth; HuPrP-Tg340-Met129:TgMet129; Immunohistochemistry: IHC; midbrain: Midb; neurologically preserved: NP; obligated carrier: OP; Periodic Acid-Schiff: PAS; peptide-N-glycosidase: PNGaseF+; phenylmethylsulfonyl fluoride: PMSF; pons/medulla oblongata: PoMe; Protein Mysfolding Cyclic Amplification: recPMCA; proteinase K: PK; proteinase K–resistant PrP^{Sc}: PrP^{res}; striatum: Stri; thalamus: Thal; Val129-PrP^{C} variant: TgVal129

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Author's contributions

RSV and JMT designed the study. AMM, HE, JMC, EG, PP and MR performed experiments. JCE, PP, JC, RRG, EG, JMT and RSV interpreted the results. JCE, HE, JMC, IH, CR, DA, EGR, IA, LMP, PP, MR, RRG and EG contributed to the sample collection and characterization. EG, JMT, JC, LMP and RSV provided extensive feedback to the manuscript to improve it significantly. TX and AMM wrote the manuscript draft. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by the ethical committee of the Hospital Clínic de Barcelona.

Animal experiments were conducted in accordance with the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 2010/63/EU) and every effort was made to minimize animal suffering. Transmission experiments were developed at Centro de Investigación en Sanidad Animal (CISA-INIA, Madrid, Spain) and they were evaluated by the Committee on the Ethics of Animal Experiments of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria and approved by the General Directorate of the Madrid Community Government (permit nos. PROEX 291/19 and PROEX 094/18).

Consent for publication

The participants or their next-of-kin provided written informed consent for the use of samples for diagnostic and research purposes.

Competing interests

RSV reports personal fees from Wave pharmaceuticals and Ionis Pharmaceuticals for attending Advisory board meetings, personal fees from Roche diagnostics, Janssen and Neuraxpharm for educational activities, and research grants to her institution from Biogen and Sage Therapeutics outside the submitted work. The other authors report no competing interests.

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Figure legends

Figure 1 Genetic pedigrees (A) Visual representation of the R136S Spanish family history. **(B)** Visual representation of the homozygous R136S Portuguese family (inferred from Pacheco et al.[14]). Created with BioRender.com

Figure 2 Magnetic resonance imaging scan of the index patient.

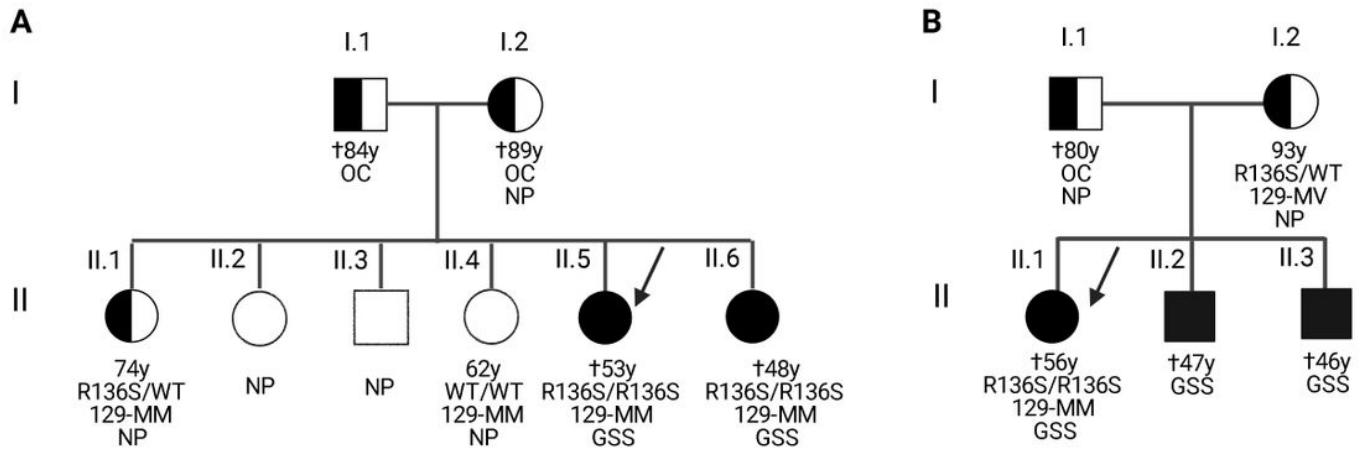
Figure 3 Neuropathological characterization. (A1) Frontal cortex shows prominent neuronal loss and gliosis with loss of cortical structure and mild superficial spongiosis (H&E stain). **(A2)** anti-PrP immunohistochemistry (12F10 antibody) shows a very high density of multicentric PrP plaques covering the whole cortical thickness. **(A3)** These large multicentric plaques are well identified in the PAS stain and the clusters of large amyloid plaques are observed by immunohistochemistry with the 12F10 anti-PrP antibody **(A4)**. **(A5)** Immunohistochemistry for hyperphosphorylated tau shows marked dystrophic neurites surrounding the plaques in the hippocampus (AT8 immunohistochemistry). **(A6)** Abundant multicentric plaques are detected in the molecular layer of the cerebellum (anti-PrP immunostaining, 12F10 antibody).

Figure 4 Western blotting with the 3F4 antibody of fresh-frozen tissue from the $PrP^{resR136S}$ index patient. Multiple bands with low molecular weight bands of approximately 8kDa in contrast to sCJDMM1. Westerns blots of CTR - PK+ (1), CTR – PK- (2), PrPresr136s Spanish proband frontal cortex (3), temporal cortex (4), parietal cortex (5), occipital cortex (6), hipppothalamus (7), thalamus (8), cerebellum (9), frontal cortex with PNGaseF+ (10), temporal cortex with PNGaseF+ (11) and sCJDMM1 (12) demonstrate that all fractions (3-11) have abnormal patterns of PrPres. All regions except for occipital cortex display a multiple band pattern.

Figure 5 *In vitro* human misfolded PrP propagation by recPMCA using R136 vs. S136 human as substrates. Human 129M 136R (wt) rec-PrP, human 129M 136S rec-PrP and a mix of both rec-PrPs in ratio 1:1 were complemented with chicken brain homogenate, seeded with different dilutions of recPMCA-adapted CJD MM1 misfolded rec-PrP (10-1 – 10-8) and subjected to a unique 24 h round of standard recPMCA. Amplified samples were digested with 50 µg/ml of Proteinase-K and analyzed by western blot using monoclonal antibody D18 (1:5,000). No significant differences were found in the propagation ability of the R136 (wt) vs. the mutant R136S rec-PrP or the mix 1:1 of both proteins.

Figure 6 Spontaneous generation of human misfolded recombinant proteins. Graphical representation of the emergence of spontaneous protein misfolding evaluated through Western blot analysis in SDS-PAGE for each round of recPMCA (outlined as R01–R15). Different 129M human recombinant proteins were grouped according to the substitution. Every experiment contained four tubes (intra-experimental duplicates) and was performed in duplicate as shown. The percentage of positive tubes (tubes showing a protease resistant signal after digestion with 80 mg/ml of PK) after each round of recPMCA was noted with different intensities of grey, as shown in the legend below the figure. Neither wild-type (wt) 129M not R136S 129M were able to misfold spontaneously. WT: wild type human rec-PrP^{Sc}.

Figures



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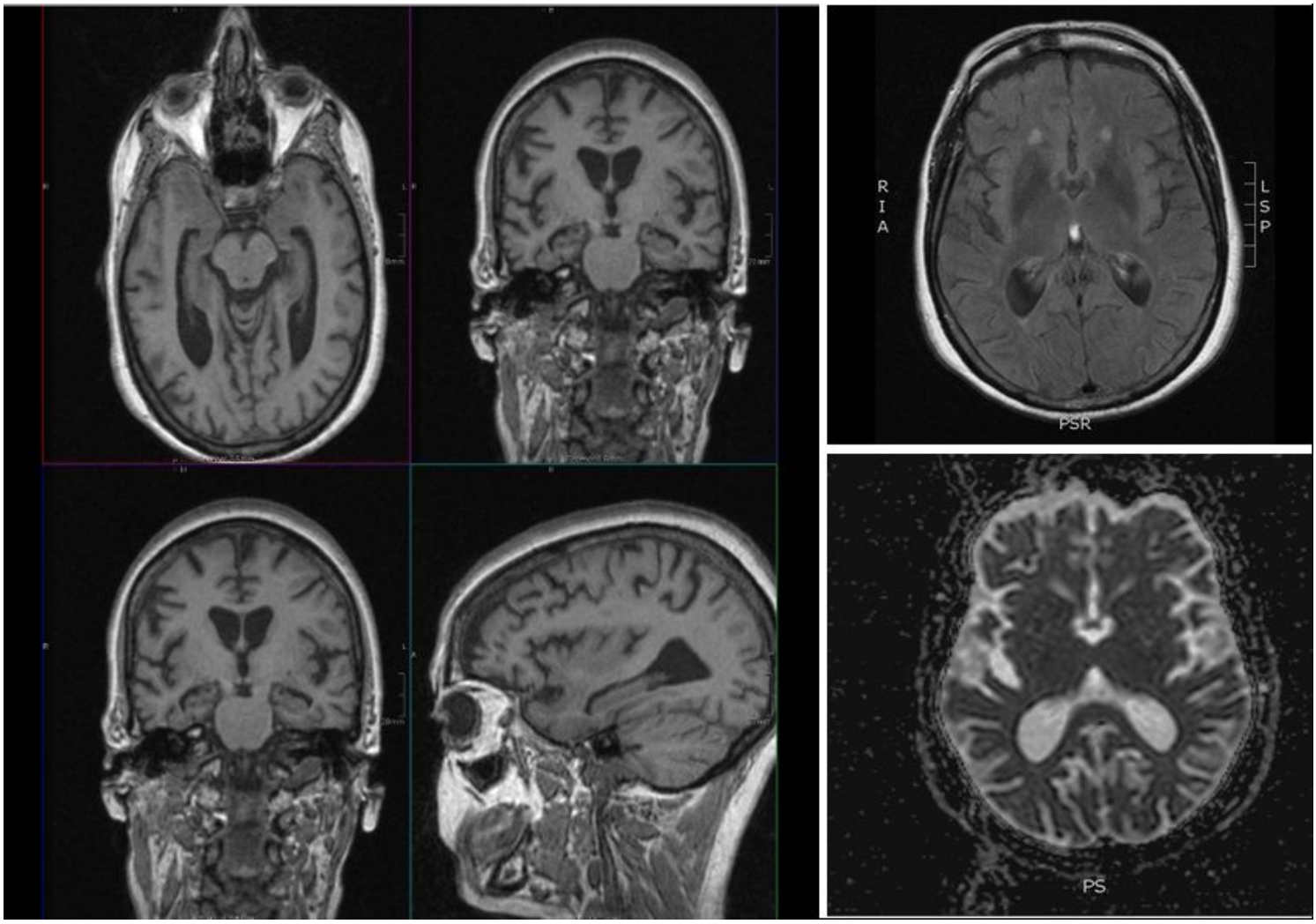

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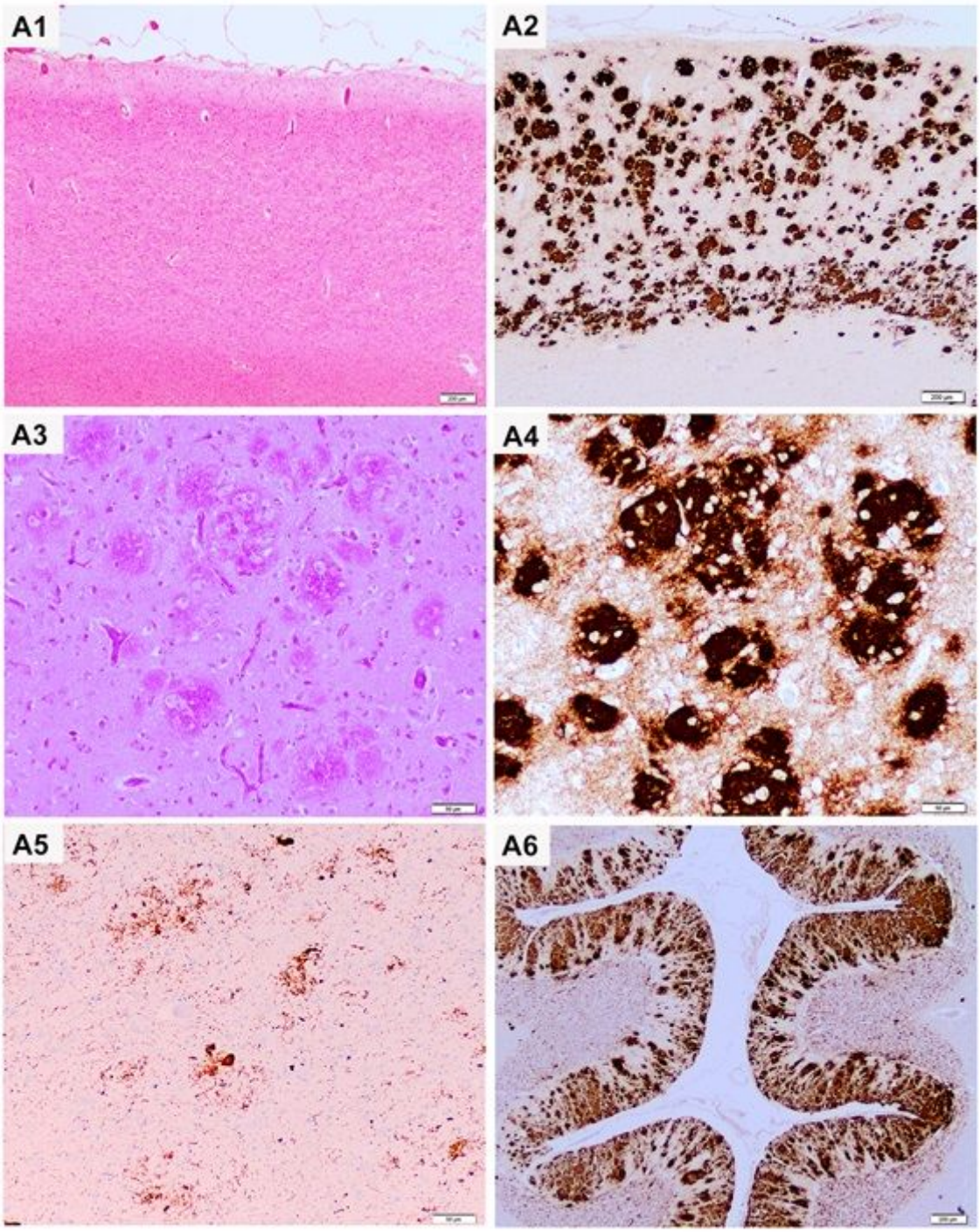

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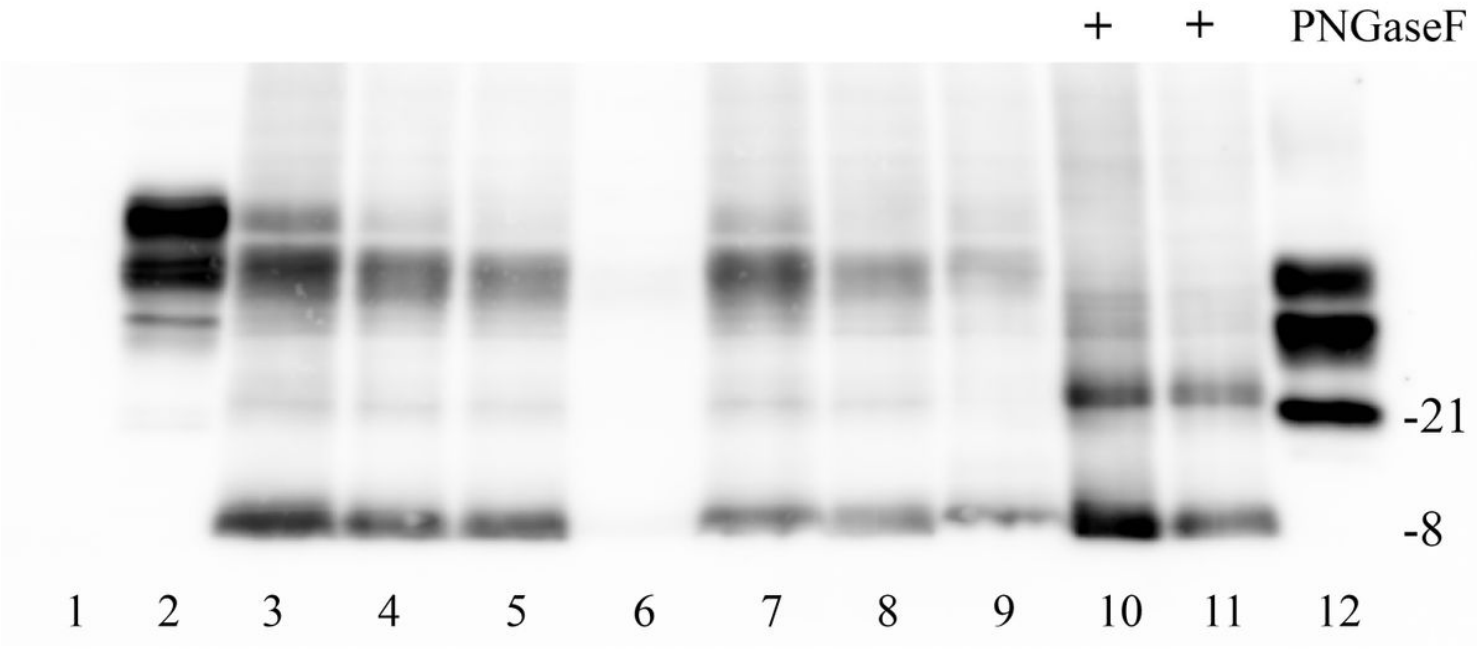

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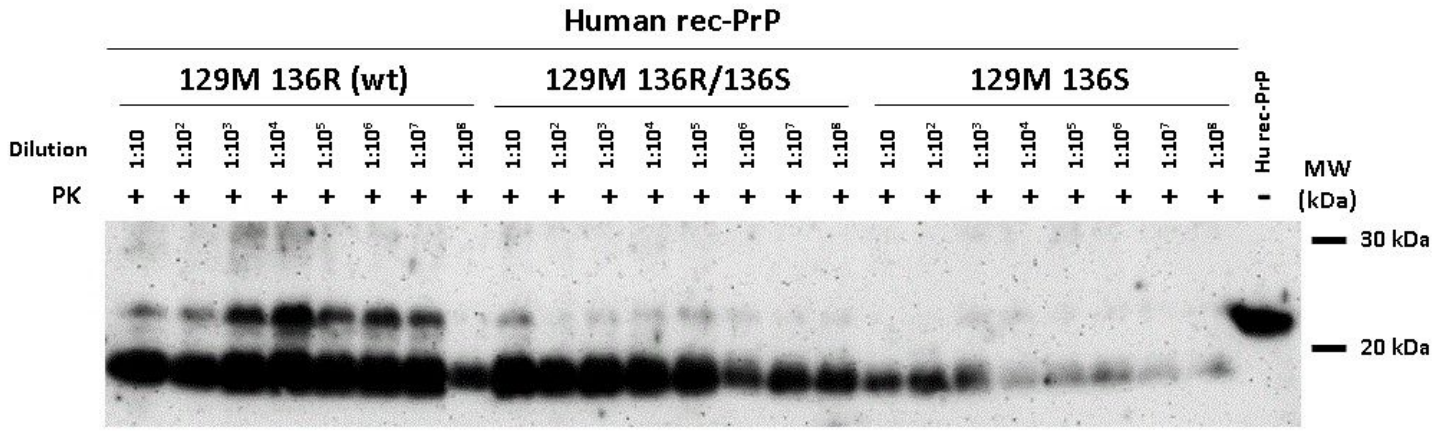

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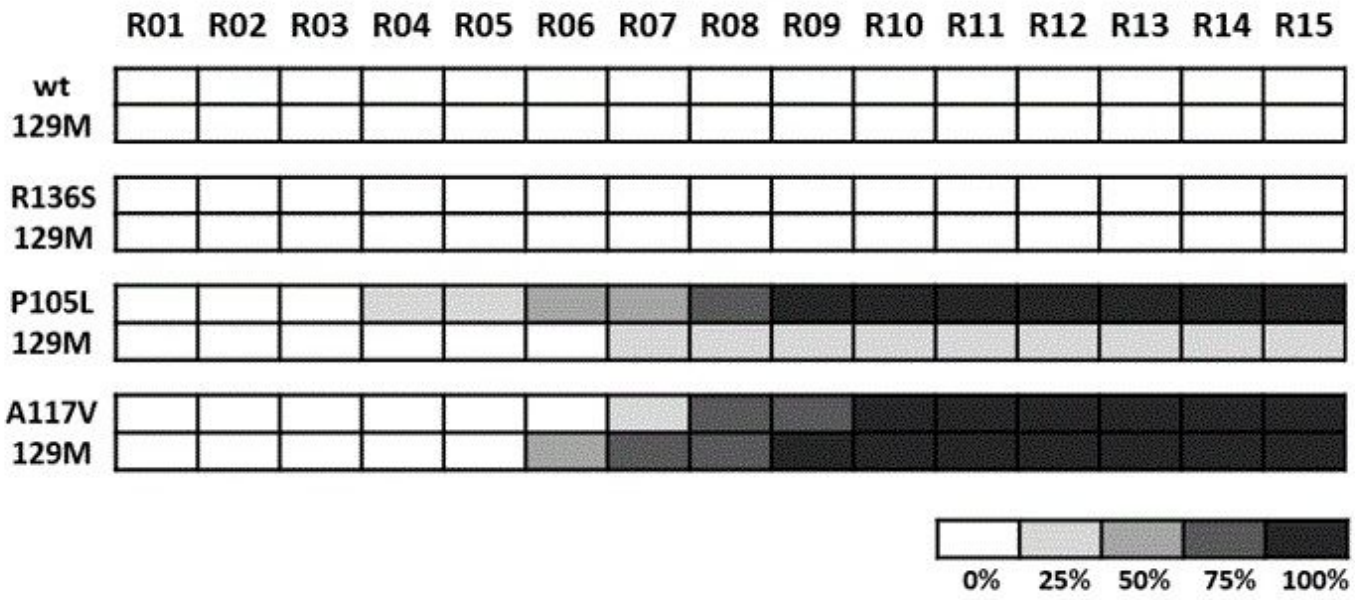

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