Effects of peptidyl-prolyl isomerase 1 depletion in animal models of prion diseases

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ABSTRACT
Pin1 is a peptidyl-prolyl isomerase that induces the cis-trans conversion of specific Ser/Thr-Pro peptide bonds in phosphorylated proteins, leading to conformational changes through which Pin1 regulates protein stability and activity. Since down-regulation of Pin1 has been described in several neurodegenerative disorders, including Alzheimer’s Disease (AD), Parkinson’s Disease (PD) and Huntington’s Disease (HD), we investigated its potential role in prion diseases. Animals generated on wild-type (Pin1+/+), hemizygous (Pin1+/−) or knock-out (Pin1−/−) background for Pin1 were experimen tally infected with RML prions. The study indicates that, neither the total depletion nor reduced levels of Pin1 significantly altered the clinical and neuropathological features of the disease.

Introduction
Prion diseases, or Transmissible Spongiform Encephalopathies (TSEs), are a group of neurological disorders that affects different animal species and humans. They can have sporadic, genetic or infectious origins [1,2]. All of them are characterized by the conformational conversion of the normal prion protein (PrPSc) to a pathological isoform named PrPSc which propagates in the brain by recruiting and converting new PrPSc molecules [2]. Compared to PrPSc, PrPSc is rich in β-sheet structures, is partially resistant to digestion with proteinase K (PK) and is prevalently insoluble in detergent. In contrast, PrPSc is predominantly composed of α-helix structures, is soluble in detergent and is completely digested by PK [3,4]. Both proteins have two N-linked glycosylation sites that enable the generation of unglycosylated, monoglycosylated and diglycosylated isoforms. The relative abundance of each PrP species represents the glycoform ratio. Once misfolded, PrPSc can acquire different abnormal conformations [5] also referred to as prion strains. Each conformer is associated with peculiar clinical, neuropathological and biochemical alterations [6]. A prion strain can be identified either neuropathologically considering (i) the incubation and survival time [7], (ii) the clinical signs, (iii) the pattern of PrPSc deposition [8] and the severity of spongiform changes [9]; or biochemically after PK digestion according to: (i) the electrophoretic mobility of the unglycosylated PrP band, (ii) the glycoform ratio [10], (iii) the resistance to PK digestion [11] and (iv) the conformational stability using chaotropic agents (e.g. Guanidine-hydrochloride) [12]. The mechanism of conversion is not fully understood, but several factors, like glycosaminoglycans, lipids, nucleic acids, metals and phosphorylations are supposed to play an important role in this process [13–20]. An important technique named Protein Misfolding Cyclic Amplification (PMCA) has been developed in 2001 with the aim of mimicking the process of prion conversion in vitro in an accelerated manner [21]. PMCA consists of cycles of sonication and incubation of a sample containing minute amount of PrPSc and an excess of PrPSc [22]. During the incubation phase PrPSc recruits and converts PrPSc to form polymers that are fragmented during the sonication phase whereby new PrPSc species are created and can induce further conversion of PrPSc. PMCA enables exponential amplification of PrPSc and has been extensively used to study the potential role of cellular cofactors in PrPSc to PrPSc conversion [17,23–28]. These studies highlighted the fact that different anionic conditions (anionic detergents, RNA and
Phosphorylation of PrPSc indeed provides anionic conditions that could severely affect its conformation [20]. Neuronal Cdk5 is a kinase able to phosphorylate PrPSc at Ser-43 position which induces the protein to change conformation and determines its conversion to PrPSc as showed either in vitro or in vivo [20]. Phosphorylated proteins can be recognized by different proteins, usually polypeptides containing either WW domains or SH2 domains such as scaffold proteins or enzymes. Among others, Pin1, a member of to the peptide-prolyl cis/trans isomerase (PPIase) family. Pin1 is a 18 kDa protein that catalyzes phospho-Serine/Threonine-catalyzed PMCA to mimic the process of prion conversion in vitro to verify whether reduced levels of Pin1 or its depletion could modulate the efficiency of RML replication. These results might help at identifying any interplay between Pin1 and prions. Establishing whether Pin1 has any role in PrPSc formation would lay the basis for developing innovative therapies for these devastating disorders.

Wild-type (Pin1+/+), hemizygous (Pin1+/−) or knock-out (Pin1−/−) mice for Pin1 express similar levels of PrPSc

Since differences in PrPSc levels can significantly alter the efficiency of prion propagation, we have initially evaluated the expression level of PrPSc in wild-type (Pin1+/+), hemizygous (Pin1+/−) or knock-out (Pin1−/−) mice for Pin1. Western blot analysis of 10% (weight/volume) brain homogenates collected from three mice per group were performed. Samples were immunoblotted with the 6D11 antibody and resulting signal underwent to densitometric analysis normalized against α-tubulin. As shown in Fig. 1A, the PrPSc signal was similar in all mice, regardless of their Pin1 genetic background. Densitometric evaluations revealed the presence of small differences that were not statistically significant.

Brain homogenates of Pin1+/+, Pin1+/− or Pin1−/− mice were used as PMCA substrates and efficiently amplified RML

Considering that the brains of Pin1+/+, Pin1+/− and Pin1−/− mice contained similar level of PrPSc, we homogenized those brains (as reported in materials and methods) and performed PMCA experiments. In particular, RML brain homogenate was serially diluted (from 10−5 to 10−9 volume/volume) in Pin1+/+, Pin1+/− or Pin1−/− substrates and subjected to 2 rounds of amplification. After one round of amplification, all RML-PrPSc dilutions were efficiently amplified, regardless of the
substrate. Thus, using our experimental conditions, the lack of Pin1 did not significantly influence the amplification efficiency of RML by means of PMCA (Fig. 1B). Furthermore, after PK digestion, we observed that the biochemical profiles of all the amplified PrPres were identical to that of the RML used as inoculum and were characterized by the predominance of the monoglycosylated PrPres species over the other. Taken together these results suggest that PMCA faithfully propagated the RML prion strain despite the use of three different substrates.

Incubation and survival times of RML injected animals were not statistically different among groups

To assess whether different expression levels of Pin1 might have exerted any influence on prion propagation in vivo, we challenged intracerebrally Pin1+/+, Pin1+/− and Pin1−/− animals with RML prion strain. Regardless of their genetic background, all RML injected mice succumbed to prion disease with an incubation time of 129.78 ± 5, 130.8 ± 2.98 and 134.33 ± 2.9 days and a survival time of 147.44 ± 3.84, 154.27 ± 2.43 and 152.82 ± 3.64 days (mean ± Standard Error of the Mean, SEM) for Pin1+/+, Pin1+/− and Pin1−/− mice, respectively (Fig. 2A and Table 1). Statistical analysis confirmed that neither the incubation time (p = 0.9881, Logrank test) nor the survival time (p = 0.4023, Logrank test) reached statistical significance among groups. None of the animals injected with mock inocula developed prion disease and their brains were used as control.

Prion depositions, spongiform changes and glial activation were similar between groups of RML injected animals

Severe spongiform changes were found in all groups of RML injected mice (Pin1+/+, Pin1+/− and Pin1−/−), especially confined to the thalamus, hippocampus and cingulated and adjacent motor cortex (Fig. 2B). Few vacuoles were detected in the remaining areas of the brain, including cerebellum and medulla. Widespread and synaptic pattern of PK-resistant PrP deposits were observed in all infected animals, prevalently affecting the hippocampus, thalamus, cerebellar cortex and retrosplenial cortex (Fig. 3A), with occasional accumulations in septum and striatum. Prion accumulation was accompanied by severe astroglial activation, mainly found in retrosplenial cortex, hippocampus and thalamus (Supplementary Figure 1A). Strong microglial activation was also
observed in the hippocampus, but was very faint in retrosplenial cortex and thalamus (Supplementary Figure 1B). These alterations were similar in all RML injected mice, regardless of their genetic background for Pin1.

**Prion inoculations did not exacerbate tau pathology in knock-out and hemizygous mice for Pin1**

Considering the fact that Pin1 seems to protect against neurodegeneration and that its depletion increases neuron vulnerability and causes tau pathologies in knock-out animals at around 9–14 months of age [35], we assessed whether RML inoculation might exacerbate tau pathology in Pin1−/− or Pin1+/− animals at terminal stage of prion disease (4–5 months after the inoculation). Immunohistochemical analysis revealed the complete lack of hyperphosphorylated tau deposits in the brain of Pin1−/−, Pin1+/− and Pin1+/+ animals sacrificed at around 6–7 months of age. Similarly, tau deposits were not detected in the brain of all mock-inoculated mice. Brain samples collected from a transgenic mouse model of tauopathy were used as positive controls for immunohistochemical analysis and confirmed the presence of abundant hyperphosphorylated tau (Supplementary Figure 2).

**Biochemical analysis revealed no differences in PK-resistant PrP among groups of RML injected animals**

Biochemical analysis of RML injected mice showed the presence of PK-resistant PrP typically characterized by a predominance of the monoglycosylated band.

| Genotype | Number of Animals | Inoculum | Attack Rate | Incubation Time (Mean ± SEM) | Survival Time (Mean ± SEM) |
|----------|-------------------|----------|-------------|-------------------------------|-----------------------------|
| Pin1+/+  | 9                 | RML      | 9/9         | 129.78 ± 5                   | 174.44 ± 3.84               |
|          | 6                 | Mock     | 0/6         | NA                           | NA                          |
| Pin1+/−  | 15                | RML      | 15/15       | 130.8 ± 2.98                 | 154.27 ± 2.43               |
|          | 10                | Mock     | 0/10        | NA                           | NA                          |
| Pin1−/−  | 11                | RML      | 11/11       | 134.33 ± 2.9                 | 152.82 ± 3.64               |
|          | 6                 | Mock     | 0/6         | NA                           | NA                          |

Table 1. Summary of the inoculation and main clinical findings in Pin1+/+, Pin1+/− and Pin1−/− mice. This table summarizes the experimental groups of animals challenged with RML or Mock. Attack rate, incubation and survival times (Mean ± SEM) of injected mice are reported.
Densitometric analysis confirmed the lack of statistical differences in PrP<sup>Pres</sup> amount between Pin<sup>+/+</sup>, Pin<sup>+/−</sup> and Pin<sup>−/−</sup> animals (Fig. 3B). To assess whether reduced levels of Pin1 or its depletion might have affected the biochemical features of the RML prion strain, we have performed PK-resistant assays by treating samples with increasing concentration of PK (from 50 μg/mL to 400 μg/mL) and subsequently verified the remaining amount of PK-resistant PrP by Western blotting. Each animal accumulated PrP whose PK resistance was totally comparable to that of the other animal groups (Supplementary Figure 3), thus indicating that either the biochemical or the biophysical properties of the RML were retained, regardless of the genetic background of the animals for Pin1.

**Prion infection did not alter Pin1 expression levels in the brain of mice sacrificed at terminal stage of the disease**

Brains of Pin<sup>+/+</sup>, Pin<sup>+/−</sup> and Pin<sup>−/−</sup> animals injected with RML prions (or mock inoculum) were collected at terminal stage of the disease. Immunohistochemical and biochemical analysis were performed to verify whether prion infection could have modified the expression level of Pin1. In particular, immunohistochemical evaluations revealed the presence of synaptic and widespread Pin1 signal throughout the whole brain of Pin<sup>+/+</sup> and Pin<sup>+/−</sup> animals. As expected, the signal was completely missing in Pin<sup>−/−</sup> mice. Fig. 4A shows representative pictures of neocortex and hippocampus of Pin<sup>+/+</sup>, Pin<sup>+/−</sup> and Pin<sup>−/−</sup> animals injected with RML or mock inocula, and shows that Pin1 immunoreaction is similar in all groups of animals, regardless of the inoculum. Biochemical and densitometric analysis performed on the same samples confirmed that Pin1 expression levels were similar in all RML or mock injected mice, thus highlighting the fact that prion infection did not modify the expression level of Pin1 in a statistically significant manner (Fig. 4B).

**Discussion**

Neurodegenerative diseases share a common pathological hallmark represented by the accumulation of misfolded proteins in the brain. Indeed, extracellular deposits of amyloid β (Aβ) and intraneuronal accumulation of hyperphosphorylated tau forming
neurofibrillary tangles (NFT) are found in AD, α-synuclein inclusions are typical of PD while prion aggregates are found in prion disorders. The exact mechanisms leading to the formation of these aggregates is still unknown, but recent evidence suggests that these insoluble species might have a neuroprotective function by sequestering oligomeric and toxic intermediates [52].

Different causative mechanisms have been proposed to explain the phenomenon of protein aggregation, including abnormal nitration, ubiquitination and phosphorylation of physiological protein. Abnormal phosphorylation of physiological proteins might impair their functions and induce the formation of post-translationally modified proteins more prone to misfolding and aggregation. For examples, tau, neurofilaments (NF) and amyloid precursor protein (APP) are proteins constantly subjected to physiological phosphorylation. Once phosphorylated these proteins interacts with other proteins and might undergo to Pin1 mediated cis-trans isomerization. Dysregulation of both phosphorylation and Pin1 activity might alter the equilibrium between cis and trans protein conformations leading to a predominant production of the pathogenic cis isoforms. This impairment has been associated with NFTs and amyloid plaques formation in AD. Recent studies have suggested that dysregulation of Pin1 activity and abnormal phosphorylation of α-synuclein and p53 are involved in Parkinson’s disease and Huntington’s disease, respectively [49,51,53].

In this work, we have analysed whether Pin1 has any pathological role also in prion diseases. Indeed, it has been reported that the phosphorylation of the residue Ser-43 at the N-terminal of PrPSc might favour its misfolding into the abnormal conformation (PrPSc) [20]. Therefore, we wondered whether Pin1 might take part in this process thus sustaining the phenomenon of prion formation and propagation in the brain. To analyse whether Pin1 and PrPSc interact in prion diseases, we have performed in vitro and in vivo experiments aimed at verifying whether reduced levels of Pin1 or even its depletion could affect the clinical and neuropathological course of prion diseases in mice on wild-
type (Pin1+/+), hemizygous (Pin1+/−) or knock-out (Pin1−/−) background for Pin1. Initially, we evaluated whether all genetically modified animals (Pin1+/+, Pin1+/−, Pin1−/−) expressed similar levels of PrP C, the substrate for prion propagation. Our results show that regardless of their genetic background the brains of all the animals expressed similar level of PrP C. Based on these premises, we performed preliminary PMCA experiments to assess whether different levels of Pin1 could affect the amplification efficiency of the RML prion strain in vitro. We have spiked RML (from 10−5 to 10−9) in brain homogenates of Pin1+/+, Pin1+/− and Pin1−/− mice. One round of amplification was able to efficiently amplify all these dilutions thus suggesting that, under these experimental conditions, different expression levels of Pin1 did not affect the RML prions replication properties. Although these results failed to provide any evidence that Pin1 could take part in the process of PrP C misfolding, we decided to perform in vivo studies because PMCA is an artificial system for prion amplification and there are no physiological processes (e.g. protein-to-protein interaction, presence of chaperones or foldases which are known to assist the folding of PrP C, presence of phosphatases, kinases, etc.) that can finely take part and regulate the intricate process of PrP C misfolding and aggregation. Therefore, we performed intracerebral inoculations of RML prions or mock inocula in the striatum of Pin1+/+, Pin1+/− and Pin1−/− mice. As already observed in the in vitro experiment, regardless of their genetic background, all RML inoculated animals succumbed to prion disease (with 100% attack rate) with incubation and survival times comparable and typical of the RML prion strain. After sacrifice, the brains of these animals were collected and analyzed biochemically and immunohistochemically to verify whether the genetic background for Pin1 might have modified the neuropathological changes typically associated to RML. In particular, immunohistochemical analysis revealed synaptic PK-resistant PrP deposition throughout the brain accompanied by severe astroglial and microglial activation. These alterations were comparable among the different groups of mice. Similarly, spongiform changes severely affected the thalamus, hippocampus and the cingulated and adjacent motor cortex of all groups of animals. Biochemically all the animals accumulated high levels of PK-resistant PrP characterized by the typical glycoform profile of the RML prion strain (predominance of the monoglycosylated band of PrP). Compelling evidences suggest that prions can change their properties in response to changes in the environment of propagation [54], in our case represented by different expression levels (or even its deletion) of Pin1. This phenomenon is not surprising and has been already observed after pharmacological treatment of cell cultures [55] or infected animals [56,57]. After PK-digestion we found that the biochemical properties of the RML were retained in all animal groups further sustaining that, under our experimental conditions, different levels of Pin1 did not alter the process of prion propagation in vivo.

We also verified whether the injection of RML might have affected the expression of Pin1 in animals sacrificed at terminal stage of the disease (especially in Pin1+/+ and Pin1+/− mice). Neither biochemical nor immunohistochemical results showed differences in Pin1 expression levels in the brain of RML or mock inoculated animals. Densitometric analysis showed a slight reduction of Pin1 in the brain of RML injected Pin1+/+ animals compared to mock inoculated mice but this difference did not reach statistical significance. Similarly, no differences in Pin1 expression levels were found in the brains of Pin1+/− animals.

Taken together these data suggest that Pin1 does not significantly interact with PrP C in prion diseases and other pathological mechanisms should be considered. Since PrP C is rich in prolines, and considering that two proline substitutions (P102L and P105L) cause a genetic form of prion disease known as GSS [58,59], other than Pin1, different peptidylprolyl cis-trans isomerases (PPIases) may play a role in PrP C misfolding. Little is known about the mechanisms that induce PrP C to PrP Ec conversion but many enzymatically driven processes might take part in this process. Finally we assessed whether the lack of Pin1 might have exacerbated tau pathology (which spontaneously occurs in these animals at around 9–14 months of age) in RML injected mice. Specific immunohistochemical analysis revealed that RML inoculated Pin1+/+ or Pin−/− mice did not accumulate hyperphosphorylated tau in the brain. Thus, although lacking Pin1 (which is known to have neuroprotective function), tau accumulation was not exacerbated by prion inoculation.

This work provides the first evidence that, in animals experimentally infected with the RML prion strain, the impairment of Pin1 activity or its total depletion did not clearly and significantly alter the clinical and neuropathological changes associated to this disease. It would be worthy to extend the analysis to human prions collected from patients with different forms of the disease. Indeed, considering the phenotypic variability of human prion pathologies, it might still be possible that Pin1 can be involved only in some of them. If this were the case, Pin1 could be considered as disease-specific therapeutic target for such devastating diseases.
**Materials and methods**

**Ethics statement**

B6.129P2-Pin1<sup>tm1Tuc</sup> mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Mice were housed in groups of 2–5 animals in individually ventilated cages, daily fed and water provided ad libitum. Lighting was on an automatic 12 h basis. Regular veterinary care was daily performed for assessment of animal health. Animal facility is licensed and inspected by the Italian Ministry of Health. Current animal husbandry and housing practices comply with the Council of Europe Convention ETS123 (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes; Strasbourg, 18.03.1986), Italian Legislative Decree 116/92 (Gazzetta Ufficiale della Repubblica Italiana, 18<sup>th</sup> February 1992) and with the 86/609/EEC (Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes). The study, including its Ethics aspects, was approved by the Italian Ministry of Health.

**Genetic analysis**

Genetic analyses were performed to identify animals with wild-type (Pin1<sup>+/+</sup>), hemizygous (Pin1<sup>+/−</sup>) or knock-out (Pin1<sup>−/−</sup>) background for Pin1. To this aim, genomic DNA was extracted from ear biopsies using the NucleoSpin Tissue kit (Macherey-Nagel) and analyzed by PCR. Two pairs of primers were used: 1.2 L (5′-AGCACCGATCCTGTCTGCAA-3′) / WILD 1.2A (5′-AAGGGATGAGCAAGATTGCGA-3′) that specifically amplify the wild-type allele, and 1.2 L / START2 (5′-CAGAGGCCACCTTGTA-3′), specific for the Pin1 knock-out allele [60]. An initial denaturation at 95°C for 5 minutes was followed by 35 cycles of amplification (95°C for 1 min, 60°C for 1 min, 72°C for 1 min) and 10 minutes extension at 72°C. PCR products were resolved by agarose gel electrophoresis.

**Animal groups and intracerebral inoculations**

Eight-week old animals were anesthetized with 2,2,2-tribromoethanol i.p.-administered (100 µL/10 g) and stereotaxically inoculated in the striatum using these coordinates: −0.5 caudal, + 2 lateral, + 3.5 depth. All surgical procedures were performed under sterile conditions. Inoculations were performed in 3 groups of animals generated on wild-type (Pin1<sup>+/+</sup>), hemizygous (Pin1<sup>+/−</sup>) or knock-out (Pin1<sup>−/−</sup>) background for Pin1. In particular, 20 µl of 10% (weight/volume) RML brain homogenate was prepared from a pool of terminally RML-sick CD1 mouse brain and injected in Pin1<sup>+/+</sup> (n = 9), Pin1<sup>+/−</sup> (n = 15) and Pin1<sup>−/−</sup> (n = 11) mice. Similarly, 20 µl of 10% (weight/volume) mock homogenate was obtained from a pool of healthy CD1 mouse brains and was inoculated in Pin1<sup>+/+</sup> (n = 6), Pin1<sup>+/−</sup> (n = 10) and Pin1<sup>−/−</sup> (n = 6) mice.

**Behavioral testing**

Incubation time was calculated as the period between the day of prion intracerebral inoculation and the day of appearance of at least two of the following clinical signs: piloerection and kyphosis, decreased locomotor activity and curiosity, uncoordinated movements, awkwardness of movement on a metal grid, inability to hang on inverted grid. Terminally sick animals, unable to ambulate and to eat, were sacrificed and survival time was calculated as the period between RML intracerebral inoculation and the day of euthanasia. Mock injected animals were sacrificed along with RML injected animals and used as controls.

**Euthanasia and brains collection**

RML-injected mice were sacrificed at the agonic stage of disease along with age-matched mock-injected animals that were used as negative controls. All mice were euthanized by 2,2,2-tribromoethanol i.p.-injected and cervical dislocations were finally performed. Brains were harvested and half used for biochemical studies and the other half processed for histological analysis.

**Western blot analysis**

Ten percent (weight/volume) brain homogenates from frozen tissues were prepared in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% NP-40, 0.5% Sodium Deoxycholate, 10 mM Tris-HCl, pH 7.4). Samples were centrifuged at 4°C, 2000 x g, 1 minute in order to remove cellular debris. Supernatant was then collected and stored at −80°C for further use. Twenty µL of supernatant was heated at 100°C for 10 minutes and loaded into 12% Bolt Bis-Tris Plus gels. Proteins were fractionated by means of SDS-PAGE under reducing conditions and transferred onto Polyvinylidene difluoride (PVDF) membrane and incubated with 5% (wt/vol) dry nonfat milk in TBST, 0.05%-Tween20 (prepared in Tris-HCl) for 1 h at room temperature with shaking. Membranes were finally incubated with antibodies against PrP...
(Clone 6D11, Covance), Pin1 (Clone G-8, Santa Cruz) and α-Tubulin (Sigma). Blots were developed using the ECL Prime detection system (Amersham) and visualized using a G:BOX Chemi Syngene system.

**PK digestion**

Before PrP analysis, samples were digested with proteinase K (PK) (Invitrogen) [100 μg/mL] for 1 hour at 37°C. Digestion was stopped by the addition of LDS-PAGE loading buffer (Invitrogen) and subjected to Western blot analysis with 6D11 (0.2 μg/mL, Covance).

**PK resistance assay**

Five aliquots (20 μL) of brain homogenates were digested with increasing concentration of PK: 50, 100, 200, 300, 400 μg/mL) and immunoblotting was performed using the 6D11 antibody. The PK-resistant signal was quantified and normalized using ImageJ software (1.48v).

**Neuropathological analysis**

Brains were fixed in Alcolin (Diapath), dehydrated and embedded in paraplast. Seven-micrometer thick serial sections were stained with hematoxylin-eosin (H&E) or immunostained with monoclonal antibodies to PrP (6H4, Prionics), Pin1 (Pin1, Santa Cruz Biotechnology), hyperphosphorylated tau (AT8, Thermo Scientific Pierce Antibodies), polyclonal antibodies to astrocyte activation (GFAP, Dako) and microglial activation (Iba-1, Abcam). Before PrP immunostaining the sections were sequentially treated with proteinase K (VWR) (10 μg/mL, 5 min) and guanidine isothiocyanate (Carlo Erba) (3 M, 20 min), and non-specific binding was prevented using ARK kit (Dako). Immunoreactions were visualized using 3,3′-diaminobenzidine (DAB, Dako) as chromogen. Spongiform profiles were determined on H&E-stained sections, by scoring the vacuolar changes in nine standard gray matter areas (1): dorsal medulla (2), cerebellar cortex (3), superior colliculus (4), hypothalamus (5), thalamus (6), hippocampus (7), septum (8), retrosplenial and adjacent motor cortex and (9) cingulated and adjacent motor cortex, as previously described [9]. Brains collected from genetically modified mice to express the human tau with P301L mutation (JPNL3) were used as positive control for tau immunostaining [61].

**PMCA procedures**

PMCA was performed as previously described [62]. Briefly, brain samples obtained from mice on wild-type (Pin1+/+), hemizygous (Pin1+/−), and knock-out (Pin1−/−) background for Pin1 were used as substrates. The animals were intracardially perfused with PBS (containing 0.5 mM EDTA, Sigma) and brains harvested and homogenized at 10% (weight/volume) in conversion buffer (PBS containing 150 mM sodium chloride and 1% Triton X-100) with the addition of protease inhibitors (Roche). To evaluate the effect of Pin1 on RML amplification, RML (Rocky Mountain Laboratories) prion strain was spiked at different dilutions (from 10−3 to 10−9) in each substrate (Pin1+/+, Pin1+/−, Pin1−/−). Samples were then transferred in 0.2 mL tubes, positioned on an adaptor placed on the plate holder of a microsonicator (Misonix, Model S3000) and subjected to 96 cycles of PMCA. Each cycle (referred to as PMCA round) consisted of 29 min and 40 sec of incubation at 37–40°C followed by 20 sec of sonication set at potency of 260–270 Watt. After one round of PMCA, an aliquot of the amplified material was diluted 10-folds into fresh substrate and a further PMCA was performed following the same procedure. To increase PMCA efficiency, teflon beads (n = 3) were added to the samples before each round of amplification. To avoid samples cross-contamination between each round, thorough decontamination of instruments and equipment was performed using 2N sodium hydroxide (NaOH) or 4M guanidine hydrochloride (Gdn-HCl).

**Statistical analysis**

Densitometric analysis were carried out using ImageJ. Statistical analysis was performed using the GraphPad Prism 5.0 software. Kaplan-Meier survival curves were plotted and differences in incubation or survival times between groups were compared using the Logrank test. Densitometric analysis of immunoblot data were performed using a double-tailed unpaired t-test (Mann-Whitney test).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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