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The E3 Ubiquitin Ligase TRAF6 Interacts with the Cellular Prion Protein and Modulates Its Solubility and Recruitment to Cytoplasmic p62/SQSTM1-Positive Aggresome-Like Structures

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Abstract

The cellular prion protein (PrP^C) is a ubiquitous glycoprotein highly expressed in the brain where it is involved in neurite outgrowth, copper homeostasis, NMDA receptor regulation, cell adhesion, and cell signaling. Conformational conversion of PrP^C into its insoluble and aggregation-prone scrapie form (PrP^{Sc}) is the trigger for several rare devastating neurodegenerative disorders, collectively referred to as prion diseases. Recent work indicates that the ubiquitin–proteasome system is involved in quality control of PrP^C. To better dissect the role of ubiquitination in PrP^C physiology, we focused on the E3 RING ubiquitin ligase tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6). Here, we report that PrP^C interacts with TRAF6 both in vitro, in cells, and in vivo, in the mouse brain. Transient overexpression of TRAF6 indirectly modulates PrP^C ubiquitination and triggers redistribution of PrP^C into the insoluble fraction. Importantly, in the presence of wild-type TRAF6, but not a mutant lacking E3 ligase activity, PrP^C accumulates into cytoplasmic aggresome-like inclusions containing TRAF6 and p62/SQSTM1. Our results suggest that TRAF6 ligase activity could exert a role in the regulation of PrP^C redistribution in cells under physiological conditions. This novel interaction may uncover possible mechanisms of cell clearance/reorganization in prion diseases.

Keywords Cellular prion protein · TRAF6 · Ubiquitination · Aggresomes

Introduction

The cellular form of the prion protein (PrP^C) is an N-glycosylated, glycosylphosphatidylinositol (GPI)-anchored protein, present in its mature form on the surface of several cell types [1], and it is prevalently expressed in the brain. It is now widely accepted that PrP^C converts into abnormally misfolded conformers, known as PrP^{Sc} or prions, which cause neuronal spongiosis, neuronal loss, and gliosis, ultimately leading to the neurodegeneration observed in prion diseases. Large amyloids composed by misfolded and insoluble PrP^{Sc} are observed in the brain of affected individuals [2, 3]. The accumulation of these aggregates overwhelms the ubiquitin–proteasome system (UPS), primarily devoted to remove misfolded proteins and preserve cellular homeostasis [4, 5]. Increased ubiquitin immunoreactivity has been observed in animal models and in human prion diseases [6, 7]. Moreover, β-sheet-rich PrP^{Sc} amyloids can inhibit the catalytic activity of the proteasome [8, 9], and failure of the proteasome system is associated with increased in vivo ubiquitination of PrP [10]. These pathogenic mechanisms are shared with other *conformational* neurodegenerative disorders, like Alzheimer's disease, Parkinson's disease, and Huntington's disease, in which misfolded proteins tend to accumulate in ubiquitinated insoluble aggregates, further dampening the UPS.

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In prion diseases, PrP^C plays a pivotal role in the conversion and replication mechanism seeded by the pathogenic scrapie forms of the protein. Few studies have addressed UPS-mediated control of PrP^C in physiological conditions or early in aggregation events. In physiological conditions, a portion of PrP^C is subjected to ER-associated degradation (ERAD) by the proteasome after relocalization to the cytosol and ubiquitination and accumulation in cytoplasmic aggregates [11]. The ubiquitin ligase gp78 [12, 13] and the ubiquitin-specific protease 14 [14] have been shown to modulate PrP^C ubiquitination status and control its levels in mammalian cells. PrP^C can also directly interact with Stub1 E3 ligase, with yet unknown consequences [15].

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an E3 ubiquitin ligase that promotes ubiquitin chain assembly in the signal transduction pathway that activates NFkB [16]. Recently, TRAF6-mediated activation of NFkB has been shown to occur in response to viral infections within large prion-like polymers formed by mitochondrial antiviral signaling protein, or MAVS. In the cytoplasm, TRAF6 binds MAVS prion-like aggregates and promote their ubiquitination, which in turn is required to facilitate the activation of downstream signaling events [17, 18]. In the brain, TRAF6 E3 ligase activity has been associated to the transduction cascade of the neurotrophin receptors p75 and TrkA [19, 20] and to the organization, function, and plasticity of neuronal synapses [21]. Our previous work has shown that TRAF6 contributes to the ubiquitination of aggregation-prone proteins associated to neurodegenerative diseases, such as α-synuclein, mutant DJ-1, and huntingtin [22–24]. TRAF6-mediated ubiquitination enhances the aggregation propensity of these misfolded proteins, triggering inclusion into insoluble aggresome-like structures. In human post-mortem brains, TRAF6 itself accumulates in tau inclusions and Lewy bodies in brains of Alzheimer's disease and Parkinson's disease patients [24].

In the current study, we provide evidence for TRAF6 interaction with and indirect contribution to the ubiquitination of PrP^C. The interaction between endogenous TRAF6 and PrP^C occurs also in vivo in the mouse brain. TRAF6 ubiquitin ligase activity triggers the accumulation of PrP^C into insoluble cytoplasmic aggresome-like structures that co-localize with p62/SQSTM1. Altogether, our results imply a role for TRAF6 in PrP^C quality control and suggest new venues of intervention for prion diseases.

Methods

Plasmids

Human pcDNA3.1(-)-TRAF6-2xFLAG (FLAG-TRAF6), pEGFP-TRAF6 (GFP-TRAF6), GFP-TRAF6, and FLAG-TRAF6 mutants deleted of the N-terminus E3 ligase domain (GFP-TRAF6 DN and FLAG-TRAF6 DN) and pGK5-HA-Ubiquitin (HA-Ubiquitin) constructs were described previously [24]. Mouse pcDNA3.1(-)-PrP^C 1–253 (PrP^C) was kindly provided by Prof. J.R. Requena (University of Santiago de Compostela, Santiago de Compostela, Spain). The pcDNA3.1(-)-PrP^C 40–231 (cyPrP) construct was obtained from PrP^C [25]. The generation of pcDNA3.1(-)-FLAG-PrP^C 1–253 (FLAG-PrP^C) was performed with a two-step RF cloning procedure [26].

Cell Culture, Transfection, and Treatments

HEK293T cells were maintained in culture in DMEM (11,965,092, Life technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 100 units/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich) at 37 °C in a humidified 5% CO_2 incubator. Cells were transiently transfected with polyethylenimine linear (23,966, Polysciences, Valley Road Warrington, PA) or with Lipofectamine 2000 (11,668,019, Thermo Fisher Scientific, Waltham, MA, USA) and collected at 48 or 72 h after transfection.

For treatments, the following reagents were used: MG132 (C2211, Sigma-Aldrich) and Lactacystin (L6785, Sigma-Aldrich). Drugs were added for 16 h at the indicated concentrations.

Animals

All animal experiments were performed in accordance with European guidelines for animal care (European Community Council Directive, November 24, 1986 86/609/EEC) and following Italian Board of Health permissions (Law n. 116/1992). Six-month-old C57BL/6J mice were housed and bred in SISSA animal facility, with 12 h dark/light cycles and controlled temperature and humidity. Food and water were provided ad libitum.

Coimmunoprecipitation

To coimmunoprecipitate PrP and TRAF6 from mouse brain, the protocol was modified from [24], in order to clearly isolate endogenous PrP. Mice were euthanized with an excess of CO_2 and cervical dislocation. Brains were extracted and homogenized in modified TRAF6 buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP40, 10% glycerol) supplemented with protease cocktail (04,693,159,001, Roche Diagnostics, Basel, Switzerland) and centrifuged at 10,000 rpm for 10 min at 4 °C. Brain lysates were incubated overnight with anti-TRAF6 antibody (sc7221, Santa Cruz, Dallas, TX, USA) or rabbit IgG (2729, Cell Signaling, Danvers, MA,

USA), as negative control, followed by incubation with protein A/G Sepharose Resin (17,061,801, GE Healthcare, Little Chalfont, UK). Resin was washed with three subsequent solutions (buffer B: 10 mM Tris HCl pH 7.6, 2 mM EDTA pH 8.0, 3.5 mM NaCl; buffer C: 10 mM Tris HCl pH 7.6, 2 mM EDTA pH 8.0, 12.5 mM NaCl; buffer D: 10 mM Tris HCl pH 7.6). Immunoprecipitated proteins were eluted in 2X Laemmli Buffer, boiled, and analyzed by western blotting.

For coimmunoprecipitation from cells, HEK293T cells were lysed 48 h after the transfection in TRAF6 buffer (200 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP40, 10% glycerol) supplemented with protease cocktail (Roche Diagnostics) and centrifuged at 10,000 rpm for 10 min at 4 °C. Cell lysates were incubated at 4 °C incubated for 2 h with anti-FLAG M2 agarose resin (A2220, Sigma-Aldrich) or overnight with mouse anti-PrP DE10 (produced in our laboratory[27]) or with mouse anti-PrP W226 (kindly provided by professor Carsten Korth, Department of Neuropathology Heinrich Heine University, Düsseldorf [28]), followed by incubation with protein A/G Sepharose (GE Healthcare). Resin was washed with three subsequent solutions (buffer B: 10 mM Tris HCl pH 7.6, 2 mM EDTA pH 8.0, 3.5 mM NaCl; buffer C: 10 mM Tris HCl pH 7.6, 2 mM EDTA pH 8.0, 12.5 mM NaCl; buffer D: 10 mM Tris HCl pH 7.6). Proteins were eluted with 2X Laemmli Buffer, boiled, and analyzed by western blotting.

Ubiquitylation Assay

For the ubiquitylation assay, HEK293T cells were transfected with PrP^{C}, FLAG-TRAF6 or FLAG-TRAF6 DN, and HA-Ubiquitin constructs and treated with 10 µM MG132 for 3 h. HEK293T cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% Triton X-100, 1% deoxycholic acid and 0.1% SDS). Cell lysates were briefly sonicated, centrifuged at 10 000 rpm for 10 min at 4 °C, and incubated for 2 h with anti-PrP W226 antibody. Antibody bounded proteins were washed with three subsequent solutions (buffer B: 10 mM Tris HCl pH 7.6, 2 mM EDTA pH 8.0, 3.5 mM NaCl; buffer C: 10 mM Tris HCl pH 7.6, 2 mM EDTA pH 8.0, 12.5 mM NaCl; buffer D: 10 mM Tris HCl pH 7.6). Proteins were eluted with 2X Laemmli Buffer, boiled, and analyzed by western blotting.

Detergent Solubility Fractionation

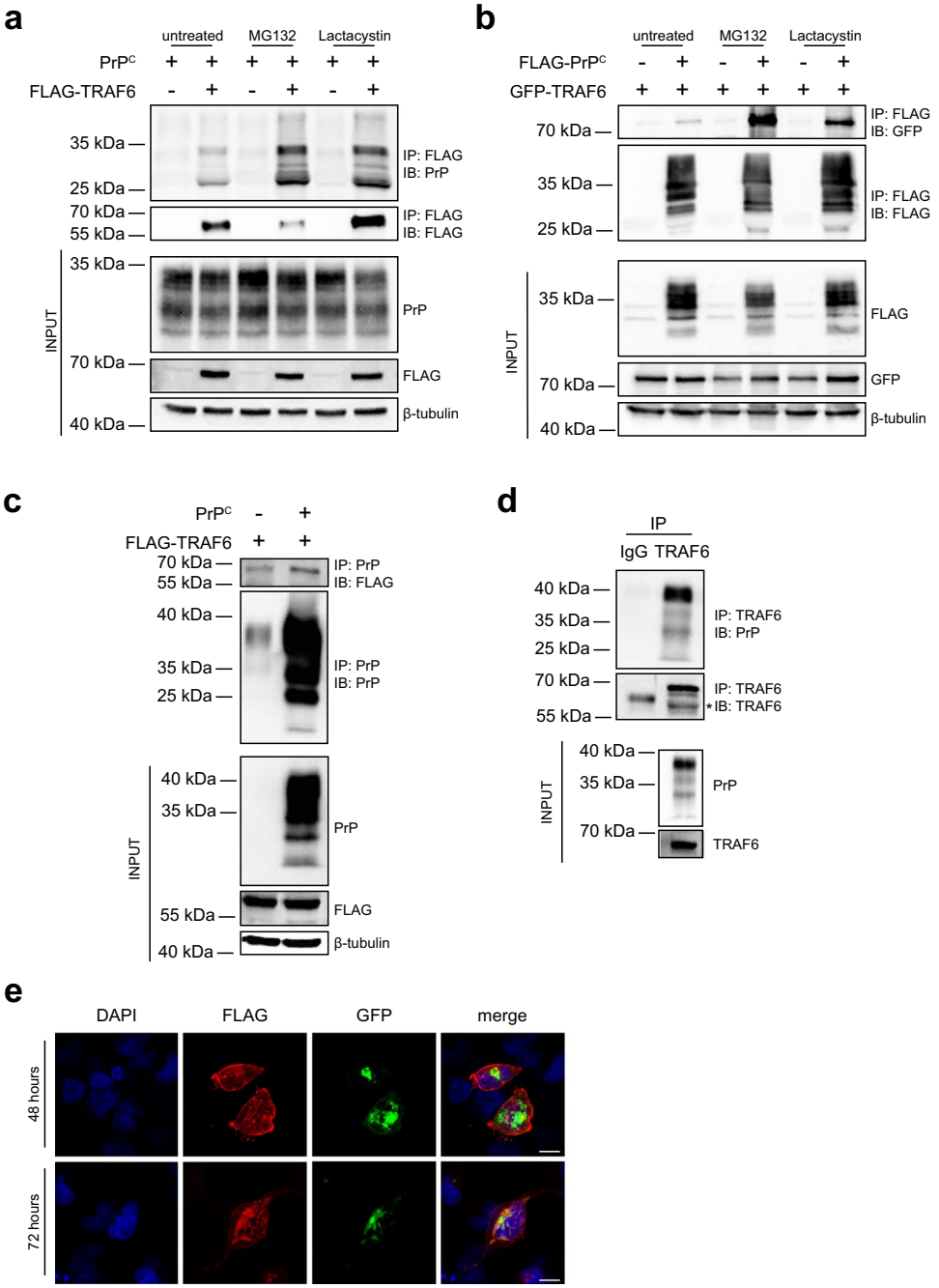
Detergent solubility was performed as previously described [29]. In detail, HEK293T cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 7.5, and 0.2% Triton X-100, supplemented with protease inhibitor cocktail (Roche Diagnostics) and 5 mM NEM (Sigma-Aldrich). Lysates were centrifuged at 20000 *g* for 30 min at 4 °C and separated into Triton X-100 soluble (supernatant) and insoluble (pellet) fractions. The pellet was dissolved in 2X Laemmli Buffer and sonicated. Samples were boiled and analyzed by western blotting.

Western Blotting

Proteins derived from adherent cells were collected, washed intensely with PBS 1X, and lysed in 2X Laemmli Buffer. Proteins were separated in 12% SDS polyacrylamide gel. After the separation on gel, proteins were transferred to nitrocellulose membrane (GE Healthcare). Membrane was blocked with 5% non-fat milk in TBST solution (TBS and 0.1% Tween20) or with 5% bovine serum albumin (BSA) in PBS solution, as needed, at room temperature for 1 h and then incubated with primary antibodies overnight at 4 °C or at room temperature for 2 h. The following primary antibodies were used: mouse anti-FLAG 1:1000 (F3165, Sigma-Aldrich), rabbit anti-FLAG 1:2000 (F7425, Sigma-Aldrich), mouse anti-GFP 1:1000 (AB290, Abcam, Cambridge, UK), rabbit anti-HA 1:1000 (71–5500, Invitrogen, Carlsbad, CA, USA), human anti-PrP D18 1:1000 (kindly provided by professor Stanley Prusiner, Institute for Neurodegenerative Diseases, UCSF, San Francisco [29]), mouse anti-PrP W226[28] (1:1000), mouse anti-β-actin peroxidase 1:5000 (A3854, Sigma-Aldrich), and rat anti-β-tubulin 1:1000 (ab6160, Abcam). For development, secondary antibodies conjugated with horseradish peroxidase (HRP) were used in combination with ECL reagent (GE Healthcare). The following secondary antibodies were used: goat anti-mouse HRP (P044701, Dako, Glostrup, Denmark), goat anti-rabbit HRP (P044801, Dako), protein A-HRP (18–160, Millipore, Billerica, MA, USA), goat anti-human HRP (31,410, Thermo Scientific), and goat anti-rat HRP (31,470, Thermo Scientific). Image acquisition was performed using the Alliance 4.7 software (UVITEC, Cambridge, UK). Quantification of protein bands from western blotting scans was performed with the ImageJ software (National Institute of Health, Bethesda, MD, USA).

Immunofluorescence

HEK293T cells were cultured on 15-mm glass coverslips and fixed in 4% paraformaldehyde at 48 or 72 after transfection. Cells were blocked in 5% NGS (005–000-121, Jackson Immunoresearch, West Grove, PA, USA) and 0.3% Triton X-100 in PBS and incubated at 4 °C overnight primary antibody. The following primary antibodies were used: mouse anti-FLAG 1:1000 (Sigma-Aldrich), rabbit anti-FLAG 1:1000 (Sigma-Aldrich), mouse anti-PrP W226[28] (1:1000), rabbit anti-HA 1:1000 (Invitrogen), and mouse anti-p62 1:500 (610,833, BD Biosciences, San Jose, CA, USA). After washes in PBS 1X, cells were



incubated with fluorophore-conjugated secondary antibodies or biotin-labeled for 60 min at RT, followed by 60 min incubation in streptavidin. The following secondary antibodies were used: anti-Mouse IgG (H + L) Alexa Fluor® 488 1:500 (A-11001, Life Technologies), anti-Mouse IgG (H + L) Alexa Fluor® 594 conjugate 1:500 (A-11005, Life Technologies), anti-rabbit IgG (H + L) Alexa Fluor® 594 1:500 (A-11012, Life Technologies), anti-Mouse IgG (H + L) Biotin 1:100 (SAB4600004, Sigma-Aldrich), anti-Rabbit IgG (H + L) Biotin 1:100 (SAB4600006, Sigma-Aldrich), and Alexa Fluor® 647 Streptavidin 1:100 (S21347, Life Technologies). GFP was detected by auto fluorescence at 488 laser. For nuclear staining, cells were incubated with 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) (D9432, Sigma-Aldrich) for 5 min. Cells were washed and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images (1024 × 1024 pixels) were acquired with confocal microscope (Nikon D-Eclipse C1, Tokyo, Japan) with a 40 × Oil N.A: 1.30 objective and additionally magnified 4 ×.

◂**Fig. 1** TRAF6 interacts with PrP^C. **a** HEK293T cells were transfected with PrP^C alone or with FLAG-TRAF6 constructs and left untreated, treated with 5 μM MG132 or with 10 μM Lactacystin for 16 h. Lysates were immunoprecipitated (IP) with anti-FLAG agarose resin, and bound proteins were revealed by immunoblot (IB) with anti-PrP D18 and anti-FLAG antibodies. Lysates were tested for the expression of PrP^C and FLAG-TRAF6 proteins. β-tubulin was used as loading control ($n=11$). **b** HEK293T cells were transfected with GFP-TRAF6 alone or with FLAG-PrP^C constructs and left untreated, treated with 5 μM MG132 or with 10 μM Lactacystin for 16 h. Lysates were immunoprecipitated (IP) with anti-FLAG agarose resin, and bound proteins were revealed by immunoblot (IB) with anti-GFP and anti-FLAG antibodies. Lysates were tested for the expression of FLAG-PrP^C and GFP-TRAF6 proteins. β-tubulin was used as loading control ($n=6$). **c** HEK293T cells were transfected with FLAG-TRAF6 alone or with PrP^C constructs and treated with 5 μM MG132 for 16 h. Lysates were immunoprecipitated (IP) with N-terminal anti-PrP DE10 (epitope 41–56) ($n=3$) antibody, and bound proteins were revealed by immunoblot (IB) with anti-FLAG and subsequently anti-PrP D18 antibodies. Lysates were tested for the expression of PrP^C and FLAG-TRAF6 proteins. β-tubulin was used as loading control. **d** C57BL/6 J mouse brains were lysed and used for immunoprecipitated endogenous TRAF6 and PrP^C proteins. Lysates were immunoprecipitated (IP) with anti-TRAF6 antibody, and bound proteins were revealed by immunoblot (IB) with anti-PrP D18 antibody. Lysates were tested for the expression of PrP^C and TRAF6 proteins. β-tubulin was used as loading control. IgG were used as negative control. The heavy chains band is marked with an *asterisk* ($n=3$). **e** HEK293T cells were transfected with FLAG-PrP^C and GFP-TRAF6 constructs and treated with 5 μM MG132 for 16 h. Images were acquired at 48 or 72 h after transfection. Immunofluorescence was performed with anti-FLAG (PrP^C) antibody. GFP-TRAF6 was revealed by GFP autofluorescence. Nuclei were marked by DAPI (4,6-diamidino-2-phenylindole). Scale bar 20 μm ($n=3$). For clarity, Fig. 1 displays cropped images of blots; each crop is performed on one individual gel. Molecular size markers are indicated

Statistical Analysis

All data were obtained by at least three independent experiments. Data represent the mean ± S.D.; each group was compared individually with the reference control group using Student's *t*-test (Microsoft Excel software, Microsoft, Redmond, WA, USA). Statistical significance differences to reference samples were indicated.

Results

The E3 Ubiquitin Ligase TRAF6 Interacts with PrP^C

To investigate whether TRAF6 might have a role in PrP^C homeostasis, we performed immunoprecipitation experiments to evaluate the ability of the two proteins to interact. We transfected HEK293T cells with a construct encoding for PrP^C and FLAG-TRAF6 or a FLAG-empty vector, as control. Although the level of interacting proteins is low, we could find a specific binding between TRAF6 and full-length PrP^C (Fig. 1a, untreated). Previously, we have shown that proteasome inhibition enhances the association of TRAF6 to proteins associated to neurodegenerative diseases [23, 24]. In addition, it is known that PrP^C levels are regulated by the proteolytic activity of the proteasome [10]. Therefore, we analyzed the binding capability of TRAF6 and PrP^C in conditions of proteasome block, with reversible (MG132) and irreversible (Lactacystin) inhibitors. Coimmunoprecipitation in cells treated with MG132 revealed an enhanced binding of TRAF6 to PrP^C (Fig. 1a). Similar results were obtained using Lactacystin (Fig. 1a). Interaction data, in untreated and treated conditions, were confirmed by reverse coimmunoprecipitation, using FLAG-tagged PrP^C and GFP-tagged TRAF6 (Fig. 1b). To avoid possible artefacts due to the FLAG insertion within PrP^C, we repeated the reverse experiments using an untagged version of PrP^C, fully confirming the results (Fig. 1c).

To further validate the significance of TRAF6 binding to PrP^C, we analyzed the interaction of endogenous proteins in vivo. Using brain from C57BL/6 J 6-month-old mice, we found that endogenous TRAF6 binds to endogenous PrP^C also in these conditions (Fig. 1d). Interestingly, in all our experiments (overexpression or endogenous), all glycosylated as well as unglycosylated forms of PrP^C can associate to TRAF6.

It is known that a portion of PrP^C normally relocates from the secretory pathway to the cytosolic space [30–32], and this event is increased upon PrP^{Sc} infection or when the proteasome activity is impaired [32–36]. Since TRAF6 is physiologically expressed in the cytosol [37–40], we followed by immunofluorescence where the two proteins could interact. To help visualization, we used TRAF6 fused to autofluorescent GFP and the FLAG-tagged version of PrP^C (Fig. 1e). We monitored protein localization at 48 and 72 h after transfection. As expected, we observed signals for PrP^C at the plasma membrane and for TRAF6 in the cytosol, mainly in foci. Interestingly, we found that FLAG-PrP^C is also located in the cytoplasm, where it accumulates in foci co-localizing with TRAF6 (Fig. 1e). Furthermore, every time we transfected both GFP-TRAF6 and FLAG-PrP^C constructs, we observed a co-localization of PrP^C and TRAF6. Similar results were obtained with an untagged version of PrP^C (data not shown).

Altogether, these results indicate that the E3 ligase TRAF6 and PrP^C are close partners in the cells.

TRAF6 Interacts with a Cytosolic Form of PrP^C

To further support the cytoplasmic route for TRAF6 and PrP^C interaction, we forced cytoplasmic localization using a 40–231 mutant (cyPrP). This form of cyPrP is devoid of ER translocation [41, 42] and plasma membrane exposure [43] due to the selectively deletion of the N-terminal ER-sequence and of the C-terminal GPI-anchor sequence [44]. We transfected HEK293T cells, which do not express endogenous full-length PrP, with untagged PrP^C and FLAG-TRAF6.

Immunofluorescence indicated that, as expected, cyPrP mutant is excluded from the plasma membrane and localized into the cell cytoplasm, where it concentrates in perinuclear regions. At this site, partial co-localization occurs with aggregated structures containing TRAF6 (Fig. 2a). We used this experimental setting to perform coimmunoprecipitation experiments. cyPrP appears as a unique unglycosylated band of approximately 15KDa. In untreated conditions, its binding to TRAF6 is detectable at low but reproducible levels. Treatment with proteasome inhibitors enhanced TRAF6-cyPrP association, mirroring the pattern observed for the full-length protein (Fig. 2b). The interaction was further confirmed by reverse coimmunoprecipitation using N-terminal specific DE10 anti-PrP antibody that specifically recognizes epitope aa41-aa52 (Fig. 2c).

TRAF6 Facilitates PrP^C Ubiquitination Independently of Its E3 Ligase Activity

The interaction between PrP^C and TRAF6 and their co-localization in the cytoplasm raises the possibility that PrP^C might be a substrate of TRAF6 E3 ubiquitin ligase activity. To test this hypothesis, we performed ubiquitination assays in HEK293T cells. Cells were transfected with HA-Ubiquitin and PrP^C with or without FLAG-TRAF6, in conditions of proteasome inhibition. A dominant-negative TRAF6 mutant deleted of the N-terminal E3 RING domain (DN) and incapable of mediating substrate ubiquitination, thus incapable of mediating downstream signaling, was also included as control [45, 46]. First, we observed that, in the presence of an excess of free ubiquitin, the PrP appears mildly ubiquitinated, as shown by the presence of high molecular weight forms of the protein (Fig. 3). This is compatible with the activity of endogenous E3 ubiquitin ligases in the cells. Overexpression of TRAF6 enhanced the accumulation of poly-ubiquitinated PrP^C. This effect was only partly repressed when TRAF6-DN mutant was used (Fig. 3). These results indicate that TRAF6 appears to control the ubiquitination of PrP^C only partially, as ubiquitinated species of the PrP^C can still be detected when TRAF6 ligase-deficient mutant is used.

TRAF6 Promotes the Accumulation of PrP^C into Insoluble Fraction

Since TRAF6 has been previously shown to alter solubility of Parkinson's disease-associated α-synuclein, synphillin,

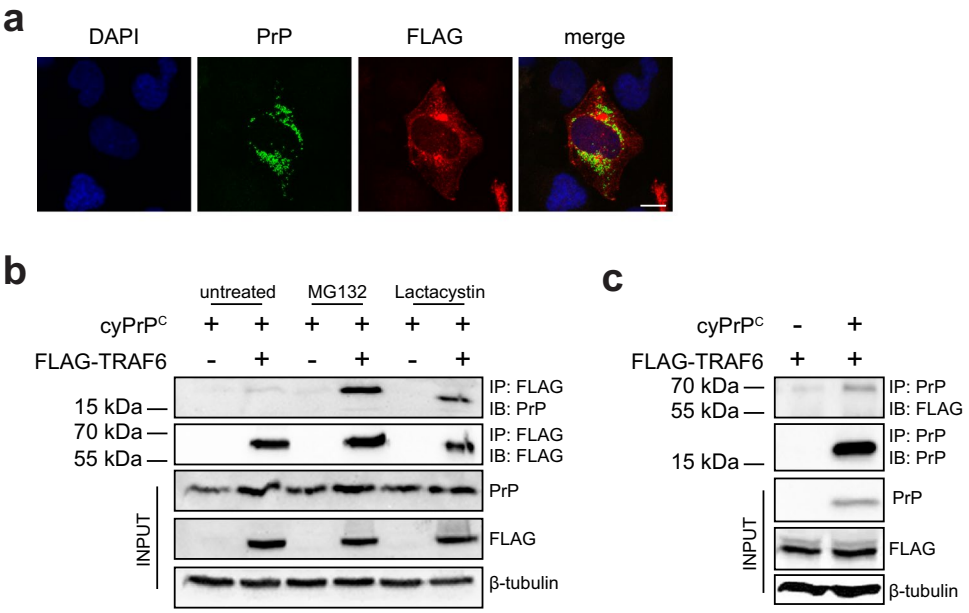


Fig. 2 TRAF6 interacts with cyPrP. **a** HEK293T cells were transfected with cyPrP and FLAG-TRAF6 constructs and treated with 5 μM MG132 for 16 h. Double immunofluorescence was performed with anti-PrP W226 and anti-FLAG (TRAF6) antibodies. Nuclei were marked by DAPI. Scale bar 20 μm ($n=3$). **b** HEK293T cells were transfected with cyPrP alone or with FLAG-TRAF6 constructs and left untreated or treated with 5 μM MG132 or with 10 μM Lactacystin for 16 h, as indicated. Lysates were immunoprecipitated (IP) with anti-FLAG agarose resin, and bound proteins were revealed by immunoblot (IB) with anti-PrP D18 and anti-FLAG antibodies. Lysates were tested for the expression of cyPrP and FLAG-TRAF6 proteins. β-tubulin was used as loading control ($n=6$). **c** HEK293T cells were transfected with FLAG-TRAF6 alone or with cyPrP constructs and treated with 5 μM MG132 for 16 h. Lysates were immunoprecipitated (IP) with N-terminal anti-PrP DE10 (epitope 41–56) antibody. Bound proteins were revealed by immunoblot (IB) with anti-FLAG and anti-PrP D18 antibodies. Lysates were tested for the expression of cyPrP and FLAG-TRAF6 proteins. β-tubulin was used as loading control ($n=2$) For clarity, Fig. 2 displays cropped images of blots; each crop is performed on one individual gel. Molecular size markers are indicated

and mutant DJ-1 [24, 47, 48], we investigated its effect on PrP^C biochemical status. We performed detergent-solubility fractionation assay in HEK293T cells. Cells were transfected with FLAG-PrP^C and HA-Ubiquitin in the presence or absence of GFP wild-type TRAF6 or DN mutant. Lysates were separated in Triton X-100 soluble and insoluble fractions. We found that PrP^C is mainly present in the insoluble fraction, compatible with its association to plasma membranes and/or cytoplasmic aggregated structures. Overexpression of ubiquitin and TRAF6 strongly enhanced the accumulation of FLAG-PrP^C in this compartment (Fig. 4a). TRAF6 effect is reversed when E3 ligase DN mutant is used. Densitometric analysis indicates that there is a statistically significant increase of FLAG-PrP^C in the presence of HA-Ubiquitin and GFP-TRAF6, but not with its ligase-deficient form (Fig. 4b). These results imply a role for TRAF6 and its ubiquitin ligase activity in modulating PrP^C biochemical properties.

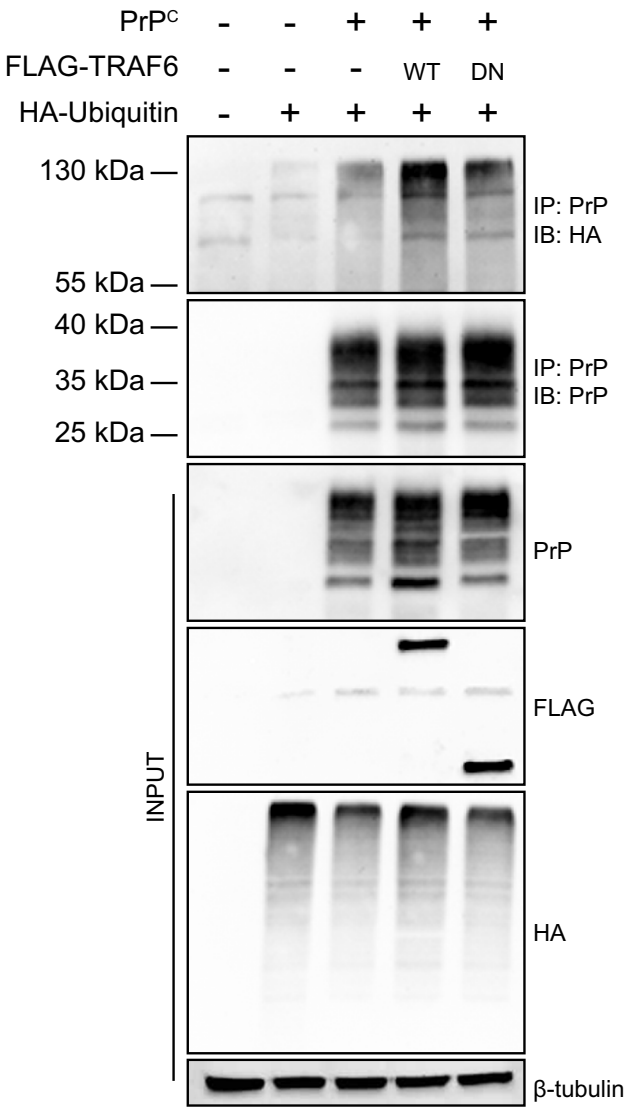


Fig. 3 TRAF6 facilitates PrP^C ubiquitination independently of its E3 ligase activity. HEK293T cells were transfected with PrP^C, FLAG-TRAF6 or FLAG-TRAF6 DN, and HA-Ubiquitin constructs and treated with 10 µM MG132 for 3 h. Ubiquitined PrP^C was visualized with anti-HA antibody after immunoprecipitation with anti-PrP W226 antibody. Immunoprecipitated PrP^C was verified with anti-PrP D18 antibody. Lysates were tested for the expression of HA-Ubiquitin, PrP^C, and FLAG-TRAF6 proteins. β-tubulin was used as loading control ($n=4$) For clarity, cropped images of blots are displayed. Molecular size markers are indicated

TRAF6 E3 Ligase Activity Contributes to Recruit PrP^C into Cytoplasmic Aggresome-Like Structures Containing p62/SQSM1

In cellular models of Parkinson's disease and Huntington's disease, TRAF6-mediated ubiquitination controls the aggregation propensity of disease-associated misfolded proteins [23, 24]. To functionally link TRAF6 and PrP^C aggregates, we monitored protein localization by immunofluorescence. HEK293T cells were transfected with HA-Ubiquitin, FLAG-PrP^C, and GFP-TRAF6 or DN mutant and treated with proteasome inhibitor. Aggregates were analyzed by double immunofluorescence coupled with GFP autofluorescence (Fig. 5a). When transfected alone, FLAG-PrP^C was prevalently localized on the cellular membrane, as expected. Interestingly, overexpression of HA-ubiquitin did not alter PrP^C localization, in conditions where a low level of PrP^C ubiquitination was observed. This suggests that a certain threshold of ubiquitination needs to be obtained to effectively trigger aggregates assembly. This was further confirmed by a diffuse staining of ubiquitin itself within the cytoplasm. Addition of ligase competent TRAF6 promoted the coalescence of PrP^C into discrete perinuclear aggregates. These were positively stained for TRAF6 itself and ubiquitin. No aggregates were observed when TRAF6 DN was used, proving that inclusion formation requires ligase competent TRAF6 to recruit ubiquitin and PrP^C (Fig. 5a).

When UPS is disrupted, ubiquitinated and misfolded proteins tend to accumulate into perinuclear region where they coalesce in larger structures termed aggresomes [49–52]. The ubiquitin-binding p62/SQSTM1 protein has been shown to play a pivotal role in aggresome formation. Indeed, p62 has been involved in most neurodegenerative diseases, including prion disease [53, 54]. Protein aggregates isolated from Alzheimer's disease brains contain ubiquitin, p62, and TRAF6 [55], and p62 co-localizes with PrP^{Sc} in infected cells through ubiquitin binding [56]. To define the nature of PrP^C aggregates formed by TRAF6, HEK293T cells were transfected with FLAG-PrP^C and GFP-TRAF6 and stained for endogenous p62/SQSTM1 (Fig. 5b). In absence of TRAF6, p62 staining is mainly cytoplasmic, with some localization also at the plasma membrane, where PrP^C is also located. Upon TRAF6 overexpression, but not with ligase

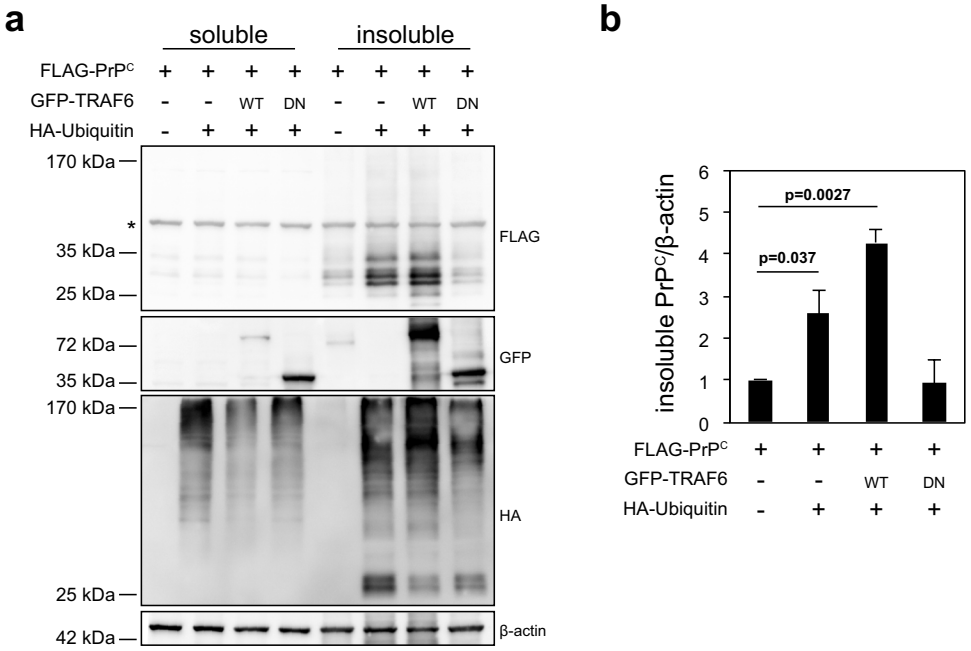


Fig. 4 TRAF6 promotes the accumulation of ubiquitined PrP^C in insoluble fraction. **a** HEK293T cells were transfected with FLAG-PrP^C, GFP-TRAF6, and HA-Ubiquitin constructs and treated with 10 µM MG132 for 3 h. Western blotting analysis of soluble and insoluble fractionation was carried out with anti-FLAG (PrP^C) antibody. Lysates were tested for the expression of GFP-TRAF6 and HA-Ubiquitin proteins. β-actin was used as a loading control. Asterisk indicates unspecific background ($n=3$). For clarity, cropped images of blots are displayed. Molecular size markers are indicated on the left. **b** Densitometric analysis of insoluble FLAG (PrP^C) level. Insoluble FLAG-PrP^C level was normalized to β-actin. Cells transfected with FLAG-PrP^C alone was used as reference and set to 1. Values are mean ± S.D. Data were evaluated statistically by Student's *t*-test. Resulting *P*-values are indicated ($n=3$)

incompetent mutant, all components coalesce into larger cytoplasmic aggregates, stained for PrP^C, TRAF6, and p62 and fully resembling aggresome-like structures observed in prion-infected cells (Fig. 5b) [56].

Together, these data provide evidence that TRAF6 E3 ligase activity is involved in controlling the formation and composition of PrP^C cytoplasmic aggresome-like structures.

Discussion

In the present study, we show that the E3 ubiquitin ligase TRAF6 is a novel interaction partner of PrP^C, an intrinsically disordered protein whose scrapie conformational isoforms are the causative agents of prion diseases. Results with N-terminally truncated variant of PrP^C suggest that binding to TRAF6 should occur in the cytoplasm where the two proteins can co-localize. The presence of all glycosylated isoform of PrP^C in our immunoprecipitation experiments and the membrane localization of PrP^C upon overexpression suggest that cytosolic localization could result, at least in part, from the cell membrane recycling. Additional portion of PrP^C can originate from ER retro-translocation [42, 55, 57]. Indeed, in both cases, it is more likely that free cytosolic-PrP^C rather than vesicle-contained PrP^C interacts with TRAF6, even if a sub-membrane localization of TRAF6 has been shown for TRAF6 signaling recruitment to lipid rafts [58–62]. Involvement of TRAF6 in the internalization of PrP^C, normally associated to lipid rafts [63], should not be excluded.

Using a cytosolic variant of PrP^C and anti-PrP^C antibodies targeting different regions of the protein, we show that the C-terminal globular region of PrP^C is responsible for the interaction with TRAF6. Interestingly, the same region has been functionally implicated in PrP^C ubiquitination by the gp78 E3 ligase, as PrP^C C-terminal deletion mutants show compromised ubiquitination even if gp78 is still capable of binding [12]. Since lysine residues, required for covalent attachment of ubiquitin to the substrate, are not present in PrP^C C-terminal region, it is likely that this domain regulates partially PrP^C ubiquitination through direct binding to E3 ligases (as in the case of TRAF6) or by controlling its accessibility (as for gp78).

To our surprise, while binding TRAF6 in vitro and in vivo, PrP^C is not a direct substrate of TRAF6 E3 ligase activity, as poly-ubiquitinated forms of PrP^C are observed even in the presence of a ligase-deficient mutant. Indeed, overexpression of ubiquitin alone is sufficient to trigger covalent linkage to PrP^C, demonstrating that additional endogenous E3 ligases are active, at least in HEK cells.

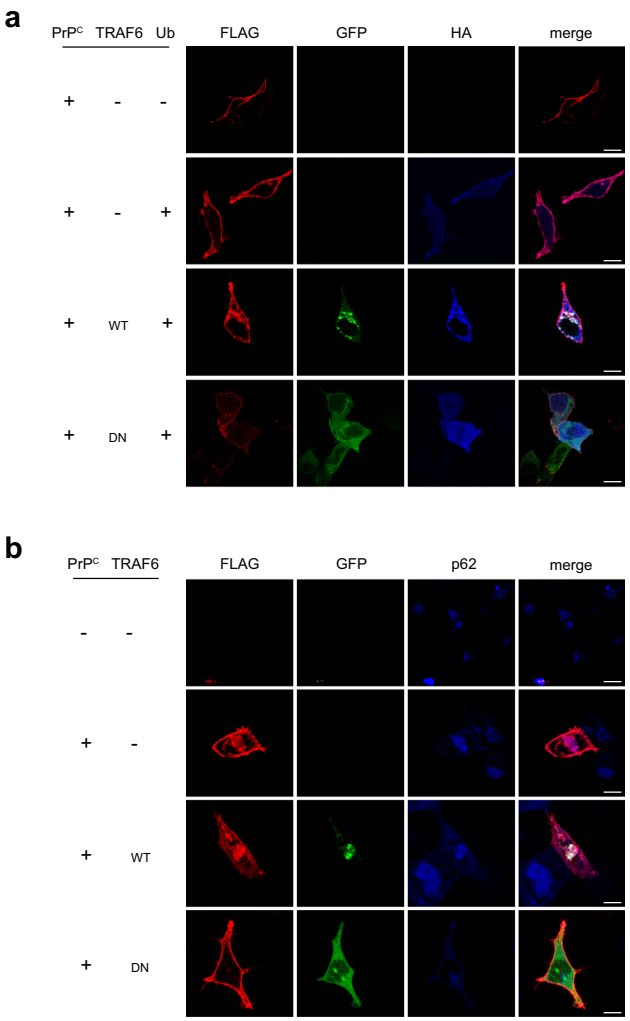


Fig. 5 TRAF6-mediated ubiquitination recruits PrP^C in aggresome-like structures. **a** HEK293T cells were transfected with FLAG-PrP^C alone or with GFP-TRAF6 and HA-Ubiquitin constructs and treated with 5 µM MG132 for 16 h. Double immunofluorescence was performed with anti-FLAG (PrP^C) and anti-HA (Ubiquitin) antibodies. GFP-TRAF6 was revealed by GFP autofluorescence. Nuclei were marked by DAPI. Scale bar 20 µm ($n=2$). **b** HEK293T cells were transfected with FLAG-PrP^C alone or with GFP-TRAF6 and treated with 5 µM MG132 for 16 h. Double immunofluorescence was performed with anti-FLAG (PrP^C) and anti-p62 antibodies. GFP-TRAF6 was revealed by GFP autofluorescence. Nuclei were marked by DAPI. Scale bar 20 µm ($n=3$)

These results are peculiar for PrP^C relative to what we had previously found for other misfolded proteins associated to neurodegenerative diseases [23, 24]. Interestingly, while TRAF6 modulates pathological aggregates containing mutant DJ-1 and mutant HTT, this protein can also interact and ubiquitinate physiological forms of α-synuclein and HTT, with yet unknown consequences unrelated to inclusion formation.

Diverse signaling modes are operated by TRAF6 by direct and indirect means in an E3 ligase dependent and independent manner. For instance, in the brain, TRAF6 mediates non-proteolytic ubiquitination of PSD-95 and controls scaffolding and clustering at the synapse. Direct ubiquitination of PSD-95 is required for proper recruitment of synapsin and glutamate receptors at the synapse [21]. Intriguingly, both PSD-95 and glutamate receptors directly interact with PrP^C, thus representing intriguing candidates for a functional interplay with TRAF6 at the plasma membrane. Indirect signaling mediated by TRAF6 has been shown to operate downstream of the TLRs (Toll-like receptors) and IL-1R to induce nontranscriptional priming of the NLRP3 (NLR family pyrin domain containing 3) inflammasome [64]. In this case, TRAF6 E3 ligase activity is required for multi-protein complex oligomerization and NLRP3 activation, but downstream signaling occurs without any direct interaction or active ubiquitination of NLRP3 by TRAF6. At the same time, transcriptional responses downstream TLRs and IL-1R in the MyD88 signaling network also involve TRAF6 but are only partly dependent on its E3 ligase activity. Overall, these and other results suggest a "scaffolding" role for TRAF6 in mediating activation of various pathways depending on the cellular context. In the case of PrP^C, TRAF6 binding facilitates prion protein ubiquitination in an E3-independent mode, while ubiquitin ligase activity is required for promoting its recruitment to aggresomes. We can envision a model in which TRAF6 E3 ligase activity towards itself and/or a yet unknown protein is required for a cascade of events that ultimately controls PrP^C partitioning within the cytoplasm. We presently cannot establish the exact temporal sequence of these events, but clearly a two-hit model is in place. In accordance, we observe a potent dominant-negative effect on PrP^C solubility when TRAF6 E3 ligase mutant is used. It is possible that TRAF6-mediated ubiquitination represents the first triggering event that occurs prior to PrP^C ubiquitination and its sequestration in already partially formed aggresomes. But it is equally conceivable that partially ubiquitinated PrP^C is necessary to activate TRAF6 and further reinforce its ubiquitination and aggregation. This latter mechanism has been shown in IRF3 activation in response to viral infection [17, 18]. Pre-formed MAVS prions/oligomers are required to activate TRAF6 signaling. MAVS bind TRAF6 but are not substrate of its enzymatic activity. Instead, aggregated MAVS form a docking site to recruit multiple ubiquitin E3s, and each ligase targets ubiquitination of distinct proteins. The formation of this multi-protein complex containing prion-like aggregates, ubiquitin, and E3 ubiquitin ligases functions as cellular "signalosome."

The functional consequences of TRAF6-mediated control of PrP^C solubility and localization and its relevance to prion pathology are presently unknown. Sequestration of neurodegenerative related proteins into aggresome structures is known to have a cytoprotective response acting to sequester

potentially toxic misfolded proteins [50, 65]. Moreover, p62/SQSTM1-positive aggregates are closely involved in the macroautophagy process in neurodegeneration [66]. Our observation of p62/SQSTM1-positive PrP^C aggregates leads to the hypothesis of a cellular clearance mechanism that function also in physiological conditions and that implies PrP^C ubiquitination and binding to TRAF6 prior to aggregation/sequestration in p62/SQSTM1 aggresomes. This finding is also supported by our previous evidence of TRAF6 relation with the physiological form of synuclein and HTT proteins [23, 24]. Altogether, our results suggest the involvement of TRAF6 in the aggregation and ubiquitylation of normal and mutated neurodegenerative-associated proteins.

It remains to be elucidated if TRAF6 represent a sort of "priming" signal for endogenous PrP^C to be susceptible to prion conversion or, alternatively, whether it is a way to sequester the prion protein and protect cells from prion infection. Interaction of endogenous proteins occurs in the brain of mice, where both neurons and immune cells reside. TRAF6 is known to mediate signaling in immune cells, which are one of the primary routes for PrP^{Sc} infection in iatrogenic prion diseases. Also, TRAF6 is known to modulate formation of aggregates of proteins associated to neurodegenerative diseases and accumulate into diseased neurons. In the future, it will be important to further dissect the functional interplay between PrP^C and TRAF6 in immune cells and neurons in physiological conditions and under PrP^{Sc} infection.

Our results reveal a novel functional interplay between TRAF6 and PrP^C and suggest that TRAF6-mediated signaling events might contribute to designing alternative strategies for prion diseases.

Abbreviations PrP^C: Cellular prion protein; PrP^{Sc}: Scrapie prion protein; TRAF6: Tumor necrosis factor receptor-associated factor 6; DN: Deleted of the N terminus

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Author Contribution LM and MC designed and performed the experiments, analyzed the data, and wrote the manuscript; FP and SG analyzed the data and the manuscript; SZ designed the experiments, analyzed the results, and wrote the manuscript; GL designed the experiments, analyzed the results, and wrote the manuscript.

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Availability of Data and Materials Data and materials used in this study and supporting the conclusions of this article are included within the article.

Code Availability Not applicable.

Declarations

Ethics Approval and Consent to Participate All animal experiments were performed in accordance with European guidelines for animal care (European Community Council Directive, November 24, 1986 86/609/EEC) and following Italian Board of Health permissions (Law n. 116/1992).

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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