Original Article

Praja1 RING-finger E3 ubiquitin ligase suppresses neuronal cytoplasmic TDP-43 aggregate formation

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Transactivation response DNA-binding protein of 43 kDa (TDP-43) is a major constituent of cytoplasmic aggregates in neuronal and glial cells in cases of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). We have previously shown neuronal cytoplasmic aggregate formation induced by recombinant adenoviruses expressing human wild-type and C-terminal fragment (CTF) TDP-43 under the condition of proteasome inhibition in vitro and in vivo. In the present study, we demonstrated that the formation of the adenoviral TDP-43 aggregates was markedly suppressed in rat neural stem cell-derived neuronal cells by co-infection of an adenovirus expressing heat shock transcription factor 1 (HSF1), a master regulator of heat shock response. We performed DNA microarray analysis and searched several candidate molecules, located downstream of HSF1, which counteract TDP-43 aggregate formation. Among these, we identified Praja1 RING-finger E3 ubiquitin ligase (PJA1) as a suppressor of phosphorylation and aggregate formation of TDP-43. Co-immunoprecipitation assay revealed that PJA1 binds to CTF TDP-43 and the E2-conjugating enzyme UBE2E3. PJA1 also suppressed formation of cytoplasmic phosphorylated TDP-43 aggregates in mouse facial motor neurons in vivo. Furthermore, phosphorylated TDP-43 aggregates were detected in PJA1-immunoreactive human ALS motor neurons. These results indicate that PJA1 is one of the principal E3 ubiquitin ligases for TDP-43 to counteract its aggregation propensity and could be a potential therapeutic target for ALS and FTLD.

Key words: adenovirus, amyotrophic lateral sclerosis, frontotemporal lobar degeneration, Praja1, TDP-43.

INTRODUCTION

Formation of neuronal and glial cytoplasmic aggregates containing transactivation response DNA-binding protein of 43 kDa (TDP-43), a DNA/RNA-binding protein that localizes predominantly to the nucleus and shuttles between the cytoplasm and nucleus, is one of the pathological hallmarks of amyotrophic lateral sclerosis (ALS) or frontotemporal lobar degeneration with TDP-43-positive inclusions (FTLD-TDP).1–5 In pathological situations, full-length wild-type (WT) TDP-43 is cleaved to form a 25-kDa C-terminal fragment of TDP-43 (CTF TDP-43) lacking nuclear localization signal (NLS) that is localized to the cytoplasm. The C-terminal region of TDP-43 contains a prion-like domain that have aggregation-prone properties. Cytoplasmic TDP-43 aggregates are phosphorylated, ubiquitinated, and likely composed of WT and CTF TDP-43.1–5 The formation of cytoplasmic TDP-43 aggregates has been attributed to multiple factors, including impaired RNA and/or protein metabolism, stress granule formation, and oxidative stress.6 Among these, impairment of proteolytic machineries, including proteasome, endosome and autophagy systems, has been shown to play a key role in TDP-43 aggregate formation.1,7 We have previously shown that proteasome inhibition enhanced the formation of adenovirally expressed TDP-43 cytoplasmic aggregates both in vitro and in vivo, suggesting that impairment of protein degradation...
pathways accelerates the formation of TDP-43-positive aggregates. In vitro time-lapse imaging analyses revealed growing cytoplasmic aggregates in the TDP-43 adenovirus-infected neuronal and glial cells, followed by collapse of the cells. These TDP-43 aggregates remained insoluble in culture media, consisted of sarkosyl-insoluble granular materials, and contained phosphorylated TDP-43. The released aggregates were incorporated into neighboring neuronal cells, indicating cell-to-cell spreading that is in line with the prion-like propagation hypothesis that explains progression of neurodegenerative diseases such as prion disease, Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease, as well as ALS/FILDL.

Recently, several reports have demonstrated that heat shock transcription factor 1 (HSF1), a master regulator of heat shock response, and its downstream heat shock proteins, including heat shock protein 70 (HSP70), HSPB8, HSP40/DNAJB2a, HSP40/DNAJB1, HSP27 and clusterin, mediate clearance of aggregated TDP-43 species.

In the present study, we demonstrate that the formation of the adenoviral TDP-43 aggregates was markedly suppressed by co-infection of an adenovirus expressing HSF1. DNA microarray analysis was performed to identify candidate molecules located downstream of HSF1 that counteract TDP-43 aggregate formation. Among these, we identified Pja1 RING-finger E3 ubiquitin ligase (PJA1) as a suppressor of phosphorylation and aggregate formation of TDP-43.

MATERIALS AND METHODS
Adult rat neural stem cell line 1464R

All experiments were performed in accordance with Japanese National Guidelines and Regulations, and were approved by the Biosafety Committee and the Animal Care and Use Committee of the Kyorin University Faculty of Health Sciences. The 1464R adult rat neural stem cell line was established as previously described (Applied Biological Materials, Richmond, Canada) and cells were cultured in growth medium composed of Neurobasal medium (#21103-049; Thermo Fisher Scientific, Waltham, MA, USA) containing 2 mM L-glutamine (#25303-081; Thermo Fisher), 2% B27 supplement (#17504-044; Thermo Fisher), 10 ng/mL fibroblast growth factor 2 (FGF2) (#FGF2; Sigma, St. Louis, MO, USA), 10 ng/mL epidermal growth factor (EGF) (#E9644; Sigma), 50 units/mL penicillin and 50 μg/mL streptomycin (#15070-063; Thermo Fisher) on 10-cm dishes coated with poly(2-hydroxyethylmethacrylate) (PHEMA) (#P3932; Sigma) to prevent cell attachment. Cells were maintained in 5% CO2 at 37°C. The 1464R cells formed typical neurospheres, and were mechanically dissociated, serially passaged in the same medium twice a week, and cultured further for more than 50 passages with no obvious morphological alterations. To differentiate 1464R cells into neuronal and glial cells, dissociated 1464R cells were seeded on poly-L-lysine (PLL) (#P1524; Sigma) -coated 12-well plates or 9-mm ACLAR round coverslips (Allied Fibers & Plastics, Pottsville, PA, USA) at a density of 4–5 × 10^5 cells per well and 1–2 × 10^4 cells per coverslip, respectively, and maintained in differentiation medium composed of F12 medium (#11765-054; Thermo Fisher) containing 5% fetal bovine serum (Moregate, Australia), 0.5% N2 supplement (#17502-048; Thermo Fisher), 1% B27 supplement, 1 μM all-trans retinoic acid (ATRA) (#R2625; Sigma), 50 units/mL penicillin, and 50 μg/mL streptomycin in 5% CO2 at 37°C. For transient transfection of plasmids, 1464R cells dissociated and seeded on coverslips in differentiation medium were immediately transfected with plasmids as described below using Fugene 6 Transfection Reagent (#E2691; Promega, Madison, WI, USA) according to the manufacturer’s instructions. Two days after, the cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and examined under an Olympus AX80TR microscope equipped with a DP70 CCD camera.

Adenovirus infection

Preparation of recombinant adenovirus vectors encoding DsRed-tagged full-length human WT (AxDsRhWT TDP43; RIKEN DNA Bank Japan; #RDB15499) and CTF (208-414aa of TDP-43) (AxDsRhCTFTDP43; RIKEN #RDB15500) human TDP-43 cDNAs have been previously described. Adenovirus vectors encoding DsRed-tagged WT and CTF human TDP-43 containing FLAG tag (5′-CTTTCATCGTCGTTTAGTGC-3′) at the 3′-ends (AxDsRhWT TDP43FLAG, AxDsRhCTFTDP43FLAG) were newly constructed for co-immunoprecipitation (Co-IP) experiments as described below. For the construction of adenovirus encoding EGFP-tagged WT and dominant negative human HSF1 and candidate genes (Table 1), cDNAs obtained from HEK 293 and 1464R cells by reverse transcription-polymerase chain reaction (RT-PCR) using primers as listed in Table 2 were successively cloned into pEFGPN1-3 (Clontech, Palo Alto, CA, USA) and adenovirus cassette cosmid pAxCAtwit2 (TaKaRa, Osaka, Japan) as described. Human UBE2E3 cDNA was cloned into pcDNA3.1MycHis (V8020; Thermo Fisher) and the resulting UBE2E3MycHis fragment was subcloned into pAxCAtwit2. The cosmids were then transfected to 293 cells and recombinant adenovirus vectors were propagated and isolated from 293 cells, and purified using a ViraBind Adenovirus Purification Kit (#VPK-100; Cell Biologics, San Diego, CA, USA). Neurons and glial cells differentiated from 1464R cells on PLL-coated 6 or 12 well plates or 9-mm coverslips were infected with adenoviruses at a multiplicity of infection of 10. PJA1 suppresses TDP-43 aggregation

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Twenty-four hours later, the cells were refed with the media with or without MG-132 (#474790; Merck Millipore/Calbiochem, Burlington, MA, USA) and further incubated for 24 h. In some experiments, proteasome inhibitors lactacystin (#426100; Calbiochem), epoxomicin (#426100; Calbiochem) and bortezomib (#021-18901; FUJI FILM Wako, Osaka, Japan) were used instead of MG-132, and the effects of autophagy inhibitors 3-methyladenine (#M9281; Sigma), bafilomycin A1 (#B1793; Sigma) and wortmannin (#W1628; Sigma) were also examined. The treated cells on the dishes or coverslips were lysed for Western blotting or fixed for immunocytochemistry, respectively, as described below. DsRed or EGFP fluorescence in living cells was examined under an Olympus IX70 inverted fluorescence microscope equipped with a DP72 CCD camera prior to cell lysate preparation or fixation.

Cell fractionation, co-immunoprecipitation, and Western blot analysis

For cell fractionation, cultured cells in 12-well plates (4.5 × 10^5 cells/well) were lysed in 50 μL of...
radioimmunoprecipitation assay (RIPA) buffer, consisting of 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), supplemented with complete protease inhibitor (#11697498001; Sigma) and PhosSTOP phosphatase inhibitor (#4906845001; Sigma) cocktails, sonicated for 15 s twice, and incubated for 30 min on ice followed by centrifugation at 20 000 g for 90 min at 4°C. The supernatants and pellets were collected as RIPA-soluble and insoluble fractions, respectively, lysed in 1× SDS-sample buffer containing 2-mercaptoethanol (ME), and treated for 5 min at 95°C. Protein concentration of each supernatant fraction was determined with a Qubit Protein Assay Kit (Thermo Fisher).

For Co-IP, 1464R cells in 6-well plates (6 × 10⁵ cells/well) were lysed in 200 μL of IP buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5% glycerol) containing cOmplete, PhosSTOP inhibitor cocktails and de-ubiquitination inhibitor PR-619 (#16276; Cayman, Ann Arbor, MI, USA), incubated for 30 min on ice followed by centrifugation at 12000 g for 30 min at 4°C. Ten percent of the resulting supernatants was analyzed as the input cell lysate, and the remaining 90% of supernatants was incubated with anti-FLAG M2 affinity gel (#A2220; Sigma) for 3 h at room temperature by rotator shaker. The affinity gel was washed three times with Tris buffered saline (TBS) consisting of 50 mM Tris–HCl (pH 7.4) and 150 mM NaCl. The inputs and immunoprecipitates were lysed in 1× SDS-sample buffer containing 2-ME and treated for 5 min at 95°C.

Fractionated cell lysates (10–20 μg) or immunoprecipitates were electrophoresed on 4–20% SDS/polyacrylamide gels under reduced conditions and transferred to PVDF membrane (Atto, Tokyo, Japan). The blotted membrane was blocked with 3% skim milk and incubated overnight with rabbit anti-FLAG (#7425; Sigma), rabbit anti-TDP-43 C-terminus (405-414) (#TIP-TDP09; Cosmo Bio, Tokyo, Japan), rabbit anti-phosphorylated TDP-43 (pSer409/S410) (#TIP-PTD-P02; Cosmo Bio), rabbit anti-PJA1 (#17687-1-AP; ProteinTech Japan, Tokyo, Japan), rabbit anti-Myc Tag (#2278; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-ubiquitin K48 (#05-1307; Sigma), rabbit anti-UBE2D2/3 (#HPA003921; Sigma), rabbit anti-UBE2E3 (#15488-1-AP; ProteinTech), rabbit anti-UBE2K (#11834-3-AP; ProteinTech), and mouse monoclonal anti-GAPDH (#ab8245; Abcam, Cambridge, UK) antibodies at dilutions of 1:1000, followed by incubation with biotinylated anti-rabbit or anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA; 1:1000) for 1 h and streptavidin-alkaline phosphatase (#11089161001; Sigma; 1:1000) for 1 h. Reactions were visualized by color development using nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyolphosphate p-toluidine salt (BCIP) (#11681451001; Sigma). In some experiments, immunoblotted TDP-43 CTF and GAPDH (as loading control) bands were quantified by ImageJ software (NIH, USA).

Cell viability assay
For cell viability assay, differentiated 1464R cells were seeded on PLL-coated 96-well plates (5 × 10⁴ cells/well) and cultured in differentiation medium. After 4 days, the cells were infected with adenoviruses at an MOI of 50. Twenty-four hours later, the cells were refed with the media with or without 0.5–2 μM MG-132 (Sigma) and further incubated for 24–48 h. The cells were then incubated with cell counting kit-8 (CCK-8) solution (#CK04; DOJINDO, Tokyo, Japan; 10 μL/200 μL culture medium) for 4 h, and the absorbance at 450 nm was measured with a microplate reader.

Immunocytochemistry
Cells were fixed with 4% PFA in PBS, permeabilized with 100% methanol, washed with PBS, and immunostained overnight at 4°C with mouse monoclonal TuJ1 (#MAB1195; R&D systems, Minneapolis, MN, USA) at a dilution of 1:200. The cells were then incubated with Alexa Fluor 350-conjugated goat anti-mouse IgG (Thermo Fisher) at 1:400 dilution for 1 h at room temperature, followed by incubation for 15 min with 2 μg/mL Hoechst 33342 (Thermo Fisher). After washing, coverslips were mounted on glass slides with Gelvatol (20% glycerol/10% polyvinyl alcohol in 0.1 M Tris–HCl pH 8.0). Immunostained cells were examined under the Olympus AX80TR microscope equipped with a DP70 CCD camera.

Animal surgery and histological analysis
The experimental protocols were approved by the Animal Care and Use Committee of the Kyorin University Faculty of Health Sciences. Adult ICR male mice (8–10 weeks old, 40–45 g) were anesthetized with intraperitoneal injection of a combination anesthetic (M/M:B: 0.3/4/5) prepared with 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol.24 Under a dissecting microscope, the right facial nerve was exposed and 6 μL solution in total of recombinant adenoviruses (1 × 10⁹ plaque-forming units [pfu] each for combined injection) was slowly injected into the main trunk (marginal branch) of the facial nerve using a 33G microsyringe (Hamilton, Reno, NV, USA). The virus suspension was mixed with Evans Blue (0.01% final; Sigma) to confirm visually that the injection had been successfully performed.

Four days after operation, mice were anesthetized by inhalation with a lethal dose of isoflurane and transcardially perfused with 0.1 M phosphate buffer, pH 7.4 (PB) followed by 4% PFA in 0.1 M PB. The brain stem tissue containing facial nuclei was dissected,
immersion fixed in the same fixative, and cryoprotected in 30% sucrose in 0.1 M PB. Serial 10-μm-thick transverse sections were made by cryostat. To enhance EGFP detection in PJA1EGFP adenovirus-treated samples, cryostat sections were permeabilized with 100% methanol, washed with PBS, preincubated with 5% normal goat serum (Invitrogen) in PBS, and immunostained overnight at 4°C with rabbit anti-GFP (#ab6556; Abcam) at a dilution of 1:1000, followed by incubation with Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen) at a 1:1000 dilution for 1 h at room temperature. The sections were counterstained with 2 μg/mL Hoechst 33342 (Thermo Fisher), mounted with Gelvatol and examined under the Olympus AX80TR microscope equipped with a DP70 CCD camera. For motor neuron cell counting (n = 4), every fifth section at a thickness of 10 μm was picked up and at least 250 facial motor neurons having Hoechst 33342-counterstained nuclei were counted in five sections, so that these neurons were counted only once in every fifth section with a 40-μm interval.

Immunohistochemistry on human tissue sections

All experiments were approved by and performed under the guidelines of the Kyorin University Ethics Committee, the Tokyo Woman’s Medical University Ethics Committee and the Niigata University Ethics Committee. Informed consent was obtained from all individuals or their guardians before the autopsy analysis. Postmortem lumbar spinal cords from five patients definitely diagnosed as having sporadic ALS and from four non-diseased control cases were fixed with 10% buffered formalin and embedded in paraffin. Deparaffinized and rehydrated sections were processed with microwaving in citrate buffer, pH 6.0 for antigen retrieval.

For light microscopic immunohistochemistry, serial 6-μm-thick sections were quenched in 3% hydrogen peroxide, blocked with 5% skim milk/PBS and incubated overnight with rabbit anti-PJA1 (#HPA000595; Atlas Antibodies, Bromma, Sweden; 1:100), mouse monoclonal anti-phosphorylated TDP-43 (#TIP-PTDM01; Cosmo Bio; 1:2000), followed by incubation with a mixture of Alexa 488-conjugated goat anti-rabbit IgG and Alexa 568-conjugated anti-mouse IgG antibodies (Molecular Probes, Eugene, OR, USA) at a dilution of 1:1000 for 1 h. The sections were treated with Autofluorescence Eliminator Reagent (#2160; Merk Millipore, Burlington, MA, USA) according to manufacturer’s instructions to eliminate endogenous lipofuscin autofluorescence, mounted with VECTASHIELD mounting medium with 4’,6-diamidino-2-phenylindole (DAPI) nuclear stain (Vector), and examined under a Carl Zeiss LSM700 confocal laser scanning microscope.

Statistics

Values in each group were expressed as mean ± SD. To compare between two groups, the unpaired Student’s t-test and Mann–Whitney’s U-test were used for measured results. Values in three to eight groups of measured results were compared by one-way analysis of variance followed by post hoc Bonferroni correction. Statistical significance was considered when P-values were less than 0.05.

RESULTS

HSF1 suppresses cytoplasmic TDP-43 aggregate formation in 1464R neurons

In the present study, we used an adult rat neural stem cell line 1464R that we established to examine aggregate formation of adenovirally-induced TDP-43 as described previously. The 1464R cells differentiate predominantly (> 80%) into TuJ1-positive neurons and, to a lesser extent (< 20%), into glial fibrillary acidic protein (GFAP)-positive astrocytes and O4-positive oligodendrocytes in the presence of ATRA. These differentiated 1464R neurons and glial cells formed cytoplasmic aggregates of TDP-43 following co-infection of adenoviruses expressing DsRed-tagged WT and CTF TDP-43 in the presence of proteasome inhibitors. These cytoplasmic TDP-43 aggregates were strongly immunoreactive for phosphorylated TDP-43, p62, and ubiquitin, were ultrastructurally composed of non-membrane bound, electron-dense fine granular materials, and were detected in sarkosyl-insoluble or RIPA-insoluble fraction by Western blot analysis, which recapitulates pathological features of TDP-43 aggregates observed in human ALS and FTLD.

In this study, we first examined the suppressing effects of an adenovirus expressing HSF1 on adenovirus-induced cytoplasmic TDP-43 aggregate formation in the differentiated 1464R neurons. The 1464R cells were differentiated in the presence of ATRA for 4 days in differentiation medium and infected with adenoviruses expressing DsRed-tagged human WT (AxDsRhWT) and CTF
DNA microarray analysis of TDP-43 aggregate formation in 1464R-derived neurons

It has been known that HSF1 acts as a master transcriptional regulator of heat shock response and governs expression of hundreds of underling proteins, including those distinct from heat shock.27 We therefore performed DNA microarray analysis to identify candidate molecules located downstream of HSF1 that counteract TDP-43 aggregate formation. The differentiated 1464R cells were co-infected with adenoviruses expressing WT and CTF hTDP-43, and hHSF1 in the presence of proteasome inhibitor MG-132 with the same protocols as indicated in Fig. 1A. Total RNA were isolated, DNA microarray analysis was performed, and candidate genes were cloned and examined by fluorescence microscopy and Western blot analysis. We found 64 genes that were upregulated more than twofold by HSF1 transduction, and 393 genes that were upregulated more than twofold in association with aggregate inhibition by HSF1 transduction. Notably, genes for most heat shock proteins were not included in these upregulated genes associated with inhibition of TDP-43 aggregate formation by HSF1 transduction. We then selected 50 genes and PCR-cloned them into EGFP-expressing plasmids, transfected these plasmids to 1464R cells, and examined their suppressing effects on adenoviral DsRed-positive TDP-43 aggregate formation under fluorescent microscopy (Fig. 2A); we chose approximately 20 genes expressing EGFP that did not co-localize with adenoviral DsRed-positive TDP-43 aggregates (Fig. 2B) and prepared adenoviruses expressing these genes. We then screened...
(Fig. 2C) and narrowed down the candidates that suppress TDP-43 aggregate formation to the following five molecules by fluorescence microscopy and Western blot analysis: acid phosphatase 5, tartrate resistant (ACP5); adhesion molecule with Ig-like domain 2 (AMIGO2); cAMP responsive element binding protein 3-like 1 (CREB3L1); Praja RING-H2 finger E3 ubiquitin ligase 1 (PJA1); and heme binding protein 2 (HEBP2) (Table 1). Western blot analysis using adenoviruses expressing these five molecules showed mild to strong suppressive effects on adenoviral soluble or insoluble TDP-43 expression (Fig. 2D, E). Among these, PJA1 adenovirus definitively suppressed phosphorylation and aggregate formation of TDP-43. Based on Western blot analysis, HSP40/DNAJB2a adenovirus also showed strong suppressive effects on TDP-43 aggregate formation (Fig. 2D), as previously described, although we observe faint induction of DNAJB2a by HSF1 in our DNA microarray analysis (Table 1). We therefore focused on PJA1 protein for further analysis.

**PJA1 suppresses phosphorylation and aggregate formation of TDP-43**

PJA1 is a RING-H2 finger E3 ubiquitin ligase abundantly expressed in human and rodent brains and four types of alternative splicing variants have been identified (Fig. 3A). PJA1 is known to be a short living-protein that...
is self-ubiquitinated by its RING-H2 domain and degraded by the proteasome.\textsuperscript{31} Consistent with our DNA microarray results as above, we confirmed upregulation of endogenous PJA1 protein induced by adenoviral HSF1, where suppression of ubiquitin-mediated degradation by proteasome inhibitor MG-132 enhanced the detection of native PJA1 bands, predominantly in its shortest form (variant X4), as verified with its migration status by

**Fig. 3** Legend on next page.

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Western blot analysis (Fig. 2B). We constructed PJA1 adenoviruses and examined adeno-virally-induced rat and human PJA1 proteins in differentiated 1464R neurons by Western blot (Fig. 3C) and fluorescence microscopy (Fig. 3D, E). We confirmed that adenoviral PJA1 is also self-ubiquitinated by its RING-H2 domain and rapidly degraded by proteasome; deletion of RING-H2 domain (ΔR) stabilized the protein resulting higher expression levels under Western blot and fluorescence microscopic analyses (Fig. 3C, D). In contrast, WT PJA1 adenoviruses strongly suppressed adenoviral TDP-43 phosphorylation and aggregate formation under proteasome inhibition, while PJA1 lacking its RING finger domain (PJA1ΔR) did not show these suppressive effects (Fig. 3E, F). These results indicate that PJA1 controls phosphorylation and proteasomal degradation of TDP-43 in our culture system.

To investigate whether adenoviral expression of HSF1 or PJA1 affects cell survival after adenoviral TDP-43 expression and proteasome inhibition, we performed a cell viability assay using CCK-8 reagent. The results showed no significant difference in cell survival between control, HSF1 and PJA1 groups (Fig. 3G), indicating that inhibition of TDP-43 aggregate formation by HSF1 and PJA1 does not support cell survival in our experimental paradigm.

To exclude the possibility that MG-132 has specific effects on HSF1 and PJA1 function besides proteasome inhibition, we examined other proteasome inhibitors, including lactacystin, epoxomicin, and bortezomib. These proteasome inhibitors equally enhanced formation of RIPA-insoluble phosphorylated TDP-43, which was suppressed by adenoviral PJA1 (Fig. 4A), confirming the common suppressive effects of PJA1 on phosphorylation and aggregate formation of TDP-43 under the condition of proteasome inhibition.

Because it has been shown that induction of autophagy also facilitates suppression of phosphorylation and aggregate formation of TDP-43, we examined the effects of autophagy inhibitors; that is, 3-methyladenine, bafilomycin A1 and wortmannin on adenoviral TDP-43 aggregate formation. Western blot analysis showed none of the significant effects of these inhibitors on TDP-43 aggregate formation nor their antagonizing effects on HSF1 or PJA1 (Fig. 4B), suggesting that protein metabolism of adenoviral TDP-43 aggregate formation in the present experiments is largely controlled by proteasome function. Adenoviral overexpression of PJA1 should, therefore, functionally antagonize proteasomal dysfunction to degrade TDP-43 in its aggregation process in the present study.

In addition, we demonstrated that adenoviral HSF1 lacking its transcriptional activation domain (HSF1Δ [380–529]) and PJA1ΔR are similarly lacking in suppressive effects on TDP-43 phosphorylation and aggregation (Fig. 4C). The HSF1Δ, acting as a dominant-negative form as previously described, blocked HSF1 activity by competing with WT HSF1 to bind target DNA and inhibited the suppressing effects of adenoviral WT HSF1 on TDP-43 aggregate formation (Fig. 4C, lane 2–4). In a similar manner, PJA1ΔR would attenuate the suppressive effects of HSF1 and PJA1 on TDP-43 aggregate formation, provided that PJA1 is a principal factor located downstream of HSF1 that counteract TDP-43 aggregate formation and PJA1ΔR lacking RING-H2 domain acts in a dominant-negative manner to compete with wild-type PJA1 to bind TDP-43. However, in the present study, PJA1ΔR did not act in a dominant-negative

Fig. 3 Suppression of phosphorylation and aggregate formation of TDP-43 by PJA1. (A) Schematic presentation of the primary structure of rat, mouse, and human PJA1. RING-H2 domains located in the C-terminal region are depicted with dark gray boxes. h, human; m, mouse; N/I, not identified; r, rat. (B) Western blot analysis of endogenous PJA1 induction by the HSF1 adenovirus in 1464R cells. The 1464R cells are infected with TDP-43 and HSF1 adenoviruses as indicated in Figure 1A. PJA1 expression is enhanced by adeno-viral HSF1 and 0.5 μM MG-132 treatments, predominantly in its shortest form (variant X4; asterisk approximately 50 kD). (C) Western blot analysis of EGFP-tagged adenosinoviruses expressing rat variant X4 (AxrPJA1x4EGFP), rat variant X1 (AxrPJA1x1EGFP), rat RING-H2-lacking variant X1 (AxrPJA1x1ΔREGFP), human variant V1 (AxhPJA1v1EGFP), and human RING-H2-lacking variant V1 (AxhPJA1v1ΔREGFP). Increased expression of wild-type (WT) PJA1 in the presence of 0.5 μM MG-132 is due to inhibition of proteasomal degradation of self-ubiquitinated PJA1, while expression of PJA1 lacking RING-H2 domain is independent of proteasomal activity. (D) EGFP fluorescence of 1464R-derived neurons infected with PJA1EGFP adenosinoviruses in the absence or presence of MG-132. The nucleus is counterstained with Hoechst 33342. WT PJA1ΔREGFP fluorescence is enhanced in the presence of MG-132 due to proteasomal inhibition, while EGFP expression of PJA1 lacking RING-H2 domain is not affected by MG-132 treatment. (E) Fluorescence microscopy of 1464R-derived neurons infected with adenosinoviruses expressing DsRed-tagged human WT and CTF TDP-43 and EGFP-tagged PJA1 constructs in the presence of MG-132. Adenosinoviruses expressing rat and human WT PJA1 (AxrPJA1x4EGFP, AxrPJA1x1EGFP, AxhPJA1v1EGFP) suppress DsRed-positive cytoplasmic TDP-43 aggregate formation, but those lacking RING-H2 domain (AxrPJA1x4ΔREGFP, AxhPJA1v1ΔREGFP) do not. (F) Western blot analysis of 1464R-derived neurons infected with adenosinoviruses expressing DsRed-tagged human WT and CTF TDP-43 and EGFP-tagged HSF1 and PJA1 constructs in the absence or presence of 0.5 μM MG-132. Adenosinoviruses expressing rat and human HSF1 and WT PJA1 (AxrPJA1x4EGFP, AxrPJA1x1EGFP, AxhPJA1v1EGFP) suppress formation of RIPA-insoluble phosphorylated TDP-43, but those lacking RING-H2 domain (AxrPJA1x4ΔREGFP, AxhPJA1v1ΔREGFP) do not. (G) Cell viability assay using CCK-8 reagent. The y-axis depicts cell survival ratio relative to untreated control. There was no significant difference in cell survival between EGFP, HSF1EGFP, and hPJA1v1EGFP adenosinoviruses infections followed by MG-132 treatment, while both longer (48 versus 24 h) incubation and higher (0, 0.5, 1, versus 2 μM) concentration of MG-132 exacerbate the cell survival.

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manner to suppress WT PJA1 or HSF1 function (Fig. 4C, lanes 5–8); whether PJA1 is an exclusively dominant effector molecule located downstream of HSF1 that counteracts TDP-43 aggregate formation remains elusive.

**PJA1 binds to TDP-43 and E2 ubiquitin conjugating enzyme UBE2E3**

We next examined whether PJA1 binds to and ubiquitinates TDP-43. We constructed adenoviruses expressing DsRed (on 5'-end)-tagged and FLAG (on 3'-end)-tagged TDP-43 WT and CTF for Co-IP experiments. The CTF TDP-43 adenovirus preferably induced RIPA-insoluble phosphorylated TDP-43, and the adenovirally induced PJA1 is also consistently localized in both RIPA-soluble and insoluble fractions (Fig. 5A) as described above (Fig. 3F). The Co-IP assay revealed that adenoviral PJA1 preferentially binds to CTF TDP-43 (Fig. 5B; Lanes 5, 7), while CTF TDP-43 was consistently ubiquitinated irrespective of adenoviral PJA1.
overexpression (Fig. 5B; ubiquitin K48 Western blot analysis, lanes 4-8), probably due to concomitant presence of endogenous PJA1 and/or other unidentified E3 enzymes to ubiquitinate CTF TDP-43 in this Co-IP preparation. Because it has been reported that PJA1 works robustly with the E2-conjugating enzymes UBE2D2/3, UBE2E3, and UBE2K, we examined E2 enzymes in Co-IP assay by Western blot and found that

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both endogenous and adenovirally-induced UBE2E3 were co-immunoprecipitated with TDP-43 and PJA1 (Fig. 5B, C), which strongly suggests that UBE2E3 is a preferential partner of PJA1 to ubiquitinate TDP-43.

Fluorescence microscopic observation of differentiated 1464R neurons infected with DsRed-tagged TDP-43 and EGFP-tagged PJA1 also revealed co-localization of cytoplasmic TDP-43 and PJA1. Higher magnification showed two types of co-localization; that is, perinuclear round fluorescence and cytoplasmic amorphous aggregates (Fig. 5D). The former perinuclear fluorescence appeared to localize to stress granules34–37 found in cells infected with adenovirus expressing both WT and RING finger domain lacking (ΔR) PJA1, while the latter cytoplasmic aggregates were observed only in cells expressing PJA1ΔR adenovirus, which would be degraded by WT PJA1 (Fig. 5D). These fluorescence results suggest that PJA1 binds to TDP-43 in stress granules and suppresses the formation of cytoplasmic TDP-43 aggregates.

**PJA1 suppresses TDP-43 aggregate formation in mouse facial motor neurons in vivo**

To investigate the suppressive effects of adenoviral HSF1 and PJA1 expression on TDP-43 aggregate formation in adult mouse motor neurons in vivo, we co-injected DsRed/TDP-43 (WT + CTF), HSF1/EGFP and PJA1/EGFP adenoviruses into the facial nerve and let the viruses transfer to the facial motor neurons via retrograde axonal transport, and express the virus-induced foreign genes in the motor neurons (Fig. 6). After the adenovirus injection (n = 4), 50–60% of mouse facial motor neurons were strongly labeled with DsRed and/or EGFP, which is much more efficient than rat facial motor neurons (10–30% labeled), as observed in our previous study.8 In the current mouse experiments, 30–40% of DsRed/TDP-43 (WT + CTF)-labeled motor neurons contained DsRed-positive cytoplasmic granular aggregates without proteasome inhibition (Fig. 6A, D). This was performed in previous experiments with rats,8 but probably due to the much higher transduction efficiency of adenoviral TDP-43 in mice, the degrading capacity of endogenous proteasome function was more overwhelmed than in rats. These mouse motor neurons bearing cytoplasmic aggregates were significantly decreased to <10% by co-infection of HSF1 or PJA1 adenovirus (Fig. 6B–D). The cells bearing cytoplasmic DsRed/TDP-43 aggregates partially colocalized to PJA1/EGFP fluorescence were infrequently observed (Fig. 6C), which may represent the degrading process of DsRed/TDP-43 by PJA1/EGFP. Together with the cell culture experiments as described above, the present study indicates the significant suppressive effect of PJA1 on TDP-43 aggregate formation both in vitro and in vivo.

**Phosphorylated TDP-43 aggregates in PJA1-immunoreactive human amyotrophic lateral sclerosis motor neurons**

Finally, we examined expression of PJA1 in motor neurons of human control and ALS spinal cords. Serial lumbar spinal cord sections were immunostained with PJA1, phosphorylated TDP-43, and HSF1. In non-diseased control cases, the great majority of neuronal cells that include spinal motor neurons were constitutively immunoreactive for PJA1 and HSF1 but not for phosphorylated TDP-43 (Fig. 7A–C). In ALS cases, cytoplasmic round inclusions immunoreactive for phosphorylated TDP-43 were observed in PJA1- and HSF1-immunopositive atrophic spinal motor neurons (Fig. 7D, E). Double immunofluorescence staining demonstrated cytoplasmic round and skein-like inclusions immunoreactive for phosphorylated TDP-43 in PJA1-immunoreactive ALS motor neurons, although these inclusions were not immunostained for PJA1 (Fig. 7G–L).

**DISCUSSION**

We have previously demonstrated adenovirally-induced TDP-43 cytoplasmic aggregate formation in 1464R-

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**Fig. 5** PJA1 binding to TDP-43 and E2 ubiquitin conjugating enzyme UBE2E3. (A) Western blot analysis of suppressive effects of adenovirus expressing human PJA1v1, but not PJA1ΔR, on phosphorylation and aggregate formation of TDP-43 after DsRed- and FLAG-tagged wild-type (WT) and CTF TDP-43 adenovirus infection in the presence of 0.5 μM MG-132 (lanes 3–8). The CTF TDP-43 adenovirus preferentially induces RIPASoluble phosphorylated TDP-43 (lanes 4, 6). Adenovirally-induced hPJA1v1 and hPJA1ΔR is consistently localized in both RIPASoluble and insoluble fractions (lanes 3, 5, 7, 8), and adenoviral hPJA1v1, but not hPJA1ΔR, decreases RIPASoluble phosphorylated CTF TDP-43 (lanes 5, 7) in a similar manner to the experiment shown in Figure 3F. Endogenous UBE2E3 is also detected both in RIPASoluble and insoluble fractions. (B) The co-immunoprecipitation (Co-IP) assay shows that PJA1 preferentially binds to CTF TDP-43 rather than WT TDP-43 (PJA1 blot; lanes 3, 5, 7, 8), while CTF TDP-43 is consistently ubiquitinated irrespective of adenoviral PJA1 induction (Ubiquitin K48 blot; lanes 4–8). Both native and adenoviral UBE2E3 are co-immunoprecipitated with WT and CTF TDP-43 (UBE2E3 and Myc-Tag blots). (C) Co-IP assay indicates that TDP-43 and/or PJA1 bind to UBE2E3 but not UBE2D2/3 or UBE2K. (D) Fluorescence micrographs of TuJ1-immunoreactive differentiated neurons co-infected with DsRed-tagged WT and CTF TDP-43 adenoviruses and EGFP-tagged hPJA1v1 (top row) or hPJA1ΔR (bottom row) adenovirus in the presence of 0.5 μM MG-132. The nucleus is counterstained with Hoechst 33342. Two types of co-localization (i.e., perinuclear round fluorescence [arrows] and cytoplasmic amorphous aggregates [arrowheads]), are observed.

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derived neuronal and glial cells under proteasome inhibition, and established time-lapse imaging analysis of these 1464R cells. The 1464R cell line can be cultured for more than 50 passages with no obvious morphological alterations and, in the presence of ATRA, ceases dividing and differentiates into neuronal and glial cells that recapitulate primary cultured brain cells. Most of these differentiated cells formed insoluble cytoplasmic TDP-43 aggregates that were phosphorylated and ubiquitinated and consisted of electron-dense granules when the cells were treated with proteasome inhibitor MG-132 following adenoviral transduction with WT and CTF TDP-43. Time-lapse imaging analyses revealed growing cytoplasmic aggregates in the transduced neuronal and glial cells, followed by collapse of the cell.

In the present study, we observed remarkable suppressing effects of adenoviral HSF1 overexpression on TDP-43 aggregate formation in 1464R-derived neurons, confirming previous reports. To investigate candidate molecules locating downstream of HSF1, we performed DNA microarray analysis of TDP-43 aggregate formation using adenovirus-induced 1464R cultures. Unexpectedly, we failed to verify known heat shock proteins that include HSP70, HSPB8, and HSP40/DNAJB2 as potential suppressors of TDP-43 aggregate formation in our DNA microarray analysis. In contrast, we found PJA1 E3 ubiquitin ligase to be the most promising molecule to counteract TDP-43 aggregate formation, although adenoviral overexpression of PJA1, as well as HSF1, does not support cell survival in our experimental paradigm.
PJA1 has been identified as a RING-H2 finger E3 ubiquitin ligase abundantly expressed in the brain, and was initially reported to bind to MAGED1/NRAGE/Dlxin1 in vitro and in vivo. Subsequently, PJA1 has been shown to impair the TGF-β/SMAD signaling-dependent tumor-suppressing pathway in diverse cancer cell lines and promote tumorigenesis of glioblastoma cell lines by degrading a transcriptional repressor CIC, while PJA1 promotes skeletal myogenesis through degradation of enhancer of zeste homologue 2 (EZH2). PJA1 has also been described to be upregulated in amygdala during the formation of fear memory, and is suggested to be one of the deleted genes in craniofrontonasal syndrome. However, there have been no reports concerning the association of PJA1 with neurodegenerative disorders, although Neurodap1 showing structural similarity to PJA1 was downregulated after facial nerve axotomy. In addition, although PJA1 was reported to suppress replication of DNA viruses such as hepatitis B virus (HBV) and herpes simplex virus 1 (HSV-1), PJA1 adenoviruses apparently did not suppress replication of all the recombinant adenoviruses including themselves used in the present study.

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**Fig. 7** Phosphorylated TDP-43 aggregates in PJA1-immunoreactive ALS motor neurons. (A-F) Immunohistochemistry of serial sections of lumbar spinal cord of non-diseased control (A-C) and ALS (D-F) cases immunostained for PJA1 (A, D), phosphorylated TDP-43 (B, E), and HSF1 (C, F) reveals that both control (A-C; arrows) and ALS motor neurons bearing phosphorylated TDP-43-positive cytoplasmic inclusions/aggregates (D-F; arrowheads) are immunoreactive for PJA1 (A, D). (G-H) Double immunofluorescence of PJA1 (G, J) and phosphorylated TDP-43 (H, K) of ALS motor neurons bearing cytoplasmic round (G-I; arrowheads) and skein-like (J-L; arrows) inclusions reveals no colocalization of these inclusions to PJA1 immunolabeling. The nucleus is counterstained with DAPI.
In this study, we demonstrated that PJA1 binds to TDP-43 and E2 ubiquitin-conjugating enzyme UBE2E3 and suppresses formation of cytoplasmic TDP-43 aggregates, which strongly suggests that PJA1 ubiquitinates and degrades TDP-43 through the ubiquitin-proteasome pathway, although whether PJA1 undoubtedly ubiquitinates TDP-43 awaits further investigation, such as through in vitro ubiquitination assay using purified PJA1 and TDP-43 proteins. Several reports have previously described ubiquitinating enzymatic activities against TDP-43. Parkin ubiquitinates TDP-43, but conversely promotes cytoplasmic TDP-43 aggregate formation.46 Uchida et al. described how the von Hippel Lindau (VHL)/cullin-2 (CUL2) E3 complex ubiquitinates misfolded TDP-43 and promoted clearance of fragmented forms of TDP-43.47 More recently, Znf-179 E3 ubiquitin ligase was shown to ubiquitinate TDP-43 and attenuate insoluble pathologic TDP-43 aggregates.48 Interestingly, Hans et al. has previously described UBE2E3 as the E2 conjugating enzyme for TDP-43,49 although the E3 ligase was not identified in their report. PJA1 as demonstrated in the present study might have been the E3 ligase missing in their report. In addition, we observed two types of co-localization of TDP-43 and PJA1 under fluorescence microscopy: perinuclear round fluorescence putatively representing stress granules34-37 and cytoplasmic amorphous fluorescence found only in cells infected with PJA1ΔR adenovirus, which were considered to be degraded by WT PJA1. We speculate that PJA1 binds to TDP-43 in stress granules and suppresses the formation of cytoplasmic TDP-43 aggregates, which should be investigated further using stress granule markers to identify co-localization of PJA1 and TDP-43 in these structures.

We also investigated in vivo suppressive effects of HSF1 and PJA1 on TDP-43 aggregate formation in adult mouse facial motor neurons by means of retrograde transport of recombinant adenoviruses injected into facial nerves as described previously in rats.8 We observed significant suppressive effects of both HSF1 and PJA1 adenoviruses on cytoplasmic TDP-43 aggregate formation in adult mouse motor neurons in vivo, again indicating that PJA1 as well as HSF1 plays a prominent role in ameliorating cytoplasmic TDP-43 aggregate formation in adult motor neurons in vivo. It has been suggested that HSF1 activity is reduced in affected spinal cord in patients with sporadic ALS, which leads to enhanced accumulation and aggregation of TDP-43 in ALS patients.20 Furthermore, we observed that, in humans, the great majority of neurons that include spinal motor neurons were immunoreactive for PJA1, and, in ALS spinal cord, phosphorylated TDP-43-immunoreactive cytoplasmic round and skein-like inclusions/aggregates were observed in PJA1-immunoreactive motor neurons, although co-localization of PJA1 in these inclusions/aggregates was not demonstrated. These results may indicate that PJA1 is constitutively expressed in the motor neurons where cytoplasmic TDP-43 aggregates are formed, and fully developed cytoplasmic TDP-43 aggregates such as round and skein-like inclusions are no longer the targets of PJA1-initiated proteasomal degradation machinery, although whether PJA1 counteracts development of TDP-43 oligomers or more mature cytoplasmic aggregates awaits further investigation. However, because TDP-43 is ubiquitously expressed in all cells, PJA1 binding to TDP-43 may not be specific to certain neuronal species that include motor neurons or hippocampal granule cells in that cytoplasmic TDP-43 aggregates are predominantly observed in ALS and FTLD cases. We are still unable to explain what causes the cell type specificity of cytoplasmic accumulation and aggregation of TDP-43, which may attribute to the cell type-specific microenvironment harboring aggregation-prone protein species.

There is accumulating evidence that shows that the transmission of pathological aggregates such as amyloid β, tau, α-synuclein, huntingtin, and TDP-43, is the principal causative event in the progression of Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, ALS, and FTLD, respectively.10-12 This notion is recognized as prion-like propagation, and cell-to-cell spreading of TDP-43 aggregates is a plausible hypothesis for explaining the progressive nature of neurodegeneration observed in ALS and FTLD. It is, therefore, noteworthy that the suppressing effect of PJA1 on formation of TDP-43 aggregates may halt disease progression of ALS and FTLD, while PJA1 did not support cell survival under proteasome inhibition in the present study. In contrast, use of ubiquitin ligase inhibitors has been shown to be an effective strategy in human cancer therapy.50 Recent reports also described a strategy to discover activators of E3 ligases with ubiquitin variants,51 which may be attractive therapeutic candidates for neurodegenerative diseases, including ALS and FTLD.

In summary, we performed DNA microarray analysis of the suppressing effect of HSF1 on TDP-43 aggregate formation in rat 1464R-derived neuronal cells. One of the upregulated candidate molecules, PJA1, has a significant capability of suppressing phosphorylation and aggregate formation of TDP-43 in vitro and in vivo, which may have therapeutic potential in ALS and FTLD.

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DISCLOSURE
The authors declare no competing financial interests.

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