Increased expression of p62/SQSTM1 in prion diseases and its association with pathogenic prion protein

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Prion diseases are neurodegenerative disorders characterized by the aggregation of abnormally folded prion protein (PrPSc). In this study, we focused on the mechanism of clearance of PrPSc, which remains unclear. The levels of p62 protein increased in prion-infected brains and persistently infected cell cultures. Upon proteasome inhibition, p62 co-localized with PrPSc, forming a large aggregate in the perinuclear region, hereafter referred to as PrPSc-aggresome. These aggregates were surrounded with autophagosome marker LC3 and lysosomes in prion-infected cells. Moreover, transient expression of the phosphomimic form of p62, which has enhanced ubiquitin-binding activity, reduced the amount of PrPSc in prion-infected cells, indicating that the activation of p62 could accelerate the clearance of PrPSc. Our findings would thus suggest that p62 could be a target for the therapeutic control of prion diseases.

Results

Up-regulation of p62 was observed following prion infection. To investigate the expression levels of p62 protein in prion-infected mice, we intracerebrally inoculated 22 L prions into ddY mice (n = 3) and
examined the levels of p62 protein in the brains of terminally sick mice by immunoblotting. Significantly up-regulated p62 was observed in the brains of 22L-inoculated mice, compared with that of the control mice (Fig. 1a). Up-regulated LC3-II was also observed, suggesting the involvement of autophagic degradation.

We next intraperitoneally inoculated 263 K prions into Syrian hamsters and examined the levels of p62 protein in the brains of terminally sick hamsters by immunoblotting. The levels of p62 also increased in the brains of 263K-inoculated hamsters, compared with those of the control hamsters (Fig. 1b). The presence of PrPSc was identified in all infected brains.

For further experiments, we used ScN2a58 cells, persistently 22L-infected N2a58 cells, containing high amounts of PrPSc. Immunoblotting revealed that the levels of p62 protein also increased in ScN2a58 cells compared with uninfected N2a58 cells (Fig. 2a). Moreover, the levels of p62 mRNA also significantly increased in ScN2a58 cells (Fig. 2b).

As microglia are known to play a major role in initiating the pathological changes in prion disease, we investigated the effects of prion infection on p62 using a microglial cell line (MG20). We added 0.1% brain homogenates from 22L-infected mice (22 L BH) or normal mice (nBH) to MG20 cells. After serial passages, the presence of PrPSc and up-regulation of p62 were identified in the 22L-infected cells from passage 3 (Fig. 2c), indicating that the phenomenon was not neuron-specific. Taken together, these data showed that up-regulation of p62 subsequently occurs in vivo and in vitro due to prion infection.

**p62 co-localizes with PrPSc in prion-infected cells.** It has been reported that PrPC is retrogradely transported from the endoplasmic reticulum (ER) and accumulates in the cytosol as protease-resistant and detergent-insoluble PrP upon proteasome inhibition26, and that aggregates may contain ubiquitinated proteins27. Another reports suggest that some of the PrP aggregates contain uncleaved signal peptides after proteasome inhibition, indicating the existence of ERAD-independent mechanism in which PrP fails to translate into the ER lumen28-30. To determine whether such PrP aggregates are recognized by p62, we investigated the subcellular localization of total PrP and p62 in N2a58 and ScN2a58 cells by immunofluorescence staining. Under normal conditions, immunofluorescence for total PrP was greater in ScN2a58 cells than in N2a58 cells [Fig. 3a, MG132 (-)]. In both cell types, endogenous p62 was distributed throughout the cytoplasm [Fig. 3a, MG132 (-)], consistent with previous observations11,14,31. When we treated N2a58 and ScN2a58 cells with a chemical proteasome inhibitor, MG132 (10 μM, 24 h) to see how the impairment of UPS affects the distribution of PrP and p62, singular p62-containing inclusions were frequently induced at the perinuclear region in both cell types [Fig. 3a, MG132 (+)]. Interestingly, total PrP was dramatically localized to p62 complex in ScN2a58 cells, suggesting that misfolded PrP tends to promote the formation of large aggresomes in prion-infected cells. To investigate whether PrPSc is sequestered in the p62 complex, we visualized PrPSc by immunofluorescence staining in combination with prior treatment with guanidium salts, which significantly increases the PrPSc signal32. After treatment with guanidine thiocyanate, PrPSc was detected in ScN2a58 cells, but not in N2a58 cells, and partially co-localized with endogenous p62 (Fig. 3b). Upon proteasome inhibition, a large PrP aggregate formed in the perinuclear region, hereafter referred to as “PrPSc-aggresome”, and importantly, p62 relocalized to PrPSc-aggresome. (Fig. 3b).

We next investigated whether the knockdown of p62 affects the formation of PrPSc-aggresome. We treated p62-specific siRNA or control siRNA to ScN2a58 cells (50 nM, 48 h), following MG132 treatment. Immunoblotting revealed that the levels of p62 protein also increased in ScN2a58 cells compared with uninfected N2a58 cells (Fig. 2a). Moreover, the levels of p62 mRNA also significantly increased in ScN2a58 cells (Fig. 2b).

**Figure 1 | Up-regulation of p62 in prion-infected brains.** (a) The levels of p62 protein in the brains of control (Cont.) or 22 L inoculated ddY mice (n = 3, per group). The amount of p62, LC3 and PK-resistant PrP (M20) was analyzed by immunoblotting. β-actin was used as a loading control. Statistical significance was evaluated using Student’s t-test. (b) The levels of p62 protein in the brains of control (Cont.) or 263 K inoculated Syrian hamsters. The amount of p62, LC3 and PK-resistant PrP (3F4) was analyzed by immunoblotting. β-actin was used as a loading control. Statistical significance was evaluated using Student’s t-test.
ScN2a58 cells were analyzed by Real time PCR. Asterisks indicate used as a loading control. (b) The levels of p62 mRNA in N2a58 and ScN2a58 cells. The amount of p62 and p62 is known to interact with ubiquitinated proteins through its C-terminus ubiquitin-associated domain (UBA). To investigate whether or not the co-localization of p62 and PrPSc was dependent whether phosphorylation of the UBA domain, missing amino acids 388–442, was introduced into ScN2a58 cells and visualized p62 and PrPSc. As expected, HA-tagged p62 localized to PrPSc-aggresomes upon proteasome inhibition. In contrast, HA-tagged p62AUBA did not relocalize to PrPSc-aggresomes, suggesting that the UBA domain plays a critical role in p62 recruitment to PrPSc-aggresomes. The levels of p62 protein increased by MG132 (10 μM, 24 h), and ubiquitinated proteins with high-molecular-weight (HMW, ~250 kDa), which are likely to derive from the aggresomes, were evident in ScN2a58 cells (Fig. S1), suggesting that PrPSc-aggresomes contain large ubiquitinated proteins.

**PrP is co-immunoprecipitated with p62.** We next performed co-immunoprecipitation experiments using ScN2a58 cell lysates. Endogenous p62 was co-immunoprecipitated with anti-PrP antibody (SAF32) both without (Fig. S2, top left) and with proteasome inhibition (Fig. S2, top right). The presence of PrP in SAF32 immunoprecipitates was also identified in both lysates (Fig. S2, bottom).

p62-positive aggresomes in prion-infected cells are surrounded by lysosomes. We next investigated the localization of such aggresomes and lysosomes in ScN2a58 cells, and we showed that p62-positive aggresomes were surrounded by lysosomes (Fig. 6a). Similar result was obtained in PrP-positive aggresomes (Fig. 6b). We also observed that LC3 was concentrated in the aggresomes (Fig. 6c). These results suggest that such aggresomes are potentially degraded in lysosomes.

**p62 is involved in the degradation of PrPSc.** To investigate whether overexpression of p62 affects the degradation of PrPSc, a HA-tagged p62 was introduced into N2a58 cells or ScN2a58 cells, and the amount of PrPSc was analyzed. Transient expression of p62 had no effect on the amount the amount of PK-resistant PrP (Fig. 7a). It has been shown that the activity of p62 appears to be controlled by phosphorylation at serine 403 (S403) of the human p62 UBA domain due to enhancement of its affinity to polyubiquitin, promoting efficient autophagic degradation of ubiquitinated proteins.

To investigate whether phosphorylation of the UBA domain affects the clearance of PrPSc, a HA-tagged phosphomimic mutant, with the substitution of mouse p62 serine 405 for glutamate (corresponding to S403 in human p62) (Fig. 7b), was introduced into ScN2a58 cells and the amount of PrPSc was analyzed. Transient expression of phosphomimic p62-HA reduced the amount of PrPSc in ScN2a58 cells (Fig. 7c). To confirm whether the mutation enhanced the affinity between p62 and ubiquitinated proteins, the lysates from transfected cells were immunoprecipitated with anti-HA antibody, and ubiquitinated proteins in the immunoprecipitates were detected by immunoblotting. As expected, the amount of ubiquitinated proteins in phosphomimic p62 transfected cells was greater than those in mock or wild-type transfected cells (Fig. 7d). In addition, after the blocking of lysosomal degradation by ammonium chloride, phosphomimic p62 was largely localized in lysosomes (Fig. S3).

Taken together, these results suggest that phosphomimic p62 accelerated the clearance of PrPSc in lysosomes.

**Discussion**

Our study revealed the following. (1) p62 increased in association with prion-infection in cell cultures and brains (Fig. 1 and 2); (2) Large PrPSc aggregates encircled by p62 form when the UPS is disrupted (Fig. 3b); (3) The interaction of p62 with polyubiquitin is crucial to the formation of PrPSc-aggresomes (Fig. 5); and (4) the constitutively active form of p62 could reduce the amount of PrPSc in infected cells (Fig. 7c). These findings suggest that p62-mediated aggresome-formation could be important in the host-defense response against prion infection.

Contrary to our data, Xu et al. previously reported that the levels of p62 protein and ubiquitinated proteins were decreased in the brains of 263K-infected hamsters. This discrepancy is unknown, but may be related to the differences in the experimental conditions.
Several different reports have described that the levels of p62 protein are known to be increased when proteasome function is inhibited. In prion-infected animal brains, proteasome activities are known to be reduced, and it has been clearly evidenced that PrPSc binds to the 20S core particle of proteasomes in vitro and inhibit proteasome activity in neurons. Actually, it has recently been reported that transcription factor NF-E2-related factor 2 (Nrf2), which enhances the expression of p62, was activated when the UPS is impaired. Furthermore, as p62 interacts with Keap1, which regulates the turnover of Nrf2, the up-regulated p62 may competitively inhibit the Nrf2–Keap1 interaction, resulting in stabilization of Nrf2 followed by transcriptional activation of p62. This positive feedback loop may contribute to the up-regulation of p62 demonstrated in the study in the large PrPSc-aggresomes forming in infected cells when proteasomes were inhibited by MG132. These findings suggest that direct inhibition of the UPS by PrPSc prompts the up-regulation of p62 as a compensatory mechanism of the UPS in prion diseases.

It is well known that p62 can bind to polyubiquitinated proteins and play a role in the degradation of misfolded proteins such as the polyglutamine-expanded huntingtin. Relatively large PrPSc-aggresomes encircled by p62 were observed in infected cells when the UPS was disrupted, suggesting that p62 mediates PrPSc-aggresome formation (Fig. 3b). Because it is not clear whether PrPSc itself is ubiquitinated or not, we cannot exclude the possibility that p62 binds indirectly with PrPSc. It is likely that the formation of p62-PrPSc

![Figure 3](image-url) Co-localization of p62 and PrP after proteasome inhibition. (a) Total PrP (SAF32: green) and p62 (red) were visualized in N2a58 and ScN2a58 cells with (+) or without (−) MG132 (10 μM, 24 h). Bars: 20 μm. (b) After the treatment of guanidine thiocyanate, PrPSc (SAF61: green) and p62 (red) were visualized in N2a58 and ScN2a58 cells with (+) or without (−) MG132 (10 μM, 24 h). Bars: 20 μm.

![Figure 4](image-url) Knockdown of p62 disrupts the formation of PrPSc-aggresome. ScN2a58 cells were treated with p62-specific siRNA or control siRNA (50 nM, 48 h), following MG132 treatment (10 μM, 24 h). After the treatment of guanidine thiocyanate, PrPSc (SAF61: green) and p62 (red) were visualized. Bars: 20 μm.

![Figure 5](image-url) UBA of p62 is required for the formation of PrPSc-aggresome. (a) Scheme of HA-tagged p62 or UBA domain-deleted p62 (p62ΔUBA, missing amino acids 388–442). (b) HA-tagged p62 or p62ΔUBA expression vectors were transiently expressed in ScN2a58 cells for 48 h, following with MG132 (10 μM, 24 h). After the treatment of guanidine thiocyanate, PrPSc (SAF61: green) and HA (red) were visualized.
complexes is dependent upon the presence of other ubiquitinated proteins in the PrP*-aggresomes because these are often observed in PrP plaques in CJD brains. Based on the fact that the phosphomimetic p62, which has enhanced ubiquitin-binding activity, promoted the clearance of PrP* (Fig. 7c), we came to the conclusion that activation of p62 is important for the efficient clearance of PrP Sc (Fig. 7c), we came to the conclusion that activation of p62 is important for the efficient clearance of PrPSc (Fig. 7c), we came to the conclusion that activation of p62 is important for the efficient clearance of PrPSc.

In vivo infection experiments. Four-weeks-old ddY mice were purchased from SLC (Hamamatsu, Japan) and were intracerebrally inoculated with 20 μL of a 10⁻⁴

Figure 6 | Aggresomes in prion-infected cells are surrounded by lysosomes. (a) p62 (green) and lysosome (red) were visualized in ScN2a58 cells treated with MG132 (10 μM, 24 h). Bars: 20 μm. (b) Total PrP (SAF32: green) and lysosome (red) were visualized in ScN2a58 cells treated with MG132 (10 μM, 24 h). Bars: 20 μm. (c) Total PrP (SAF32: green) and LC3 (red) were visualized in ScN2a58 cells treated with MG132 (10 μM, 24 h). Bars: 20 μm.

In conclusion, disruption of the UPS may be involved in prion pathogenesis, while the enhancement of p62-activity is a possible therapeutic target for the induction of the autophagic clearance of pathogenic prion proteins.

Methods

Antibodies. Anti-p62/SQSTM1 (MBL, PM045), anti-β-actin (Sigma-Aldrich), anti-HA (Invitrogen), anti-ubiquitin (Santa Cruz Biotechnology, P4D1), anti-LC3B (Cell Signaling Technology, #2775), and anti-PrP (Santa Cruz Biotechnology, M20; SPI-Bio, SAF61 and SAF32; SIGNET, 3F4) antibodies were purchased from the indicated vendors. Horseradish peroxidase-conjugated anti-goat (Santa Cruz Biotechnology), anti-mouse and anti-rabbit IgG antibodies (GE Healthcare Life Sciences) were used for immunoblotting. Alexa Fluor® 488-conjugated anti-mouse IgG and Alexa Fluor® 594-conjugated anti-rabbit IgG antibodies (Invitrogen) were used for immunofluorescence analysis.

Cell cultures. The mouse neuroblastoma Neuro 2a cells were obtained from the American Type Culture Collection (CCL 131). N2a58 cells are mouse PrP® overexpressing Neuro 2a cells, and ScN2a58 cells originated from N2a58 cells infected with a mouse-adapted scrapie strain, 22 L, as previously described. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen). MG20 cells (a gift from Dr. Iwamari), isolated from neonatal tgα20 mice that overexpress mouse PrP®44, were cultured in DMEM containing 10% heat-inactivated FBS, penicillin-streptomycin, 2-mercapto-ethanol and insulin. All cultured cells were maintained at 37 °C in 5% CO₂ in the biohazard prevention area of Nagasaki University.

Plasmids and siRNA. Mouse p62 open reading frame was amplified from N2a58 cDNA with primers of mp62-BamHI-F (5’-ccgctcgagtcattaagcgtaatctggaacatcgtatgggtaatgtgggtatagggcagcttc-3’) and mp62-Xhol-R (5’-ccgctcgagcatctgccgagttgtctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttctgtgagttc...
dilution of brain homogenate prepared from mice terminally sick with 22 L strain. As a control, age- and strain-matched mice were intracerebrally inoculated with phosphate buffered saline. The brains of the mice were removed at the terminal stage of disease. Animals were cared for in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

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Author contributions
T.H. designed the work, produced all the data, and wrote the manuscript. T.N., K.Satoh and K.Sano contributed reagents and materials. D.I., R.A. and N.N. revised the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports.