Amyloid aggregates of the deubiquitinase OTUB1 are neurotoxic, suggesting that they contribute to the development of Parkinson’s disease

Raniki Kumari1‡, Sanjay Kumar‡, Sanjay Kumar‡, Abhishek Kumar Singh‡, Pranita Hanpude‡, Deepak Jangir†, and Tushar Kanti Maiti‡§

From the 1‡, 2‡ Functional Proteomics Laboratory, Regional Centre for Biotechnology (RCB), NCR Biotech Science Cluster, Faridabad 121001, India, the 3‡ Kalinga Institute of Industrial Technology (KIIT), Bhubaneswar, Odisha 751024, India, and the 4§ Manipal Academy of Higher Education, Manipal, Karnataka 576104, India

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Parkinson's disease (PD) is a multifactorial malady and the second most common neurodegenerative disorder, characterized by loss of dopaminergic neurons in the midbrain. A hallmark of PD pathology is the formation of intracellular protein inclusions, termed Lewy bodies (LBs). Recent MS studies have shown that OTU deubiquitinase ubiquitin aldehyde-binding 1 (OTUB1), a deubiquitinating enzyme of the OTU family, is enriched together with α-synuclein in LBs from individuals with PD and is also present in amyloid plaques associated with Alzheimer’s disease. In the present study, using mammalian cell cultures and a PD mouse model, along with CD spectroscopy, atomic force microscopy, immunofluorescence-based imaging, and various biochemical assays, we demonstrate that after heat-induced protein aggregation, OTUB1 reacts strongly with both anti-A11 and anti-osteocalcin antibodies, detecting oligomeric, prefibrillar structures or fibrillar species of amyloidogenic proteins, respectively. Further, recombinant OTUB1 exhibited high thioflavin-T and Congo red binding and increased β-sheet formation upon heat induction. The oligomeric OTUB1 aggregates were highly cytotoxic, characteristic of many amyloid proteins. OTUB1 formed inclusions in neuronal cells and co-localized with thioflavin S and with α-synuclein during rotenone-induced stress. It also co-localized with the disease-associated variant pS129-α-synuclein in rotenone-exposed mouse brains. Interestingly, OTUB1 aggregates were also associated with severe cytoskeleton damage, rapid internalization inside the neuronal cells, and mitochondrial damage, all of which contribute to neurotoxicity. In conclusion, the results of our study indicate that OTUB1 may contribute to LB pathology through its amyloidogenic properties.

Parkinson’s disease (PD) is the second most common neurodegenerative disorder that is characterized pathologically by the death of dopaminergic neurons in the substantia nigra pars compacta region of midbrain and the accumulation of intracellular proteinaceous aggregates as Lewy body (LB) (1, 2). Clinically, PD is described by a constellation of motor deficits, including rigidity, resting tremor, and bradykinesia. However, these symptoms appear at a later stage of the disease, when more than 70% of dopaminergic neurons are nonfunctional (3, 4). The pathogenesis of idiopathic PD is not well-elucidated. However, it is considered as a multifactorial disease where environmental factors contribute significantly to genetically predisposed individuals with the progression of age (5, 6). In most of the case, PD prevails as a sporadic form, and the familial form of the disease is rare (7). It has been shown that PARK genes along with many other genes regulate the complex mechanism of PD. Mutations in PARK genes like α-Synuclein (PARK1), Parkin (PARK2), UCHL-1 (PARKS), PINK1 (PARK6), DJ-1 (PARK7), and LRRK2 (PARK8) are linked with familial PD. LB formation in the brain is a pathological hallmark of PD, and α-synuclein is the major component of LB (8, 9). Classical LB is described as intraneuronal, eosinophilic inclusion with a hyaline core and a pale peripheral halo that is always positive for α-synuclein and ubiquitin (10). α-Synuclein is deposited in LB in a hyperphosphorylated (Ser-129) form with a β-sheet–rich and fibrillar structure (11). MS analysis of LB has uncovered more than 500 proteins in the human cortical LB-enriched sample, and more than 40 proteins are co-enriched with α-synuclein, including several kinases (MAPKK1/MEK1, protein kinase C, and doublecortin-like kinase), deubiquitinating enzymes (UCHL1 and a novel otubain-1 (OTUB1)), and numerous ubiquitin ligases (KPC and SCF) (12). Furthermore, spatially targeted optical microproteomics study of Alzheimer’s plaque has also identified many proteins that are deposited in Aβ plaque. OTUB1 is one of the potential proteins that is also found in the AD plaque (13). However, the molecular basis for its enrichment in LB, as well as Aβ plaque, remains unclear, and this is our immediate interest.

OTUB1 belongs to an ovarian tumor (OTU) domain cysteine protease superfamily of the deubiquitinating enzyme that specifically removes Lys-48–linked ubiquitin chains from their target sites (14, 15). OTUB1 is ubiquitously expressed in human tissues.
like brain, gastric, colorectal, and liver tissue (16, 17). The OTUB1 gene is located on human chromosome 11q13.1. It is a 271-amino residue protein with catalytic domain spanning residues 46–271. The catalytic domain is highly conserved across species from yeast to mammals (18, 19). It plays a crucial role in diverse physiological and pathological processes, including T cell anergy, virus-triggered interferon 1 induction, and stabilization of p53, estrogen receptor-α, cIAP, SMAD2/3, RhoA, FOXM1, TRAF3/6, MDMX, and Snail (20–30). It also unexpectedly plays a noncatalytic role in inhibiting the accumulation of Lys-63 polyubiquitin chain in the DNA double-strand damage response (31). The inhibition of Lys-63 ubiquitin chain formation is due to the inhibition of the activity of ubiquitin-conjugating enzyme Ubc13 (UBE2N) through tight binding with the N terminus of OTUB1. The deubiquitinating activity of OTUB1 is not essential for the inhibition of the ubiquitin-conjugating activity of Ubc13 (32). Recent studies have also demonstrated that OTUB1 is an anti-apoptotic agent and it attenuates the apoptosis of neuronal cells after intracerebral hemorrhage (33). The silencing of OTUB1 inhibits the migration of human glioma cells in vitro (34). Moreover, OTUB1 also directly controls the accumulation of Tau and phosphorylated Tau (AT8 Tau) by regulating Lys-48-linked Tau deubiquitination (35).

OTUB1 has been reported in the regulation of lung cancer development, ovarian cancer, esophageal squamous cell carcinoma metastasis, colorectal cancer, and many other malignant tumors. However, its high expression in the brain has suggested that it must have a vital role in neuronal functions. On the other hand, its presence in LB, Alzheimer’s plaque, and Tau pathology also provides strong evidence that OTUB1 might be a key player in neurodegenerative diseases.

In this study, we have demonstrated that OTUB1 is an amyloidogenic protein, which forms ordered aggregates with a β-sheet–rich structure in vitro. The oligomeric and fibril forms of OTUB1 cause cytotoxicity due to cytoskeleton disruption, pore formation in the cell membrane, internalization of oligomeric and fibrillar aggregates, the abrupt increment of reactive oxygen species (ROS), and damage to mitochondria. The oligomeric and fibril forms of OTUB1 also induce the expression of α-synuclein and its pathological form pS129-α-synuclein in vitro and co-localize with pS129-α-synuclein in vitro and in a PD mouse model. In summary, our data unambiguously demonstrate that the amyloid characteristic of OTUB1 might regulate the complex PD pathology.

**Results**

*In silico analysis identifies OTUB1 as an amyloidogenic protein*

Earlier reported MS data of LB were reanalyzed, and our analysis yielded 52 proteins that showed significant differential expression in Lewy bodies of PD compared with control. The aggregation propensity of these 52 proteins was analyzed in the Prediction of Amyloid STructure Aggregation 2.0 (PASTA 2.0) online server (36). PASTA 2.0 provides secondary structural information as well as energy value in the genome scale. PASTA 2.0 energy function evaluates cross-β pairing between amino acid sequence stretches. We plotted PASTA 2.0 energy values with the relative abundance of proteins in LB compared with control. Correlation plot analysis identified 27 proteins with a PASTA 2.0 score $>9.0$, and many of them were reported earlier as amyloidogenic proteins, as listed in Table S1. In a correlation plot, each blue dot represents the protein that was prone to aggregate or was amyloidogenic in nature, and a blue dot above the red line represents more amyloidogenicity of the protein. The red line represents a fitting of the correlation plot. The proteins with high PASTA 2.0 energy exhibited a greater amyloidogenic property, and proteins with low PASTA 2.0 energy showed a lesser amyloidogenic property (Fig. 1A). The PASTA 2.0 energy values for OTUB1 and α-synuclein were $−8.0$ and $−7.0$, respectively (Table S1). Interestingly, OTUB1 is also shown to enrich with α-synuclein in the LBs (12).
OTUB1 is an amyloidogenic protein

Figure 2. An inherent property of OTUB1 as amyloid-like protein. A, melting temperature curve. The melting temperature curve displays the stability of the OTUB1 protein. The melting temperature is 47 °C. B, detection of oligomers and fibrillar species during aggregation of OTUB1 using the dot blot method. 350 μM OTUB1 was used to induce aggregates at 37 °C. 10 μl of heat-induced aggregates of OTUB1 aliquot were used at each time point 0, 1, 2, 4, 6, 12, 24, 48, and 72 h for the anti-A11 and anti-OC reactivity assay and 1 μl of OTUB1 aliquot for anti-OTUB1 reactivity in the dot blot assay. These blots were probed with anti-OTUB1, anti-A11, and anti-OC antibodies. C, scatter plot showing the normalized intensity of A11 and OC against total OTUB1 intensity (mean ± S.E., n ≥ 3). D, ANS-binding assay displays the exposure of hydrophobic patches during aggregation of OTUB1 in a concentration-dependent manner (mean ± S.E., n ≥ 3). E, β-sheet fluorescence intensity shows the amyloid property of OTUB1 at different concentrations during aggregation (mean ± S.E., n ≥ 3). F, Congo red birefringence assay performed using cross-polarized light. Congo red dye bound with OTUB1 protein gives birefringence of an apple yellow-green color. Scale bar, 60 pixels. G, CD spectroscopy, showing the molar ellipticity ratio of α-helix (208/218 nm) and β-sheet (218/208 nm) at different time points (mean ± S.E., n ≥ 2).

Exposure β aggregation propensity (Fig. 1, D and E). Both of these stretches lie in the N-terminal and NAC domain of α-synuclein, and they play a crucial role in α-synuclein aggregation (39, 40). Our in silico analysis provided an indication of the amyloidogenic behaviors of OTUB1.

OTUB1 shows amyloid-like structure in vitro

Next, we tested the amyloidogenic potential of OTUB1 with purified recombinant protein. OTUB1 purification was performed by a standard GST purification protocol followed by gel filtration. The quality of the protein was analyzed by SDS-PAGE. The gel filtration chromatogram and SDS-PAGE image are shown in Fig. S1 (A and B). Thermal stability of OTUB1 was estimated in the thermal melting experiment in CD spectroscopy, and it was found to be 47 °C (Fig. 2A). We also performed a dot blot assay with anti-A11, anti-OC, and anti-OTUB1 antibody after incubation of OTUB1 (350 μM) at 37 °C for 0, 1, 2, 4, 6, 12, 24, 48, and 72 h. We used 10 μl of heat-induced aggregates of OTUB1 aliquot at each time point for anti-A11 and anti-OC reactivity assays and 1 μl of OTUB1 aliquot at each time point for anti-OTUB1 reactivity in a dot blot assay. The anti-A11 antibody specifically reacts with the oligomeric structure or prefibrillar structure of amyloidogenic protein, whereas the anti-OC antibody specifically reacts with fibrillar species of amyloidogenic protein. Reactivity of A11 and OC antibody was normalized to the total OTUB1. Interestingly, the anti-A11 antibody showed strong reactivity at the initial time points, which are 1-, 2-, 4-, and 6-h. Among them, A11 reactivity is maximum at 6 h time point. However, the anti-OC antibody reactivity started from 1 h, and its signal was increased with the time of incubation (Fig. 2, B and C). The reactivity against anti-A11 and anti-OC antibody demonstrated that OTUB1 produced amyloid aggregates in heat-induced conditions. Proteins that have a tendency to aggregate also expose the hydrophobic surface in heat-induced conditions (41). The hydrophobic sur-
face exposure was measured by an 8-anilino-1-naphthalene sulfonic acid (ANS)-binding assay. Incubation of OTUB1 with ANS at 37 °C showed an increase in fluorescence intensity with time (Fig. 2D). The concentration-dependent ANS binding was observed in the case of OTUB1, and it was evident from the rate of binding analysis. Thioflavin T (ThT) is a dye that binds with the amyloid proteins and shows an increase in ThT fluorescence (42). Here, we observed that OTUB1 strongly bound with ThT dye in heat-induced conditions. The normalized ThT fluorescence at different concentrations of OTUB1 showed an increased ThT fluorescence intensity with the progression of time at 37 °C (Fig. 2E). Both ANS- and ThT-binding kinetic parameters at different OTUB1 concentrations are given in Table S2. Congo red (CR) is a histologic dye that binds to amyloid proteins because of their extensive cross-β-sheet structures and produces apple yellow-green color birefringence in cross-polarized light (43). The mature amyloid-like structure of OTUB1 was confirmed by CR-binding assays. OTUB1 fibril-bound CR showed an apple yellow-green color birefringence under cross-polarized light (Fig. 2F). Far-UV CD spectropolarimetry was used to find the secondary structure component analysis of OTUB1. Dichroweb analysis (44) of the CD spectra of OTUB1 confirmed 39 ± 5% α-helix, 17 ± 3% β-sheet, and a 20 ± 0% turn in solution as indicated in Table S3. The secondary structural information obtained from CD analysis was very much consistent with the crystallographic information with α-helix 44%, β-sheet 24%, and turn 28% (45). The time-dependent molar ellipticity change of OTUB1 at 37 °C was also monitored. At 0 h of incubation, OTUB1 showed two minima, one at 208 nm and the other at 222 nm (Fig. S2). The molar ellipticity ratio of 218 nm/208 nm (β-sheet) was slowly increased, and the 208/218 nm (α-helix) ratio was decreased upon incubation of OTUB1 at 37 °C with the progression of time (Fig. 2G). Collectively, the ThT binding assay, the CR-binding assay, and secondary structural analysis have confirmed that OTUB1 undergoes amyloid-like conformation in heat-induced conditions.

**OTUB1 makes oligomeric intermediates that transform into mature fibril**

The oligomerization of OTUB1 upon heat-induced conditions was monitored in a dynamic light-scattering experiment. We found that the hydrodynamic radius \( (R_d) \) of the OTUB1 monomer at 0 h of incubation was 8 ± 0.6 nm, and with time, the hydrodynamic radius of OTUB1 species was shifted to a higher-size particle, as indicated in Fig. 3A. The hydrodynamic radius of different species at 6, 12, 24, 48, and 72 h were 1.42 ± 0.06 × 10^3, 1.79 ± 0.14 × 10^3, 1.83 ± 0.12 × 10^3, 1.99 ± 0.35 × 10^3, and 2.24 ± 0.43 × 10^3, respectively. The OTUB1 monomer-to-oligomer transition was completed within a 6-h incubation, which prompted us to perform the dynamic light scattering (DLS) study with a short time interval. Protein was incubated at 0 min, 30 min, 60 min, 90 min, 2 h, 4 h, and 6 h, and oligomeric intermediates were monitored in DLS at 37 °C. We found 4.73 ± 0.52 × 10^2-nm species as a major oligomer upon 30 min of incubation. The hydrodynamic radius of major species gradually increased in size with the time of incubation (Fig. S3A). The morphology of heat-induced aggregates of OTUB1 at 0, 6, 12, 48, and 72 h was visualized in the atomic force microscope. It did not show any characteristic aggregate at 0 h. However, we observed specific ordered oligomeric aggregates at 6 h. Longer incubation induced fibrillar morphology, as evident in the atomic force microscope (AFM) images at 12, 48, and 72 h (Fig. 3B). The crater-like OTUB1 oligomeric assemblies were found at short incubation times (2, 4, and 6 h) (Fig. S3, B and C). Quantification of height image of OTUB1 monomer, oligomers, and fibrils were performed in which the mean heights of monomer, oligomers, and fibrils were 2.5 ± 0.2, 5.8 ± 0.4, and 8.3 ± 0.2 nm, respectively (Fig. 3, C–E). Further, we analyzed the height of the peak and valley of the twisted fibril. The mean height of the peak and valley of the twisted fibrils was 7.1 ± 0.1 and 5.6 ± 0.1 nm, respectively (Fig. 3, F and G). Zoomed images of a single fiber and line profile of a single fiber are shown in Fig. 3 (H and I). Thus, DLS and AFM studies confirm that OTUB1 produces initially oligomeric species of specific geometry that eventually transform into amyloid-like fibers.

**Cytotoxicity of oligomeric and fibrillar OTUB1 species**

Our AFM results demonstrated that OTUB1 oligomer produced an annular ring-like structure. Many amyloid proteins like Huntington protein, Ataxin 3Q82, and amylin peptide are shown to have an annular-like structure, and they exhibit strong cytotoxicity (46–48). To test the toxicity of OTUB1 oligomers and fibrils, SH-SY5Y cells were exposed to different forms of OTUB1 (monomer, oligomers, and fibrils) for 48 h. OTUB1 oligomers were enriched from monomer by using a 100-kDa Amicon Ultra 0.5-ml centrifugal device. Fibrils were enriched by centrifugation of aggregates of OTUB1 at 16,000 rpm for 1 h. Fibrillar pellets were resuspended in sterile PBS. Oligomers and fibrils were confirmed by a ThT assay and AFM. Cytotoxicity was measured by annexin-FITC/propidium iodide (PI) staining followed by FACS analysis. Percentages of viable, early apoptotic, late apoptotic, and necrotic cells for control, monomer, oligomers, and fibrils are shown in Fig. 4A. The apoptosis analysis was performed in which we found that OTUB1 oligomers showed more toxicity than the OTUB1 fibrillar treatment group. (Fig. 4B and Table S4). ROS production due to exposure of various forms of OTUB1 was also estimated. OTUB1 oligomers released a huge amount of ROS compared with monomer and fibrils (Fig. 4C). The excess ROS production could be due to the alteration of mitochondrial health. Next, we investigated the effect of different forms of OTUB1 (monomer, oligomers, and fibrils) on mitochondrial function. The mitochondrial function was determined by the fluorescent microscopic examination of the mitochondrial membrane potential \( (\Delta \psi_m) \)-dependent uptake and retention of the fluorochrome MitoTracker Red (CMXRos) in the mitochondria. Fig. 4 (D and E) demonstrates the effect of different forms of OTUB1 (monomer, oligomers, and fibrils) on the \( \Delta \psi_m \) in neuronal cells. The \( \Delta \psi_m \) of OTUB1 monomer was found to be similar to the control condition. However, in the case of OTUB1 oligomers, \( \Delta \psi_m \) was reduced drastically. OTUB1 fibrils also showed reduced \( \Delta \psi_m \). Thus, our results confirm an altered mitochondrial \( \Delta \psi_m \) upon exposure of OTUB1 oligomers to SH-SY5Y cells.

OTUB1 is an amyloidogenic protein
Extracellular oligomeric OTUB1 perturbs membrane nanostructure of SH-SY5Y cells

It has been demonstrated in the literature that exposure of oligomeric species of Aβ-peptide and α-synuclein causes the membrane damage and forms the porelike structure on the cell membrane (49, 50). To test surface morphology change of neuronal cells, retinoic acid–differentiated SH-SY5Y cells were exposed to monomer, oligomers, and fibrils, and surface properties were measured by AFM. We used heat-aggregated BSA as a control in treatment conditions. Three-dimensional topological structure of control cells (BSA-treated) and OTUB1 monomer did not show any change in their morphology. The mean heights of monomer, oligomers, and fibrils are 2.5 ± 0.2, 5.8 ± 0.4, and 8.3 ± 0.2 nm, respectively. F and G, the mean height of peak and valley of twisted fibril of OTUB1 are 7.1 ± 0.1 and 5.6 ± 0.1 nm, respectively. H and I, height image of zoomed single fibril showing the twist and section line profile of twisted fibril.
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Surface roughness parameter was estimated for these conditions. In both control and monomer, treated cells showed similar surface roughness (average roughness (Ra) and mean roughness (Rq)). The oligomer treatment condition exhibited a drastic change in surface roughness (Fig. 5D). Interestingly, oligomer-treated cells also showed porelike structures in cytoplasmic regions. In summary, the extracellular oligomeric OTUB1 exposure causes severe neuronal membrane damage.

Figure 4. Analysis of apoptosis, ROS generation, and damage of mitochondria by OTUB1. A, SH-SYSY cells were exposed to 10 μM OTUB1 monomer, oligomers, and fibrils for 48 h; cell percentage showing in quadrants as early apoptosis, late apoptosis, and necrosis. B, quantification of flow cytometry (annexin V FITC/PI). A scatter plot of quantified flow cytometry data shows the cell percentage of the viable cell, early apoptotic cell, late apoptotic cell, and necrotic cell after treatment with OTUB1 monomer, oligomers, and fibrils. Values are mean ± S.E. (error bars), n = 3. C, SH-SYSY cells were exposed to OTUB1 monomer, oligomers, and fibrils at a concentration of 10 μM for 24 h. The level of intracellular ROS was determined by incubating the cells with H2DCF-DA at a concentration of 25 μM at 37 °C for 30 min. The mean fluorescence intensity of green fluorescence released by cleaved DCF was plotted for each sample. The fluorescence intensity of each treatment condition was normalized on a scale of 0–1. Values are mean ± S.E., n = 3. D, SH-SYSY cells were exposed to OTUB1 monomer, oligomers, and fibrils at a concentration of 10 μM for 24 h. Confocal images show the mitochondria (MitoTracker Red CMXRos (red)) and microtubule (α-tubulin (green)). Scale bars, 20 μm (full image) and 5 μm (zoomed regions). E, quantification of MitoTracker Red CMXRos intensity of confocal images. Values are mean ± S.E., n = 3. *, p < 0.05; ***, p < 0.0001; NS, not significant.

Oligomeric form of OTUB1 disrupts the cytoskeleton of neuronal cells

The neuronal cytoskeleton is composed of microtubules, actin filament, and neurofilaments, which play a critical role in both the establishment and maintenance of neuronal plasticity, polarity, morphology, and integrity of axons (51). However, in many neurodegenerative conditions, the cytoskeleton is altered...
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due to the accumulation of amyloidogenic proteins in cells (52). Our result showed an impairment of membrane surface roughness upon oligomeric OTUB1 treatment of neuronal cells (Fig. 5D). To investigate the effect of OTUB1 aggregates on the structure of the actin filament of neuronal cells, we exposed different forms of OTUB1 (monomer, oligomers, and fibrils). OTUB1 oligomers heavily disrupted actin filament and formed patchlike aggregates inside cells, as observed in the phalloidin-FITC staining (Fig. 6). On the other hand, OTUB1 monomer- and fibril-treated cells showed an actin filament structure similar to that observed in control cells. Our results strongly suggest that, like other oligomeric amyloid proteins, OTUB1 oligomers also perturb the cytoskeleton of neuronal cells.
Oligomeric OTUB1 rapidly internalizes inside neuronal cells

Spreading of pathogenic prion protein has been observed in pathology of many neurodegenerative disease (53, 54). Oligomeric intermediates and protofibril of amyloid proteins spread to healthy cells through the cellular internalization (55, 56). Recently, it has also been demonstrated that the fibrillary aggregate of p53 rapidly internalizes in cells and regulates gene expression (57). To check the cellular internalization of OTUB1 aggregates, SH-SY5Y cells were treated with FITC-labeled monomer, oligomers, and truncated fibrils of OTUB1 and BSA. Cells were trypsinized and treated with trypan blue prior to fluorescence microscopy to remove unwanted fluorescence signals from the cell surface due to adherence of fluorescently labeled protein. Trypan blue quenches the FITC fluorescence signal if the protein is attached to the surface (58). However, trypan blue cannot quench the fluorescence signal once the protein is internalized to the cells. OTUB1 monomer was evenly distributed throughout the cells with very high intensity. Both oligomers and truncated fibrils showed the internalization of protein with a punctate structure. However, BSA did not show any internalized signal (Fig. 7).

OTUB1 produces amyloidogenic inclusion under rotenone-induced conditions in neuronal cells

To evaluate the protein aggregate formation inside neuronal cells, we exposed SH-SYSY cells to 500 nM rotenone for 24 h. Rotenone generates an excess amount of ROS/RNS species by inhibiting the mitochondrial complex I system (59). The ROS/RNS species modify the protein and cause protein aggregation in cells (60–62). We performed an analytical gel filtration followed by an anti-OTUB1 antibody dot blot assay with rotenone-treated and DMSO-treated cells. In control cells, most of the OTUB1 protein was eluted in around 14–17 ml of peak fraction in a Superose 6 10/300 GL column. However, some amount of OTUB1 was also eluted in the void volume (8–10 ml). On the other hand, OTUB1 was eluted mostly in the void volume (8–12 ml) in rotenone-treated conditions (Fig. 8, A and B). Our result demonstrated that in oxidative stress conditions, OTUB1 produced cellular aggregates. We analyzed the inclusion formation of OTUB1 in confocal microscopy by staining SH-SYSY cells with thioflavin S and anti-OTUB1 antibody. Thioflavin S is a fluorescent dye that binds with amyloid protein in cells or tissues (63). Rotenone-treated cells showed strong co-localization of thioflavin S with OTUB1 with a Pearson’s value of 0.62 (Fig. 8, C and D). However, control cells did not show any signal of thioflavin S. These results provide evidence for cellular aggregation of OTUB1 in redox stress conditions.

OTUB1 co-localizes with α-synuclein and pS129-α-synuclein under rotenone-induced conditions in neuronal cells

It is well-documented that the expression of α-synuclein and its pathological form pS129-α-synuclein increases upon expo-
sure of rotenone to neuronal cells (64). To understand the expression of OTUB1 in neuronal cells upon rotenone treatment, we performed immunoblotting and probed with OTUB1, α-synuclein, and pS129-α-synuclein. We found that the expression of OTUB1, α-synuclein, and pS129-α-synuclein was increased up to 2-, 2.5-, and 2-fold, respectively, in rotenone treatment conditions as compared with control (Fig. 9, A and B). To verify whether OTUB1 co-localizes with pS129-α-synuclein and α-synuclein in rotenone-induced conditions, the cells were probed with anti-OTUB1, anti-α-synuclein, and anti-pS129-α-synuclein antibodies. A significant increase in co-localization of OTUB1 with pS129-α-synuclein and α-synuclein was observed in rotenone-treated conditions compared with control, with co-localization Pearson values of 0.32 and 0.36, respectively (Fig. 9, C–F). Thus, both Western blotting and confocal microscopy studies strongly suggest that under redox stress conditions, there is an enhanced expression of a pathological form of α-synuclein (anti-pS129-α-synuclein) with increased co-localization with OTUB1.

**Aggregates of OTUB1 induce the expression of α-synuclein and pS129-α-synuclein in neuronal cells**

Cell-to-cell propagation of aggregated protein plays an important role in the progression of α-synucleinopathies (65). The mechanism of amyloid fibril formation in various pathological conditions has been confirmed to occur in vitro for a wide variety of extracellular amyloids, such as Aβ peptides and prion proteins as well as intracellular proteins, such as α-synuclein and Tau (66). Thus, we examined whether the oligomeric or fibril species of OTUB1 can induce the expression of α-synuclein and pS129-α-synuclein in vitro. We exposed the different forms of OTUB1 (monomer, oligomers, and fibrils) to SH-SY5Y cells and analyzed the changes in the expression of α-synuclein and pS129-α-synuclein. We found that both

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**Figure 7. OTUB1 internalization to the SH-SYSY cells.** FITC-labeled different forms of OTUB1 (monomer, oligomers, and fibrils) and BSA were allowed to internalize into the cells. After trypsinization and trypan blue treatment, live cells were imaged by fluorescence microscopy. The first panel shows the bright field of corresponding images of FITC-conjugated OTUB1 internalized in the second panel. Scale bar, 20 μm.
OTUB1 oligomers and fibrils were able to enhance the expression of pS129-/H9251-synuclein and /H9251-synuclein 2- and 3-fold, respectively, as confirmed by immunoblotting (Fig. 10, A and B). The Western blotting data also strongly agreed with the data from confocal microscopy, where we found that both oligomers and fibrils significantly enhanced the expression of pS129-/H9251-synuclein (Fig. 10, C and D). These results indicate that OTUB1 shows a characteristic property of amyloid, and its amyloid-like form induces the disease mechanism in vitro.

OTUB1 accumulates and co-localizes with pS129-α-synuclein in a rotenone-induced PD mouse model

Next, we examined the OTUB1 expression and its co-localization with the pathological form of α-synuclein in the rotenone-induced PD mouse model with confocal microscopy. We have generated a rotenone-induced PD mouse model as described previously (67). The body weight of each mouse was measured during the study (Fig. S4A), and the PD phenotype was confirmed by the rotarod test (Fig. S4B). The brains of PD and control mice were isolated and fixed, and immunohistochemistry was performed. Further, we checked the intensity of OTUB1 and pS129-α-synuclein in control as well as in rotenone-treated mouse brains. The expression of OTUB1 was higher in the hippocampus and cortex region of rotenone-induced PD mice than in control mice. Further, we have checked the expression of pS129-α-synuclein, and we observed the enhanced expression of phosphorylated α-synuclein in the hippocampus and cortex of PD mouse brains. However, its intensity was very low or at a basal level in control mice. The yellow signal of the merged image indicate the co-localization of OTUB1 and pS129-α-synuclein in the hippocampus and cortex region of PD mouse brains. However, in control mouse brains, less yellow signal were observed in the hippocampus and cortex (Fig. 11).

Discussion

Accumulation of misfolded proteins in neurons is a common mechanism for neurodegenerative disorders, including PD (68, 69). The dysregulated proteostasis is not only implicated in the disease mechanism but also considered as an attractive therapeutic target (68). In PD pathology, α-synuclein oligomeric aggregates contribute to the disease progression and LB formation (70, 71). Interestingly, many proteins that are enriched with α-synuclein in the LB are either entrapped with the α-synuclein fibrillary network or co-aggregated due to their amyloidogenic nature (72). Aggregation propensity analysis of LB
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Figure 9. Expression of OTUB1, α-synuclein, and pS129-α-synuclein and co-localization of OTUB1 with α-synuclein and pS129-α-synuclein in SH-SY5Y cells. Cells were treated with 5 μM rotenone for 12 h in complete medium. A, immunoblot showing the expression level of OTUB1, α-synuclein, and pS129-α-synuclein. B, scatter plot showing the quantification of immunoblotting. C and E, confocal microscopy images showing the co-localization of OTUB1 with pS129-α-synuclein and α-synuclein. D and F, quantification was performed with co-localization Pearson’s value for pS129-α-synuclein and α-synuclein. Scale bar, 20 μm. Values are mean ± S.E. (error bars), n ≥ 3. *, p ≤ 0.05; ***, p ≤ 0.0001; NS, not significant.
proteins shows that OTUB1 is a candidate with PASTA 2.0 energy of -8, which is similar to the PASTA 2.0 energy of H9251-synuclein which is -7. Aggregation region analysis by multiple algorithms also confirms that OTUB1 also carries a few segments of amino acid residues (Residues number 158–184) with high aggregation propensity.

The amyloidogenic behavior of OTUB1 has been shown by biophysical methods. In heat-induced conditions, OTUB1 rapidly produces oligomeric intermediates that are reactive to the A11 antibody. Interestingly, unlike other amyloid proteins, it also shows strong anti-OC binding within a 1-h incubation. The anti-OC antibody reactivity upon brief incubation of OTUB1 at 37 °C indicates that the fibril species are generated very quickly. The ThT-binding assay and DLS studies show oligomeric species formation in a time-dependent manner. AFM results show a nice arrangement of OTUB1 oligomers with crater-like geometry. The crater-like structure has been shown in many proteins with an amyloid property, such as amylin, serum amyloid protein, and Ataxin 3Q82 (73, 74). The crater-like structure also shows toxicity as described in the literature (75, 76). Similar to Aβ and α-synuclein, OTUB1 oligomers also alter the membrane nanostructure, as observed in AFM. The membrane damage by amyloid oligomers is presumably due to the electrostatic interaction between protein assembly and lipid surfaces, as observed in many antimicrobial peptides (77, 78). Neuronal cells exposed to amyloid oligomers show a disrupted actin cytoskeletal network compared with monomer. It has been demonstrated that the A30P mutant of α-synuclein increases the rate of polymerization and disrupts the cytoskeletal network during reassembly of actin filaments (79–81). We observe similar perturbation of actin cytoskeletal network arrangement by OTUB1 oligomers. It is intriguing to speculate that OTUB1

Figure 10. Expression of pS129-α-synuclein and α-synuclein after treatment of OTUB1 aggregates to SH-SY5Y cells. Cells were exposed to 10 μM OTUB1 monomer, oligomers, and fibrils for 24 h in Opti-MEM medium. A, immunoblot showing the expression level of pS129-α-synuclein and α-synuclein. β-Actin was probed for total protein loading control. B, scatter plot showing the quantification of immunoblotting. C, confocal images showing the intensity of pS129-α-synuclein. On the y axis, percent intensity refers to the intensity of pS129-α-synuclein in the percentage of cells. Values are mean ± S.E. (error bars), n = 3–*, p ≤ 0.05; **, p ≤ 0.01; ****, p ≤ 0.0001; NS, not significant.
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oligomers may have similar interactions with actin. Here, we have demonstrated that monomeric, oligomeric, and fibrillar OTUB1 are taken up by the cells rapidly. The rapid internalization of amyloid monomeric, oligomeric, and fibril species has been proven through either membrane pore formation or clathrin-mediated endocytosis (82—84). Altered mitochondrial function is also another factor that promotes cell death caused by extracellular amyloid oligomeric exposure (85—88). In the present study, we have observed mitochondrial alteration and excessive ROS generation upon oligomeric OTUB1 treatment. Gel filtration followed by an anti-OTUB1 antibody dot blot assay with control and rotenone-induced cells demonstrates the aggregation of OTUB1. The co-localization of Thio-S and anti-OTUB1 in rotenone-induced SH-SY5Y cells showed the amyloid behavior of OTUB1 inside cells. Interestingly, OTUB1 co-localized with α-synuclein and pS129-α-synuclein in rotenone-induced cellular conditions. This indicates that aggregated OTUB1 may cross-talk with the aggregation of a pathogenic form of α-synuclein. Moreover, OTUB1 aggregates also induce the level of α-synuclein and pS129-α-synuclein, which suggests evidence that OTUB1 might regulate the pathology of PD via increased expression of a pathological form of α-synuclein. Further, we have examined the co-localization of OTUB1 and pS129-α-synuclein in a rotenone-induced PD mice model in confocal microscopy, and it provides a strong clue as to its amyloidogenic character in PD regulation.

In summary, our study demonstrates that OTUB1 is a novel amyloidogenic protein in the LB and provides its aggregation in vitro and in vivo. The oligomeric structure of OTUB1 is a specific molecular form that potentiates neuronal toxicity, cytoskeleton disruption, the abrupt release of ROS, and damage to mitochondria (Fig. 12). OTUB1 co-localizes with the pS129-α-synuclein in neuronal cells and a PD mouse model. Apart from prominent role of OTUB1 in deubiquitination activity and negative regulation of Ubc13 (89), amyloid aggregation of OTUB1 provides a new mechanism in synucleinopathies that can be further studied.

Figure 11. Co-localization of OTUB1 with pS129-α-synuclein in rotenone induced PD mice model. C57BL/6 male mice (20–25 g) were treated with rotenone (40 mg/kg body weight) for 45 days orally once per day. In the hippocampus and cortex region, co-localization was performed using anti-OTUB1 and anti-pS129-α-synuclein. In rotenone-treated mice, co-localization was greater compared with in vehicle-treated mice. Images were acquired using a Leica SP8 microscope with a ×63 oil objective. Scale bar, 20 μm.
Materials and methods

Mammalian cell culture and treatment conditions

SH-SY5Y cells were grown at 37 °C with 5% CO₂ in HiGlutaXL Dulbecco’s modified Eagle’s medium, high-glucose (HiMedia, Mumbai, India) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% antibiotic-antimycotic solution (HiMedia). A cellular AFM experiment was carried out in retinoic acid–differentiated cells. SH-SY5Y cells were seeded on a glass coverslip and were grown up to 60% confluence. The cells were differentiated by differentiation medium containing HiGlutaXL Dulbecco’s modified Eagle’s medium, high-glucose, supplemented with 2.5% FBS, 10 μM retinoic acid (Sigma–Aldrich), and 1% antibiotic-antimycotic solution (HiMedia) for 3 days. Rotenone (Sigma–Aldrich) was dissolved in DMSO in a stock concentration of 10 mM, diluted in medium, and applied to cells in a concentration of 500 nM for 24 h to induce aggregation of different proteins (OTUB1 and α-synuclein) in medium containing 5% FBS. To induce the phosphorylation of α-synuclein, rotenone was used at a concentration of 5 μM for 12 h in complete medium. Control cells were treated with diluted DMSO as a vehicle. To induce the expression level of α-synuclein and pS129-α-synuclein, 10 μM OTUB1 aggregates were applied to cells for 24 h in Opti-MEM medium.

Expression and purification of OTUB1

The full-length pOPINK-OTUB1 plasmid was a kind gift from Dr. David Komander (Addgene, plasmid 61420). Expression and purification of OTUB1 were performed according to a standard GST purification protocol. Briefly, BL21 (DE3) cells containing pOPINK-OTUB1 plasmid were grown to A₆₀₀ nm of 0.8 in lysogeny broth medium at 37 °C and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 16 h at 18 °C. Cells were harvested, and pellets were resuspended in 1/10 PBS with 400 mM KCl buffer, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 15 μg/ml lysozyme. The homogenized cells were centrifuged at 16,000 × g for 1 h, and the clear supernatant was loaded onto a GST affinity column (GE Healthcare). Protein was eluted with elution buffer containing 50 mM Tris-HCl, pH 8, 500 mM NaCl, and 20 mM reduced GSH. The GST tag was removed by Pre-scission protease (GE Healthcare). The protein was further purified in a Superdex-200 16/600 gel filtration column, and the purity of the protein was confirmed by using SDS-PAGE.
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Rotenone-induced Parkinson mouse model

All animal experiments were carried out in accordance with the guidelines of the animal care facility of the Regional Centre for Biotechnology (RCB) and were approved by the animal ethics committee at RCB. The PD mouse model was generated according to the method described previously (67) with minor modifications. Briefly, 8-week-old C57BL/6 male mice (20–25 g) were acclimated for 15 days to a 12-h light/dark cycle and maintained at 23 °C. Mice were housed in standard laboratory cages and had free access to food and water throughout the study. Rotenone (40 mg/kg) (Sigma) was suspended in 0.5% carboxymethyl cellulose sodium salt (CMC, Sigma), and a 5-ml/kg body weight volume was administered orally once a day for 45 days. CMC was used as vehicle control. Motor activity of mice was measured using a rotarod (IITC Life Science) to ensure model generation.

In vitro aggregation of OTUB1

Freshly purified OTUB1 was diluted to different concentrations (50, 100, 200, and 350 μM) in 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, and 0.01% sodium azide. The protein sample was incubated at 37 °C in a shaker incubator with rotation at 300 rpm in a 2-ml centrifuge tube containing 2-mm glass beads. The aggregation kinetics was monitored by aliquoting samples at desired time points up to 4 days.

ThT-binding assay

ThT fluorescence was measured using Hitachi-7000 spectrophotometer with excitation at 442 nm, and emitted light was collected in the 450–600 nm range. The ThT assay was performed by mixing with heat-incubated 10 μM protein samples in 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 0.01% sodium azide, and freshly prepared thioflavin T dye in a final concentration of 10 μM. The increase of ThT fluorescence for all of the samples was plotted at 482 nm with time. The data were plotted and fitted with a sigmoidal curve in Origin 8 software. Binding parameters were calculated from the fitted graphs.

ANS-binding assay

Hydrophobic surface exposure due to aggregation was analyzed by an ANS-binding assay. Heat-incubated OTUB1 and ANS solution was mixed in assay buffer (50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA) with a final concentration of 20 and 40 μM, respectively. ANS fluorescence excitation was set at 372 nm, and emission was recorded from 400 to 600 nm with a slit width of 5 nm (both for excitation and emission) using a Hitachi-7000 fluorescence spectrophotometer. ANS fluorescence obtained for all of the samples was plotted at 470 nm with time.

CD spectroscopy

The protein samples were diluted in 50 mM phosphate buffer, pH 7.2, with a final concentration of 5 μM, and far-UV spectra were recorded in a JASCO J815 CD spectrometer. The spectra were collected from 190–260 nm wavelengths with accumula-

tions of 10 scans at a speed of 200 nm/min. All of the CD experiments were performed with a 2-mm cuvette. The spectra were analyzed in the DICHROWEB server for secondary structural components.

DLS

Dynamic light scattering experiment was carried out in a Zetasizer Nano series instrument (Malvern Instruments), which was equipped with a Peltier temperature controller, and the temperature was set at 25 °C. Heat-incubated protein solution (1 mg/ml) was taken at the desired time point, and a DLS measurement was made for each sample with the detection angle of 90°.

Congo red birefringence

A Congo red birefringence binding assay was carried out as described earlier (90) with minor modifications. OTUB1 fibril (50 μM) was mixed with 200 μl of the saturated amount of the ethanolic solution of Congo red, and the mixture was incubated at 37 °C for 30 min. Congo red solution was subsequently removed by centrifugation at 20,400 × g for 30 min. The resultant pellet was washed extensively with 70% ethanol. The washed pellet was resuspended in 50 μl of sterilized Milli-Q water and placed over a glass slide. Images were acquired from an air-dried glass slide using a microscope equipped with cross-polarized light (Olympus, SZX16).

Atomic force microscopy of proteins and SH-SY5Y cells

The surface morphology of recombinant OTUB1 protein aggregates and the perturbation of SH-SY5Y cell membrane upon exposure to OTUB1 monomer, oligomers, and fibrils were visualized with an AFM, which was equipped with an AFM scanner and Zeiss optical microscope (IPK Instruments, Berlin, Germany). OTUB1 monomer, oligomers, and fibrils (10 μM) were placed on a freshly cleaved mica sheet and incubated at 25 °C for 30 min. The immobilized protein on mica sheet was washed with distilled water to remove salts and for cellular AFM, cells were washed to remove the unbound proteins/aggregates and subsequently dried under air. The AFM imaging of protein was collected in tapping mode using a silicon cantilever with drive frequency and spring constant of 300–320 kHz and 13–77 newtons/m, respectively. The surface morphology change of retinoic acid–differentiated SH-SY5Y cells due to the exposure of 10 μM OTUB1 monomer, oligomers, and fibrils was visualized in the AFM. The BSA-treated cells were considered as a control. The cells were fixed with 4% PFA for 15 min at 25 °C and imaged with a gold-coated Hydra cantilever with a resonance frequency and force constant of 17 ± 4 kHz and 0.1 newtons/m, respectively, in contact mode (APPNANO). All images for protein and cells were taken at a resolution of 256 × 256 or 512 × 512 pixels and a scan speed of 0.8–1 line/s. All images were processed with JPK software.

Gel filtration, dot blot, and immunoblot analysis

SH-SY5Y cells were treated with 500 nM rotenone for 24 h to generate oxidative stress. Oxidative stress also induced protein aggregation in cellular conditions. Rotenone-treated cells were harvested and dissolved in a buffer containing 50 mM Tris-HCl,
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Analysis of apoptosis by annexin V-FITC assay

Cell survival assay was performed using an annexin V-FITC staining apoptosis assay kit (Sigma–Aldrich) following the manufacturers' protocol. Briefly, OTUB1 monomer, oligomers, and fibrils were applied to cells for 48 h. Cells were harvested and washed with cold PBS. The cells were then resuspended in 1× binding buffer (10 mM HEPES/NaOH, pH 7.5, 0.14 M NaCl, and 2.5 mM CaCl₂). Annexin V-FITC and PI were added to the cell suspension and incubated at room temperature for 10 min. The stained cells were sorted by fluorescence using a BD FACSVersa flow cytometer (BD Biosciences) with a minimum of 10,000 events recorded per sample using BD Cell Quest Pro software. Data were displayed in two-color dot plot formats in log scale, and cells were expressed as a percentage of viable, early apoptotic, late apoptotic, and necrotic cells.

FITC labeling of OTUB1 species and cell permeability assay

Monomer, oligomers, and truncated fibrils of OTUB1 and BSA were labeled with FITC (Sigma–Aldrich). Truncated fibril was produced by sonication with a 50% amplitude for 3 min with a 10-s pulse. FITC labeling was performed according to the manufacturer's protocol. Briefly, 2 mg/ml OTUB1 protein was made in freshly prepared 0.1 M sodium carbonate buffer, pH 9. Conjugation reactions were titrated to achieve a dye/protein molar ratio of ~1:10. The reaction mixture was incubated at room temperature for 1 h in rotating conditions. Unlabeled FITC dye was removed by extensive washing of proteins with 50 mM phosphate buffer containing 150 mM NaCl in a 3-kDa cutoff Amicon Ultra-0.5 ml (Millipore) centrifugal device. SH-SY5Y cells were grown up to 70% confluence on the sterile Lab-Tek II chamber slide to visualize the live cells. The medium was replaced with Opti-MEM, and the cells were allowed to grow for another 4 h. FITC-conjugated OTUB1 species (monomer, oligomers, and truncated fibrils) and BSA at a concentration of 100 μg were added to the medium for 24 h. Cells were washed with phosphate-buffered saline and trypsinized with 0.001% trypsin (HyMedia) for 1 min to remove the extra protein from the cell surface. After trypsinization of cells, 500 μm trypsin blue was applied to cells for 10 min. Live-cell images were acquired by using fluorescence microscopy (Nikon, Japan).

Measurement of intracellular ROS generation

Reactive oxygen species generation due to exposure of extracellular OTUB1 monomer, oligomer, and fibril species was measured by FACS analysis upon treatment of H2DCF-DA dye. Briefly, SH-SY5Y cells were exposed to OTUB1 monomer, oligomers, and fibrils at a concentration of 10 μM for 24 h. After treatment, cells were washed twice with ice-cold PBS. The cells were harvested with 1× PBS, 5 mM EDTA buffer and subsequently incubated with 25 μM H2DCF-DA (Molecular Probes, Life Technologies) at 37 °C for 30 min. After incubation, cells were again washed twice with 1× PBS buffer, and DCF fluorescence intensity was estimated by FACS with an excitation and emission wavelength of 488 and 523 nm, respectively.

Tissue preparation and immunofluorescence

Control and rotenone-treated mice were perfused through the transcardial aorta with 50 ml of PBS followed by 150 ml of a cold fixative consisting of 4% paraformaldehyde in PBS buffer, pH 7.4, after deep anesthesia with ketamine/xylazine (90 mg/kg, 4.5 mg/ml; intraperitoneal). The brains were post-fixed with 0.5% Triton X-100, respectively, in PBS for 24 h. Brains were post-fixed with 1× PBS, 5 mM EDTA buffer and subsequently incubated with 25 μM H2DCF-DA (Molecular Probes, Life Technologies) at 37 °C for 30 min. After incubation, cells were again washed twice with 1× PBS buffer, and DCF fluorescence intensity was estimated by FACS with an excitation and emission wavelength of 488 and 523 nm, respectively.
nology), anti-pS129-α-synuclein (1:500, Cell Signaling Technology), anti-α-synuclein (1:200; Santa Cruz Biotechnology), and anti-α-tubulin (1:200; Invitrogen) antibodies. The washed slides were incubated with secondary antibody (A594 goat antimouse (Thermo Fisher Scientific) and A488 goat anti-rabbit (1:500) (Thermo Fisher Scientific) for 45 min at room temperature. Samples were counterstained with 4',6-diamidino-2-phenylindole and mounted using anti-fade mounting medium (ProLong Gold Antifade, Invitrogen). Immunofluorescent images were captured with the Leica TCS SP8 confocal microscope. Image processing and co-localization (Pearson’s coefficient value) analysis were carried out with the offline software LASX of Leica TCS SP8.

Statistical analysis

Data were presented as mean ± S.D. or mean ± S.E. Statistical analyses were performed using Prism 7.0 software (GraphPad Software). The significance of differences was assessed using an unpaired Student’s t test and ordinary one-way ANOVA followed by Tukey’s multiple-comparisons test. Immunoblot quantification was performed using ImageJ. Values of p ≤ 0.05 were considered statistically significant.

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