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Clearance of Mutant Aggregate-Prone Proteins by Autophagy

Brinda Ravikumar, Sovan Sarkar, and David C. Rubinsztein

Summary

The accumulation of mutant aggregate-prone proteins is a feature of several human disorders, collectively referred to as protein conformation disorders or proteinopathies. We have shown that autophagy, a cytosolic, non-specific bulk degradation system, is an important clearance route for many cytosolic toxic, aggregate-prone proteins, like mutant huntingtin and mutant α-synucleins. Induction of autophagy enhances the clearance of both soluble and aggregated forms of the mutant protein, and protects against toxicity caused by these mutations in cell, fly, and mouse models. Inhibition of autophagy has opposite effects. Thus, the autophagic pathway may represent a possible therapeutic target in the treatment of certain protein conformation disorders.

Key Words: Autophagy; aggregate-prone proteins; Huntington's disease; rapamycin.

1. Introduction

Several human diseases, referred to as protein conformation disorders (PCDs) or proteinopathies, are caused by the accumulation of misfolded, mutant proteins. These conditions include Alzheimer's disease, Parkinson's disease (PD), Huntington's disease (HD), and many of the dominant spinocerebellar ataxias ***(1)***. In many cases, the mutation confers a toxic gain-of-function on the target protein and there is a strong correlation between the accumulation of the mutant protein and disease severity ***(2)***. An attractive pharmacological approach toward treatment of these PCDs would thus be to enhance the clearance of the toxic mutant proteins.

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Autophagy is a nonspecific bulk degradation system involved in the clearance of cytosolic long-lived proteins and organelles. During autophagy, double-membraned structures called autophagic vacuoles enclose a portion of the cytosol with the contents to be degraded. These autophagic vacuoles then fuse with the lysosomes where their contents are degraded by lysosomal hydrolases *(3)*. A key regulator of mammalian autophagy is a phosphatidyl inositol (PI) kinase-related kinase, mammalian target of rapamycin (mTOR) *(4)*. Inhibition of mTOR by treatment with rapamycin results in induction of autophagy. Another kinase involved in the regulation of mammalian autophagy is the class III PI-3-kinase, Vps34. Inhibition of Vps34 by 3-methyl adenine (3-MA) leads to an inhibition of autophagy *(5)*. Furthermore, lysosomal acidification is important for the fusion of autophagic vacuoles with the lysosomes. Thus, autophagy can also be inhibited by using compounds like Bafilomycin A1, which affects the acidification of lysosomes by inhibiting the proton pump *(6)*. Autophagy occurs constitutively in mammalian cells. Inhibition of autophagy by conditional knockout of autophagy-specific genes in the brains of mice resulted in an aberrant accumulation of ubiquitinated protein aggregates reminiscent of those seen in the PCDs *(7,8)*. This suggests the importance of autophagy in the clearance of normal soluble cellular proteins.

We have shown that autophagy is an important clearance route for mutant aggregate-prone proteins, like mutant huntingtin that causes HD and mutant α-synuclein that causes forms of PD *(9–12)*. Our data suggest that autophagy is clearing soluble forms of the proteins. It is likely that the numbers of aggregates also decrease when autophagy is induced, as this process lowers the concentration of the aggregate precursors—the soluble aggregate-prone species. We used different modulators that act at distinct steps of the autophagic pathway to study aggregation/clearance of a mutant huntingtin fragment and clearance of mutant α-synuclein *(9–12)*. We also measured changes in huntingtin-induced cell death with autophagy modulators *(9)*.

2. Materials

2.1. Cell Culture

1. Cell lines: African green monkey kidney cells (COS-7), stable inducible rat pheochromocytoma (PC12) cell lines expressing a variety of proteins; enhanced green fluorescent protein (EGFP)-tagged huntingtin exon-1 fragment with 23 or 74 polyglutamine repeats (EGFP-HDQ23 or EGFP-HDQ74) *(13)*, or hemagglutinin (HA)-tagged A53T or A30P mutants of α-synuclein *(10)*.
2. Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Gillingham, Dorset, UK).
3. Fetal bovine serum (FBS) (Sigma-Aldrich, Gillingham, Dorset, UK).

4. Horse serum (Sigma-Aldrich, Gillingham, Dorset, UK).
5. L-Glutamine (Sigma-Aldrich, Gillingham, Dorset, UK).
6. Penicillin/Streptomycin solution (Sigma-Aldrich, Gillingham, Dorset, UK).
7. 1X Trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, Gillingham, Dorset, UK).
8. Geneticin/G418 sulfate (Gibco Invitrogen, Paisley, UK) is dissolved in sterile water to make a 100 mg/mL stock and frozen at –20°C in aliquots.
9. Hygromycin-B (Merck Chemicals Ltd., Nottingham, UK) solution is mixed with DMEM to a 26 mg/mL stock and aliquots are frozen at –20°C.
10. Doxycycline (Sigma-Aldrich, Gillingham, Dorset, UK) is dissolved in sterile water to make a 100 mg/mL stock and stored at –20°C (*see* **Note 1**).
11. 6-well plates (NUNC; Invitrogen, Paisley, UK).
12. 22 × 22 mm thickness No. 1 glass cover slips (VWR international, Lutterworth, UK).
13. 75-cm^2 flasks (NUNC; Invitrogen).
14. Cell scrapers (Sarstedt Ltd., Leicester, UK).

2.2. Modulators of Autophagy

1. 3-Methyl adenine (3-MA) (Sigma-Aldrich, Gillingham, Dorset, UK), an inhibitor of autophagy, is dissolved in water to make a 50 m*M* stock and stored at room temperature (*see* **Note 2**).
2. Rapamycin (Rap) (Sigma-Aldrich, Gillingham, Dorset, UK), an autophagy inducer, is dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Gillingham, Dorset, UK) to make a 0.5 µg/µL stock, and the aliquots are stored at –20°C in dark Eppendorf tubes to keep away from direct light.
3. Bafilomycin A1 (BafA1) (Sigma-Aldrich, Gillingham, Dorset, UK), an autophagy inhibitor, is solubilised in dimethyl sulfoxide (DMSO) to prepare a 100 µ*M* stock and stored at –20°C. The aliquots are kept in dark Eppendorf tubes to keep away from direct light.

2.3. Transient Transfection

1. Lipofectamine transfection reagent (Invitrogen).
2. Plasmid DNA: EGFP-tagged huntingtin exon-1 fragment with 74 polyglutamine repeats (EGFP-HDQ74) ***(2)***.
3. Plasmid DNA: EGFP tagged LC3 (EGFP-LC3; kind gift from T. Yoshimori) ***(14)***.

2.4. Immunocytochemistry

1. 1X Phosphate-buffered saline (PBS) solution.
2. Paraformaldehyde (PFA) is dissolved in 1X PBS to make a 4% stock (*see* **Note 3**).
3. Antifadent, citifluor AF1 (Citifluor Ltd., London, UK).

4. 4´,6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Gillingham, Dorset, UK) is dissolved in citifluor to make a 3 µg/mL stock and stored at 4°C.
5. Nikon Eclipse E600 fluorescence microscope (Nikon, Welwyn GC, UK).
6. Zeiss LSM 510 META confocal microscope (Carl Zeiss, Inc. UK Ltd., Kingston upon Thames, UK).

2.5. Lysis of Mammalian Cells

1. Lysis buffer (2X): 20 m*M* Tris-HCl pH 6.8, 137 n*M* NaCl, 1 m*M* ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 10% glycerol; store at 4°C. 1X lysis buffer is prepared fresh with protease inhibitor (see below) just before harvesting cells for lysis.
2. Complete protease inhibitor cocktail tablet (Roche Diagnostics Ltd., Burgess Hill, UK) is dissolved in 2 mL sterile water and stored at –20°C. While making 1X lysis buffer, complete protease inhibitor cocktail solution is added at 1:25 dilution.

2.6. Sodium Dodecyl Sulfide–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Resolving gel (12%): 30% acrylamide:bis-acrylamide solution, 1.5 *M* Tris-HCl pH 8.8, 10% SDS, 10% ammonium persulfate (APS) (*see* **Note 4**), *N,N,N´,N´*-tetramethyl-ethylenediamine (TEMED) (*see* **Note 5**) and distilled water.
2. Stacking gel (5%): 30% acrylamide:bis-acrylamide solution, 1 *M* Tris-HCl pH 6.8, 10% SDS, 10% APS, TEMED, and distilled water.
3. Water-saturated isobutanol: Take equal volumes of distilled water and isobutanol in a glass bottle, mix in a stirrer overnight, allow to separate and store at room temperature. Use the top (butanol) layer.
4. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad, Hemel Hempsted, UK).
5. 3X sample buffer: 187.5 m*M* Tris-HCl pH 6.8, 6% w/v SDS, 30% glycerol, 150 m*M* DTT, 0.03% w/v bromophenol blue.
6. 10X Running buffer: 250 m*M* Tris, 1.92 *M* glycine. While making 1X gel running buffer with distilled water, add 10% SDS at 1:100 dilution.
7. SDS-PAGE apparatus (Bio-Rad).
8. Power pack (Bio-Rad).

2.7. Western Blotting

1. 10X Transfer buffer: 250 m*M* Tris, 1.92 *M* glycine. Make 1X transfer buffer with distilled water.
2. Hybond nitrocellulose membrane (Amersham Bioscience UK Ltd., Little Chalfont, UK).
3. Trans blot SD semi-dry transfer cell (Bio-Rad).
4. Power pack (Bio-Rad).
5. Extra-thick filter paper (Bio-Rad).

6. Blocking buffer: 6% (w/v) nonfat dry milk, 0.1% (v/v) Tween 20 in 1X PBS.
7. Platform rocker (Bibby Sterilin Ltd., Pontypridd, UK).
8. Primary antibody: Mouse anti-EGFP (8362-1, Clontech Laboratories Ltd., Mountain View, CA, USA), mouse anti-HA (MMS-101P, Covance Laboratories, Harrogate, UK). Dilutions are made in blocking buffer (*see* **Note 6**).
9. Secondary antibody: Enhanced chemiluminescent (ECL) anti-mouse IgG conjugated to horseradish peroxidase (NA931, Amersham Biosciences). Dilution is made in blocking buffer.
10. ECL Western blotting detection reagent (Amersham Biosciences).
11. High-performance chemiluminescence film (Hyperfilm ECL, Amersham Biosciences).
12. Hypercassette (Amersham Biosciences).
13. RP X-OMAT Processor, Model M6B (Developer, Kodak Ltd., Watford, UK).

2.8. Stripping and Reprobing Immunoblots

1. Stripping buffer: 10% SDS, 1 *M* Tris-HCl pH 6.8, 14 *M* β-mercaptoethanol.
2. Hot block (Bibby Sterilin Ltd.).
3. Primary antibody: Rabbit anti-actin (A 2066, Sigma-Aldrich, Gillingham, Dorset, UK). Dilution is made in blocking buffer.
4. Secondary antibody: Enhanced chemiluminescent (ECL) anti-rabbit IgG conjugated to horseradish peroxidase (NA934, Amersham Biosciences). Dilution is made in blocking buffer.

3. Methods

3.1. Mammalian Cell Culture

3.1.1. Culturing of COS-7 Cells

1. COS-7 cells are maintained at subconfluent densities in DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 2 m*M* l-glutamine at 37°C, 5% carbon dioxide (CO_2).
2. The cells are grown on coverslips in 6-well plates for immunofluorescence analysis.
3. The stock cultures are maintained in 75-cm^2 flasks (NUNC; Invitrogen) and subcultured periodically as described in **Subheading 3.1.1.1**.

3.1.1.1. COS-7 Subculture

1. The medium from the flask is removed and the cells are rinsed once with DMEM (without serum).
2. Two mL of 1X trypsin-EDTA is added to cover the cell layer and the flask is incubated at 37°C for 5 min or until (no longer than 10 min) the cells are dissociated from the flask.

3. The enzyme activity is quenched by adding 8 mL of fresh supplemented medium and the cells are dissociated by pipetting the medium up and down a few times. This also mechanically frees cells from individual clumps into suspension.
4. The cells are passaged in 1:10 to 1:20 ratio in fresh supplemented medium and maintained at 37°C, 5% CO_2 incubator with humidity.

3.1.2. Culturing of Stable PC12 Cells

1. The PC12 stable cells expressing either the huntingtin exon-1 fragment tagged to EGFP with 23 or 74 polyglutamine repeats (EGFP-HDQ23 or EGFP-HDQ74) ***(13)*** or the wild-type or A53T or A30P mutant α-synuclein ***(10)*** are maintained in DMEM supplemented with 10% heat-inactivated horse serum, 5% Tet-approved FBS, 100 U/mL penicillin/streptomycin, 2 m*M* L-glutamine, 100 µg/mL G418 (to maintain tet-on element) and 75 µg/mL hygromycin (to maintain the huntingtin exon-1 or α-synuclein components) at 37°C, 10% CO_2 with humidity.
2. The cells are seeded at 1–2 × 10^5 per well in 6-well plates either directly (for Western blot analysis) or on glass cover slips (for immunofluorescence analysis).
3. The expression of the transgene is switched on with 1 µg/mL (for huntingtin) or 2 µg/mL (for α-synuclein) doxycycline.
4. The expression of the transgene is switched off by removing the medium containing doxycycline followed by a few rinses with fresh supplemented doxycycline-free medium.

3.1.2.1. PC12 Subculture

1. PC12 cells are subcultured using a protocol similar to that used for COS-7 cells **(Subheading 3.1.1.1.)**, except that a much shorter incubation period with trypsin is required. Since PC12 cells tend to form clumps, care is taken to dissociate the clumps to a fine suspension before reseeding them.

3.2. Transient Transfection

3.2.1. Transient Transfection with EGFP-HDQ74 Plasmid DNA

1. COS-7 cells are grown on coverslips in 6-well plates for 24 h prior to transfection.
2. Transfection is performed using LipofectAMINE reagent (Invitrogen) (*see* **Note 7**). 4.5–6 µL of the reagent prediluted in 100 µL of serum-free DMEM is mixed with 1.5–2 µg of plasmid DNA (empty pEGFP or EGFP-tagged HDQ74) also diluted in 100 µL of serum-free DMEM.
3. The mixture is allowed to stand at room temperature for 30 min.
4. In the mean time, cells in 6-well plates are rinsed once with serum-free DMEM and a further 800 µL of the serum-free DMEM is added to the cells.
5. The lipofectAMINE-DNA mixture is then added to the cells, making the total transfection volume up to 1 mL.
6. The transfection mixture is replaced with fresh supplemented culture medium after 3–5 h incubation at 37°C, 5% CO_2. The transfected cells are treated with

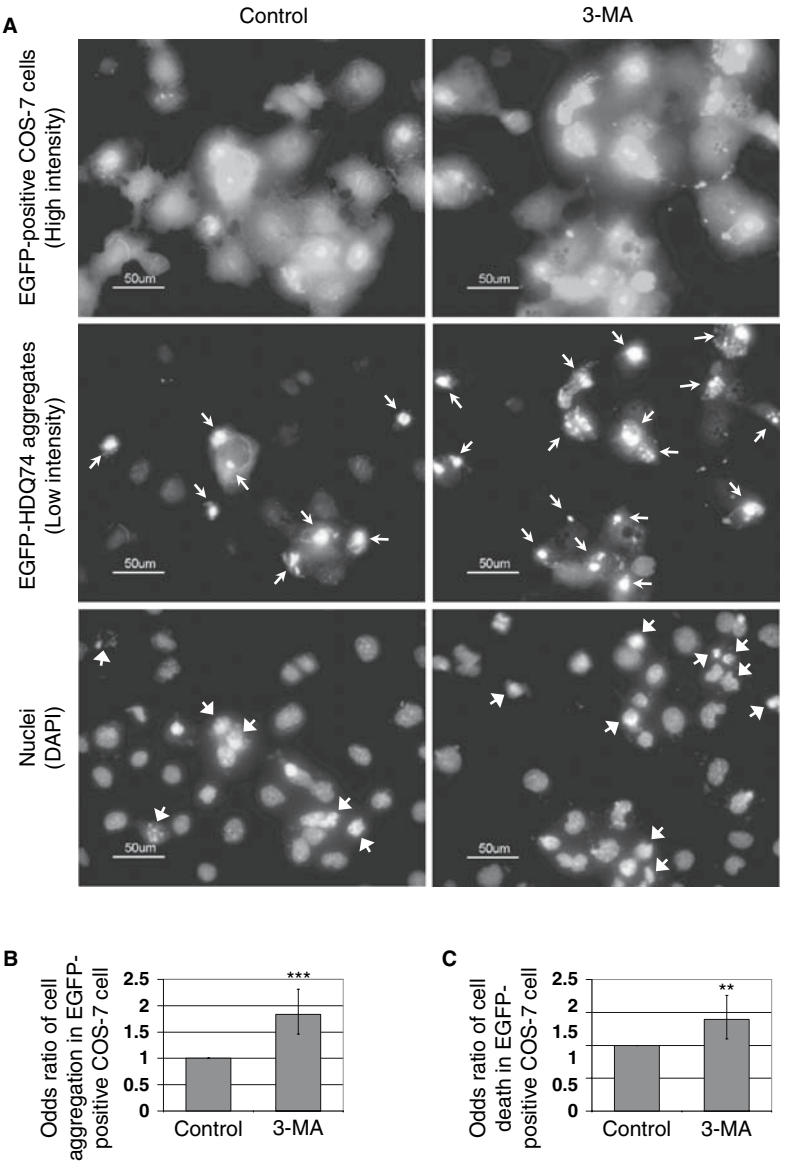

Fig. 1. Analysis of mutant huntingtin (EGFP-HDQ74)-induced aggregation and cell death in transiently transfected COS-7 cells in the presence of an autophagy inhibitor. **(A)** COS-7 cells were transfected with 1.5 μg of EGFP-HDQ74 construct for 4 h and then grown in complete media for 48 h. Cells were either left untreated or treated with 10 m*M* 3-MA (an autophagy inhibitor) for the last 15 h of the 48 h post-transfection period, and then fixed for immunofluorescence analysis. Fluorescent microscopy images of EGFP-positive COS-7 cells with EGFP-HDQ74 aggregates were

autophagy modulators (*see* **Subheading 3.2.2.**) and fixed for immunofluorescence (*see* **Subheading 3.2.5.**) after 48 h **(Fig. 1A)**.

3.2.2. Treatment of EGFP-HDQ74-Transfected COS-7 Cells with Modulators of Autophagy Pathway

1. COS-7 cells transiently transfected as above are treated with either 0.2 µg/mL rapamycin for 48 h posttransfection or 10 m*M* 3-methyl adenine or 400 n*M* bafilomycin A1 for the last 15 h of the 48 h posttransfection period prior to fixation (*see* **Subheading 3.2.5.**). Respective vehicles are used as controls.

3.2.3. Transient Transfection with EGFP-LC3 Plasmid DNA

1. COS-7 cells are grown on cover slips in 6-well plates for 24 h, followed by treatment with autophagy modulators for 24 h (*see* **Subheading 3.2.4.**) prior to transfection.
2. Transfection is performed using LipofectAMINE reagent (Invitrogen) and EGFP-LC3 construct, as described in **Subheading 3.2.1**.
3. The transfection mixture is replaced with fresh supplemented culture medium after 3–5 h incubation at 37°C, 5% CO_2. The transfected cells are treated with the autophagy modulators for a further 2 h (*see* **Subheading 3.2.4.**) and then fixed for immunofluorescence (*see* **Subheading 3.2.5.**) **(Fig. 2)**.

3.2.4. Treatment of EGFP-LC3-Transfected COS-7 Cells with Modulators of Autophagy Pathway

1. COS-7 cells are pretreated with 10 m*M* 3-methyl adenine or 0.2 µg/mL rapamycin for 24 h prior to transfection. EGFP-LC3 transfected cells are treated with these autophagy modulators for a further 2-h posttransfection period before fixing for immunofluorescence (*see* **Subheading 3.2.5.**).

Fig. 1. (*Continued*) taken at high (top panel) and low intensity (middle panel) to denote the number EGFP-positive transfected cells (top panel) and aggregates (indicated by arrows, middle panel), respectively. Nuclei were stained with DAPI (bottom panel) for analysis of cells showing apoptotic morphology (indicated by thick arrowheads). (Bar, 50 µm.). 3-MA treatment increased the proportion of transfected cells with aggregates and cell death, compared to the control. (**B** and **C**) Approximately 200 EGFP-positive COS-7 cells were counted for the proportion of cells with EGFP-HDQ74 aggregates **(B)** and cell death **(C)**. The effect of 3-MA treatment on the percentage of EGFP-positive cells with aggregates or apoptotic morphology (cell death) was expressed as odds ratios, and the control (untreated cells) was taken as 1. 3-MA significantly increased the proportion of cells with aggregates ($p < 0.0001$) and cell death ($p = 0.006$), compared to the control.

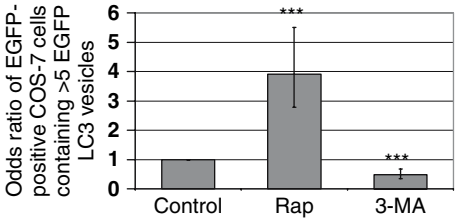

Fig. 2. Effect of autophagy modulators on EGFP-LC3 vesicles in transiently transfected COS-7 cells. COS-7 cells were pretreated with or without 0.2 μ*M* rapamycin (Rap) or 10 m*M* 3-MA for 24 h prior to transfection. Cells were then transfected with 1.5 μg of EGFP-LC3 construct for 4 h and treated with or without the autophagy modulators for a further 2 h. Cells were fixed and analyzed by fluorescence microscopy for the proportion of transfected cells with more than five EGFP-LC3 vesicles. The effects of treatment on the percentage of EGFP-positive cells with more than five EGFP-LC3 vesicles were expressed as odds ratios and the control (untreated cells) was taken as 1. Rapamycin (an autophagy inducer) significantly increased the number of cells with more than five EGFP-LC3 vesicles ($p < 0.0001$), whereas 3-MA (an autophagy inhibitor) significantly reduced the number of cells with more than five EGFP-LC3 vesicles ($p < 0.0001$).

3.2.5. Fixing Cells for Immunofluorescence Analysis

1. The cells on cover slips are rinsed once with 1X PBS and fixed with 4% paraformaldehyde in 1X PBS for 20 min.
2. The fixed cells are further rinsed three times with 1X PBS, air-dried, and mounted onto glass slides on the antifadent citifluor, supplemented with 3 μg/mL DAPI to allow visualization of nuclear morphology (*see* **Note 8**).

3.3. Quantification of Aggregate Formation and Abnormal Cell Nuclei

1. Aggregate formation and nuclear morphology are assessed using a fluorescence microscope (Nikon eclipse E600W fluorescent microscope) **(Fig. 1B,C)**.
2. Two hundred EGFP-positive COS-7 cells are randomly selected and the proportion of cells with EGFP-HDQ74 aggregates (bright fluorescent foci) is assessed. If a cell has no aggregate, a score of zero is given, while a cell having one or more aggregates is given a score of one ***(9)***. The observer is blinded to the identity of the slides and the experiments are performed in triplicate and are repeated twice.
3. Cells are considered dead if the DAPI-stained nuclei showed apoptotic morphology (fragmentation or pyknosis). Pyknotic nuclei are typically less than 50% the diameter of normal nuclei and show obvious increased DAPI intensity. These criteria are specific for cell death, as they show a very high correlation with propidium iodide staining of live cells ***(15)***. Furthermore, these nuclear abnormalities are reversed with caspase inhibitors ***(13,15)***.

3.4. Quantification of Cells with EGFP-LC3 Vesicles

1. The proportion of cells with EGFP-LC3 vesicles are assessed using a fluorescence microscope (Nikon eclipse E600W fluorescent microscope) **(Fig. 2)**.
2. Two hundred EGFP-positive COS-7 cells are counted for the proportion of cells with more than five EGFP-LC3 vesicles (green fluorescent punctuate structures). If a cell has 0–5 EGFP-LC3 vesicles, a score of zero is given, while a cell having more than 5 EGFP-LC3 vesicles is given a score of one ***(12)***. The observer is blinded to the identity of the slides and the experiments are performed in triplicate and are repeated twice.

3.5. Clearance Experiments in PC12 Stable Inducible Cell Lines

1. The transgene expression in the PC12 stable cells is induced by adding doxycycline (*see* **Subheading 3.1.2.**) for 8 h (for huntingtin cell lines) or 48 h (for the α-synuclein cell lines) ***(9,10)***.
2. The expression is then turned off by removing doxycycline from the media (*see* **Subheading 3.1.2.**).
3. Concurrent with switching off the expression, the cells are treated with the modulators of autophagy, 3-MA, Rap, or BafA1 (as in **Subheading 3.2.1.**) for 24, 48, or 72 h (for immunofluorescence analysis), or 120 h (for Western blot analysis). Equal amounts of water or DMSO are added as controls, where relevant.
4. The cells are either fixed for immunofluorescence as explained in **Subheading 3.2.2. (Fig. 3)** or collected for Western blot analysis as explained in **Subheading 3.6. (Fig. 4)**.

3.6. Western Blot Analysis for the Clearance of Aggregate-Prone Proteins

3.6.1. Preparation of Samples

1. The cells from 6-well plates are scraped into the media and collected in labeled Eppendorf tubes for each sample.
2. The cells are centrifuged at 5000 g at 4^{o}C for 5 min.
3. The supernatant is discarded and the cell pellets are washed once with 1X PBS.
4. The cells are then lysed with appropriate volumes of lysis buffer for 30 min on ice.
5. The lysed cells are centrifuged at 13,000 g for 5 min at 4^{o}C to remove cell debris and any unlysed cells.
6. The supernatant is stored at –80^{o}C until further analysis.
7. Protein assay is performed on the samples with Bio-Rad protein assay kit.
8. 30 µg of protein from each sample is mixed with 3X sample buffer (to a final 1X) and boiled at 100^{o}C for 5 min on a heating block before loading on the gel for SDS-PAGE.

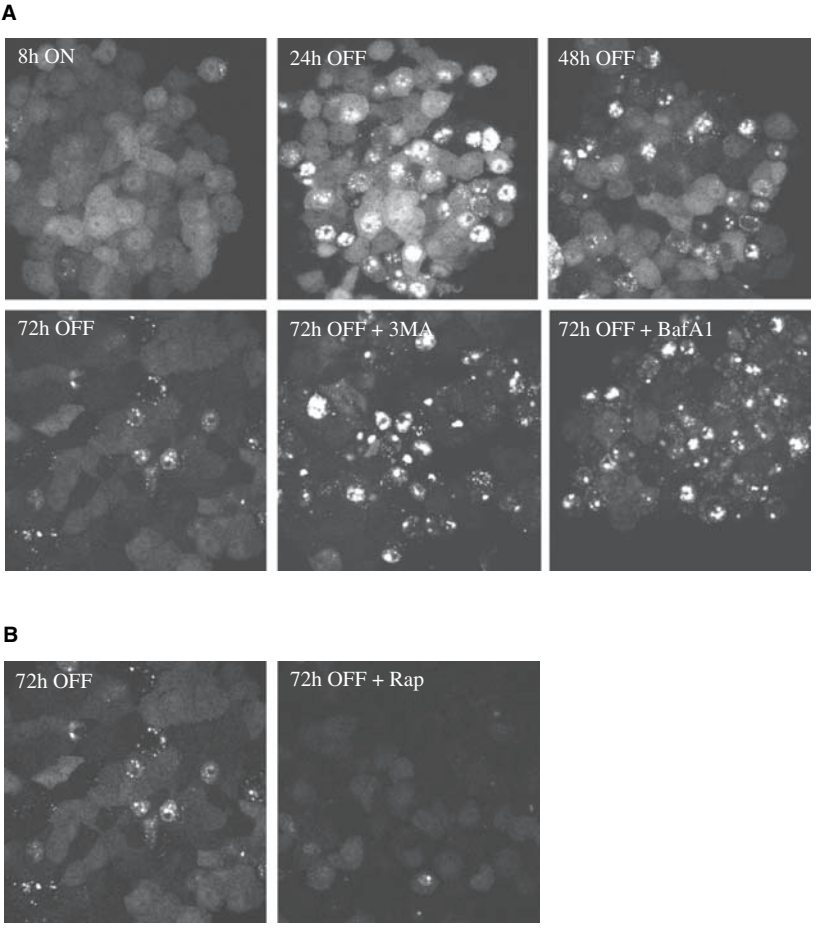

Fig. 3. Analysis of the clearance of huntingtin exon-1 with 74 polyQ repeats (EGFP-HDQ74) aggregates in a stable inducible PC12 cell line in the presence of modulators of autophagy. **(A)** The clearance of EGFP-HDQ74 aggregates was inhibited by the inhibitors of autophagy [3-methyladenine (3-MA) and bafilomycin A1 (BafA1)]. Stable PC12 cells expressing EGFP-HDQ74 were induced for 8 h (8h ON) with doxycycline. The expression was then turned off by removing doxycycline and the cells were left either untreated (24 h OFF, 48 h OFF, or 72 h OFF) or treated with 10 m*M* 3-MA (72 h OFF + 3-MA) or 400 n*M* BafA1 (72 h OFF + BafA1) for 72 h. **(B)** Enhanced clearance of EGFP-HDQ74 aggregates with induction of autophagy by 0.2 μ*M* rapamycin (Rap). Similar clearance experiment as in **(A)** was performed without (72 h OFF) or with rapamycin (72 h OFF + Rap).

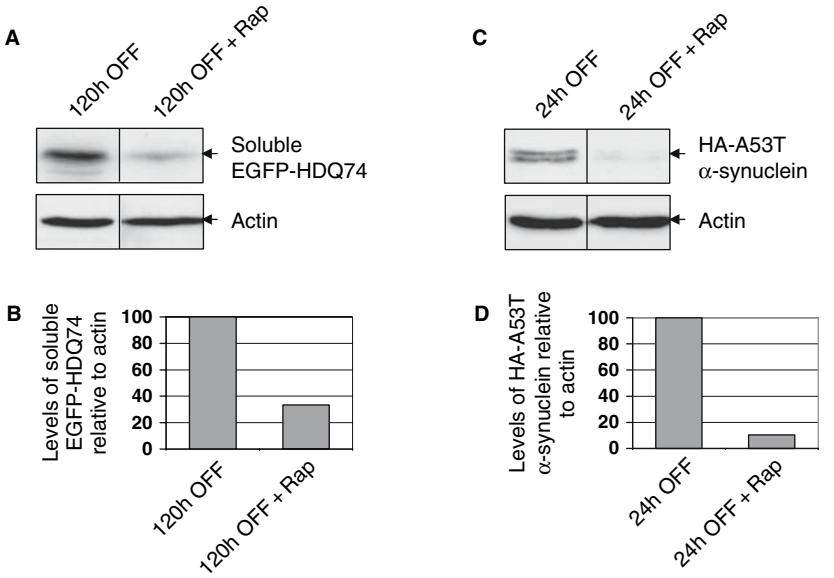

Fig. 4. Effect of rapamycin on the clearance of mutant aggregate-prone proteins in stable inducible PC12 cell lines expressing EGFP-HDQ74 or A53T α-synuclein. **(A)** Stable inducible PC12 cells expressing EGFP-HDQ74 were induced with doxycycline for 8 h and transgene expression was switched off (by removing doxycycline) for 120 h, with (+) or without (–) 0.2 μ*M* rapamycin (Rap). Clearance of soluble EGFP-HDQ74 was analyzed by immunoblotting with antibody against EGFP. Rapamycin (120 h OFF + Rap) enhanced the clearance of soluble EGFP-HDQ74 compared to the control (120 h OFF). Note that the lanes appearing as separate gel strips were derived from nonadjacent lanes from the same exposure of immunoblot from a single gel where both the samples were loaded; the intervening lanes were excised to simplify presentation. **(B)** Densitometry analysis for the clearance of soluble EGFP-HDQ74 was done by the ratio of intensity of EGFP relative to actin. Rapamycin treatment greatly enhanced the clearance of soluble EGFP-HDQ74 compared to the control condition (120 h OFF, which is set to 100%). **(C)** Stable inducible PC12 cells expressing the A53T mutant of α-synuclein were induced with doxycycline for 48 h and transgene expression was switched off (by removing doxycycline) for 24 h, with (+) or without (–) 0.2 μ*M* rapamycin (Rap). Clearance of A53T α-synuclein was analyzed by immunoblotting with antibody against HA. Rapamycin (24 h OFF + Rap) enhanced the clearance of A53T α-synuclein compared to the control (24 h OFF). Note that the lanes appearing as separate gel strips were derived from nonadjacent lanes from the same exposure of immunoblot from a single gel where both the samples were loaded; the intervening lanes were excised to simplify presentation. **(D)** Densitometry analysis for the clearance of A53T α-synuclein was done by the ratio of intensity of HA relative to actin. Rapamycin treatment greatly enhanced the clearance of A53T α-synuclein compared to the control (24 h OFF, set to 100%).

3.6.2. SDS-PAGE

1. SDS-PAGE is performed using Bio-Rad gel apparatus.
2. Resolving gel is first poured in between a gel plate with a 0.75- or 1-mm integrated spacer and a glass plate sandwich that are held together in the gel casting unit. The gel is poured up to 75% of the length of the plates, leaving space for pouring the stacking gel.
3. A small volume (500 μL) of water-saturated isobutanol is added on the surface of the resolving gel, which is later washed with distilled water when the gel has polymerised.
4. The stacking gel is poured over the resolving gel and a 10-well comb is inserted into it.
5. After polymerization of the stacking gel, the combs are removed and the wells thus created are rinsed with distilled water.
6. The casted gels are fitted into the gel apparatus and 1X gel running buffer is poured into the tank.
7. The boiled samples are then loaded into the wells along with a prestained molecular weight marker.
8. The gels are run at a constant current of 15 mA per gel until the marker bands migrate to the desired position.

3.6.3. Western Blotting

1. The proteins in the samples that have been separated by SDS-PAGE are transferred electrophoretically onto a nitrocellulose membrane using Bio-Rad semi-dry transfer apparatus.
2. Nitrocellulose membranes are cut according to the size of the gel and equilibrated in 1X transfer buffer along with extra-thick filter papers for 5–10 min. The gel is taken out and is also equilibrated in the transfer buffer.
3. The gel transfer unit is then assembled by placing a filter paper on the base plate of the transfer apparatus, then the nitrocellulose membrane, followed by the gel on top of the membrane and finally two more filter papers on the top. While placing the gel/filter papers on the membrane, the surface is rolled gently with a plastic pipet so as to remove any air bubbles.
4. After placing the compression plate and connecting the lid, the transfer is performed at a constant voltage of 15 V for approximately 1 h.
5. The membranes (immunoblots) are then removed and incubated in 25 mL of blocking buffer with gentle shaking on a rocker at room temperature for 1 h.
6. The blocking buffer is discarded and primary antibody diluted in 10 mL of blocking buffer (anti-EGFP at 1:2000 dilution for detecting mutant huntingtin and anti-HA at 1:1000 dilution for detecting mutant α-synuclein) is added to the immunoblots and incubated overnight on a rocker in a cold room at 4°C. Blotting can also be carried out by incubating the membrane with anti-EGFP antibody for 1 h at room temperature on a rocker (*see* **Note 9**).

7. The nonspecific binding of the primary antibody is then removed by washing the immunoblots with 0.1% Tween 20 in 1X PBS (PBS-T) solution, three times for 10 min each on a rocker at room temperature.
8. Secondary antibody (anti-mouse IgG conjugated to horseradish peroxidase at 1:2000 dilution) is added to the immunoblots and incubated for a minimum of 1 h (not more than 3 h) on a rocker at room temperature.
9. The nonspecific binding of the secondary antibody is then removed by washing the immunoblots in PBS-T solution, three times for 10 min each on a rocker at room temperature.
10. After the final wash and discarding the PBS-T, the following procedures are performed in a dark room under safe light conditions.
11. 1.5 mL each of the detection reagents 1 and 2 of the ECL Western blotting detection system are mixed and added to the immunoblots for 1 min, while ensuring coverage of the entire surface.
12. The detection solution is discarded and the immunoblots are then wrapped in cling films and placed in hypercassettes along with a hyperfilm with appropriate exposure times **(Fig. 4A,C)**.
13. The films are developed in an automated Kodak developer.

3.6.4. Stripping and Reprobing the Immunoblots

1. The immunoblots are stripped of the signal for EGFP or HA by immersing them in stripping buffer at 65^{o}C on a hot block for 10 min (*see* **Note 10**).
2. The immunoblots are then washed with 1X PBS twice for 5 min each on a rocker at room temperature, followed by blocking with blocking buffer for 1 h.
3. After discarding the blocking buffer, primary antibody (rabbit anti-actin at 1:2000 dilution) is added to the immunoblots and incubated either overnight at 4^{o}C or for 1 h at room temperature on a rocker.
4. The immunoblots are then washed three times with PBS-T as before, incubated with secondary antibody (ECL anti-rabbit IgG conjugated to horseradish peroxidase at 1:2000 dilution) for a minimum of 1 h on a rocker at room temperature, followed by three washes with PBS-T. The signal is detected with the ECL Western blotting detection system as before **(Fig. 4A,C)**.

3.7. Statistical Analysis

3.7.1. Odds Ratio for Quantification of Cells with Mutant Huntingtin Aggregation and Cell Death and EGFP-LC3 Vesicles

1. Pooled estimates for the changes in aggregate formation **(Fig. 1B)**, cell death **(Fig. 1C)**, or EGFP-LC3 vesicles **(Fig. 2)**, resulting from perturbations assessed in multiple experiments, are calculated as odds ratios with 95% confidence intervals [e.g., odds ratio of aggregation = (percentage of cells expressing construct with aggregates in perturbation conditions/percentage of cells expressing construct without aggregates in perturbation conditions)/(percentage of cells expressing

construct with aggregates in control conditions/percentage of cells expressing construct without aggregates in control conditions)]. Odds ratios were considered to be the most appropriate summary statistic for reporting multiple independent replicate experiments of this type, because the percentage of cells with aggregates under specified conditions can vary between experiments on different days, whereas the relative change in the proportion of cells with aggregates induced by an experimental perturbation is expected to be more consistent. Our lab has used this method frequently in the past to allow analysis of data from multiple independent experiments ***(12,13,15)***.

2. Odds ratios and p values are determined by unconditional logistical regression analysis, using the general log-linear analysis option of SPSS 9 software (SPSS, Chicago, USA). The control condition is set to one. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS, nonsignificant.

3.7.2. Densitometry Analysis on the Immunoblots for Assessing the Clearance of Mutant Aggregate-Prone Proteins

1. Densitometry analysis on immunoblots from three independent experiments is performed by Scion Image Beta 4.02 software (Scion Corporation, Maryland, USA). The control condition is set to 100%.
2. The clearance of the mutant aggregate-prone proteins is determined by the ratio of the intensity of EGFP to actin for mutant huntingtin clearance **(Fig. 4B)** or the ratio of the intensity of HA to actin for mutant α-synuclein clearance **(Fig. 4D)**.

4. Notes

1. Doxycycline is stored as a 100 mg/mL stock solution at –20°C in dark Eppendorf tubes to protect it from direct light. For experiments, it is diluted in sterile water to a 10 mg/mL working stock solution, which can also be stored at –20°C and can be used for a few months. The working stock is then diluted again in the cell culture medium to a final concentration of 1 μg/mL.
2. When 3-MA is stored at room temperature, it crystallizes. When using the stock solution for experiments, 3-MA needs to be warmed in a 37°C water bath until the solution becomes transparent. This should be started at least 30 min prior to use.
3. The PFA solution is warmed to about 50°C with constant stirring in a fume hood to help it dissolve. If the solution is cloudy, a few drops of 1 *M* NaOH may be added to make the solution clear. It is then cooled at room temperature, aliquoted, and stored at –20°C.
4. APS must be prepared fresh every time prior to pouring a gel.
5. TEMED is best stored at room temperature. As it may decline in quality after opening, it is better to buy small bottles.
6. Mouse anti-EGFP is an excellent antibody for Western blotting and can be used at 1:10,000 dilution. Mouse anti-HA antibody is diluted at 1:1000 in blocking buffer.

7. For 1 μg of DNA to be transfected in COS-7 cells in 6-well plates, use 3 μL of lipofectamine reagent.
8. The cover slips are mounted carefully onto the glass slides using forceps to prevent formation of air bubbles. Nail varnish is then applied to the edges of the cover slips to hold them firmly on the slides.
9. The primary antibodies can be reused a few times for subsequent experiments if stored in 0.02% sodium azide (done by dilution from a 10% stock solution) at –20°C. The only adjustment that is required is to increase the exposure time of the film at the ECL step.
10. Stripping of the immunoblots must be carried out in glass beakers on a hot plate inside a fume hood, as this generates a foul smell.

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