Evidence for p62 aggregate formation: Role in cell survival

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Abstract The polyubiquitin-binding protein p62 has been shown to localize in aggregates common to several types of diseases. Here, we report that p62 forms independent fibrillar aggregates in vitro in a time- and concentration-dependent manner. FTIR spectra and ThT fluorescence assay of p62 reveals increased β-sheet content as aggregates form compared to the native protein. The fibrillar nature of the aggregates was observed by transmission electron microscopy. Overexpression of p62 in HEK cells results in aggregate formation that may protect cells from apoptosis. Altogether, these results suggest that p62 fibrils may influence cell viability and indicates an important role for p62 in aggresome formation.

Keywords: p62; Protein aggregation; Alzheimer's disease; Sequestosome

1. Introduction

Sequestosome 1/p62 is a multimeric signaling protein that serves a wide variety of cellular functions, including: a scaffold in nuclear factor κB (NF-κB) activation, nerve growth factor-related signal transduction during neuronal cell differentiation, and regulation of gene transcription in the nucleus (reviewed in [1]). Through its C-terminal ubiquitin-associating (UBA) domain, p62 binds non-covalently to lysine 63 (K63)-linked polyubiquitinated proteins [2]. Traditionally, K63-linked polyubiquitin chains have been viewed as modifiers of protein function, including DNA repair, stress response, and activation of the IκBα signaling complex [3]. Recently, it has been shown that p62 binds to proteasomal subunits Rpn10/S5a and Rpt1 through its N-terminal Phox and Bem1p (PB1) domain [2]. Together, these findings suggest that p62 may serve as a shuttling factor for K63-polyubiquitinated proteins destined for degradation by the proteasome. This is a controversial observation, as the generally accepted mechanism for proteolysis through the ubiquitin–proteasome system (UPS) is the attachment of lysine 48 (K48)-linked polyubiquitin chains to the substrate, followed by the binding of the polyubiquitin chain to the proteasome in order to localize the substrate for degradation [3–5].

The accumulation of p62 has also been associated with neurodegenerative disease. The protein has been observed to localize in aggregates that are seen on analysis of brains taken from individuals with Alzheimer's disease (AD) [6,7]. p62 has also been found in Lewy bodies (LBs), a Parkinson's disease (PD)-associated aggregate, but not in precursors to LBs [8]. The association of p62 with neurodegenerative diseased tissues does not appear to be a coincidental result of accumulation of the protein, and it has been suggested that p62 plays an active role in aggregate formation by recruiting polyubiquitinated proteins through its UBA domain [2]. In addition, p62 is transcriptionally upregulated when the proteasome is inhibited, suggesting that aggregate formation may be a cellular reaction to stress conditions [8].

There are several different proteins that can aggregate when not degraded by the cell: α-synuclein in PD, amyloid-β (Aβ) proteins in AD, and mutant huntingtin in Huntington's disease (HD) are principal components of aggregates linked to neurodegenerative diseases. All of these proteins are also capable of forming aggregates in vitro when incubated at sufficient concentrations [9–11]. In response to accumulation of neurotoxic aggregates, the cell actually transports aggregates along the microtubules to the perinuclear area of the cytosol – called the microtubule organizing center – to form large inclusions called aggresomes [12]. Interestingly, in at least the case of Aβ-proteins, small protofilaments have been shown to be more neurotoxic than large aggregates, suggesting that the cell attempts to protect itself by moving all of the accumulating proteins to one place [10,12,13].

In this study, we set out to determine whether p62 expressed and purified from a bacterial system forms independent aggregates in vitro. In order to characterize these aggregates, p62 was tested to determine if the protein undergoes a conformational change during the process of aggregation. Finally, human embryonic kidney (HEK) cells that overexpress GFP-p62 were analyzed to examine the relationship between p62 aggregation and cell survival.

2. Materials and methods

2.1. GST-p62 protein preparation

Escherichia coli cells expressing GST-p62 plasmid were used for the preparation and purification of GST-p62 as previously described [2]. Protein concentrations were determined by Bradford Assay.

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Abbreviations: AD, Alzheimer's disease; GST, glutathione *S*-transferase; FTIR, Fourier transform infrared; ThT, Thioflavin T; HEK, human embryonic kidney; NF-κB, nuclear factor κB; UBA, ubiquitin associated; PB1, Phox and Bem1p; UPS, ubiquitin–proteasome system; LB, Lewy body; PD, Parkinson's disease; HD, Huntington's disease; GFP, green fluorescent protein; TEM, transmission electron microscopy

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2.2. Aggregation of GST-p62

For time-dependent assays, 25 μM GST-p62 was treated with 100 mM sodium acetate and 1 mM sodium azide [14]. The protein was incubated at 37 °C with constant agitation in a MT-360 microtube mixer (TOMY Tech USA, Inc., Fremont, CA) for up to 7 days. Aliquots were removed for analysis after 4–6 h, and at 1, 2, 3, and 7 days. For the concentration-dependent assays, GST-p62 was prepared as above at 12.5, 25, or 50 μM.

2.3. SDS–PAGE gel electrophoresis for coomassie blue stain or western blotting

Twenty-five microliter aliquots from time points described above were treated with 25 μL sample buffer, boiled, and stored at −80 °C. The proteins were separated via SDS–PAGE gel electrophoresis employing a 10% gel and stained with Coomassie blue, or transferred from the gel to nitrocellulose for Western Blot analysis employing mouse monoclonal anti-p62 (Transduction Labs, Lexington, KY) or polyclonal anti-p62 (Santa Cruz, San Diego, CA or gift of M.T. Diaz-Meco, Madrid, Spain) [2]. The blot was processed employing enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

2.4. Fourier transform infrared spectroscopy

Infrared spectra of 25 μM GST-p62 were obtained using a Nicolet 5PC FT-IR Spectrometer [9]. A small amount of protein solution was sandwiched between KBr discs, and a measurement was recorded. Percent transmittance of the peak at 1638 cm^{-1} was analyzed.

2.5. Thioflavin T fluorescence assay

Twenty microliter GST-p62 aliquots were removed at various times and 1 mL of 10 μM Thioflavin T (ThT) in 50 mM Tris–HCl (pH 8.0) was added. After mixing for 1 min, fluorescence was measured at λ_{ex} = 450 nm and λ_{em} = 482 nm on a Perkin–Elmer LS55 Luminescence Spectrometer [15].

2.6. Transmission electron microscopy

After incubation of 25 μM GST-p62 as above for 7 days, the p62 aggregates were collected by centrifugation at 14000 rpm in an Eppendorf Microcentrifuge for 5 min. The aggregates were fixed in 3% (v/v) glutaraldehyde in Millonig's phosphate buffer (0.1 M, pH 7.3), further fixed in 1% osmium tetroxide (w/v), and dehydrated in an ascending series of ethanols ending in propylene oxide. The sample was embedded in Durcupan ACM plastic (Electron Microscopy Sciences, Hatfield, PA), sectioned and stained with uranyl acetate and lead citrate. Sections were examined using a Philips 301 TEM (FEI Company, Hillsboro, OR) operating at 60 kV.

2.7. HEK cell transfection and confocal analysis

HEK 293 cells were transfected with GFP-p62 using Lipofectamine (Gibco-BRL, Grand Island, NY) and grown at 37 °C [2]. Over the course of 7 days, cells were removed and fixed. The cells were then viewed with a Bio-Rad MRC 1024 confocal microscope.

2.8. HEK cell viability assay

HEK cells were transfected with GFP-p62. After two days, cells were stained with LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Using confocal/fluorescence microscopy, cells with or without aggregates, were scored as live (green) or dead (red), respectively.

3. Results

3.1. Incubation of GST-p62 leads to aggregation

To determine if p62 could form aggregates in vitro, 25 μM GST-p62 was incubated for a period of 7 days. SDS–PAGE followed by Coomassie blue staining revealed that the GST-p62 band, at MW ≈ 70, disappeared with time, while high molecular weight p62 aggregates appeared (Fig. 1A). Western Blot analyses for p62 confirm that native GSTp62 disappeared

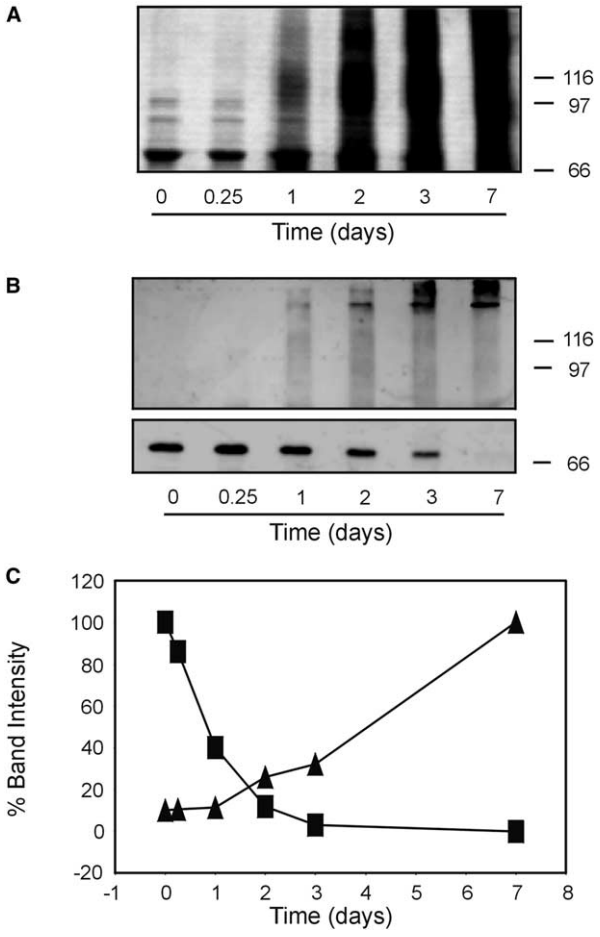

Fig. 1. p62 forms independent aggregates. (A) Coomassie blue stain of p62 aggregation. Twenty-five micromolar GST-p62 was incubated for 7 days at 37 °C. Aliquots were removed at time points shown, samples resolved by SDS–PAGE and stained with Coomassie blue. (B) Twenty-five micromolar GST-p62 was incubated as above, and proteins were separated by SDS–PAGE and Western blotted for p62. (C) Quantification of p62 aggregation. Band intensities of Coomassie blue stain in (A) were analyzed. GST-p62 band (■) was quantified (with background subtraction) and band intensity was analyzed relative to $t = 0$. HMW protein (▲) was quantified (with background subtraction), and band intensity was analyzed relative to $t = 7$ days.

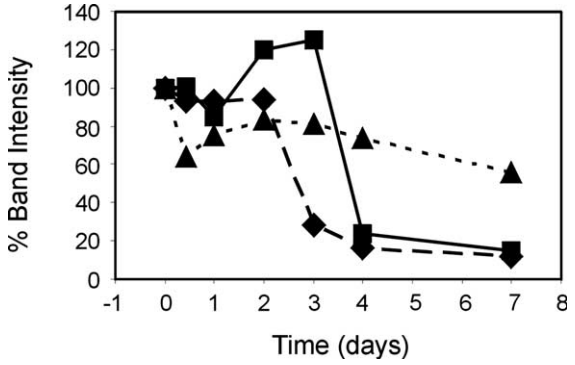

Fig. 2. Concentration-dependent aggregation of p62. GST-p62 at a concentration of 12.5 (▲----▲), 25 (■—■), and 50 (◆–-◆) μM was incubated and aliquots removed as indicated. The samples were resolved by SDS–PAGE and stained with Coomassie blue. GST-p62 bands were analyzed relative to $t = 0$.

Table 1
FTIR readings at 1638 cm^{-1}

Time (days)	% Transmittance
0	27.97
5	9.525
7	2.499

with time concomitant with formation of aggregated p62 (Fig. 1B). High molecular weight p62 aggregates were detected with both monoclonal (Fig. 1B) and polyclonal p62 antibodies (data not shown). Taken together, these data suggest that p62 forms high molecular weight aggregates in vitro (Fig. 1C). This finding is similar to aggregation observed for α-synuclein [15].

3.2. p62 aggregation is concentration-dependent

Numerous studies have shown that increased concentrations of neurodegenerative disease-related proteins lead to aggregation in vitro and in vivo [10,16,17]. To find out whether the aggregation of p62 depends on concentration, 12.5, 25, and 50 μM GST-p62 were incubated for 7 days (Fig. 2). Analysis of the GST-p62 band at each concentration showed that 12.5 μM GST-p62 did not completely aggregate by 7 days, 25 μM GST-p62 completely aggregated by day 4, and 50 μM

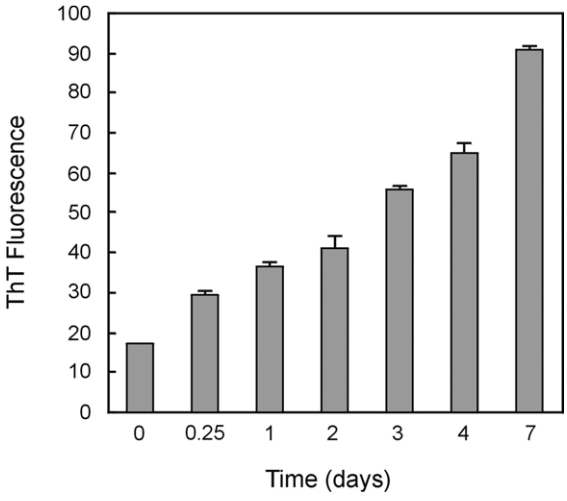

Fig. 3. Conformational change in p62 shown by ThT fluorescence. Twenty-five micromolar GST-p62 was incubated and aliquots were removed at time points shown. At these times, 10 μM Thioflavin T was added to each aliquot. After 1 min of mixing, the fluorescence of the solution was measured, with $\lambda_{ex} = 450$ nm and $\lambda_{em} = 482$ nm ($X \pm$ S.E.M of triplicate assays conducted with two separate preparations of p62).

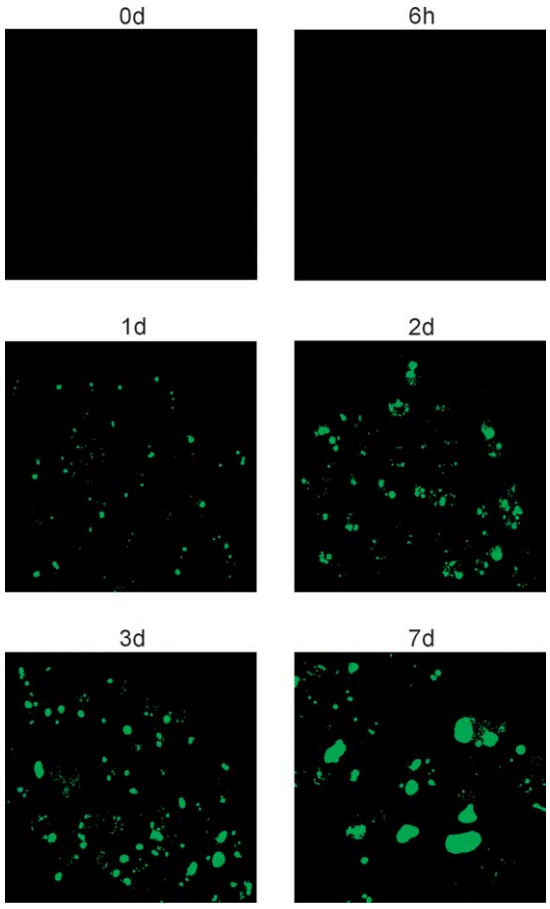

Fig. 5. Aggregation of p62 in HEK cells. HEK cells transfected with GFP-p62 were fixed at time points shown post-transfection. The cells were then viewed with a confocal microscope.

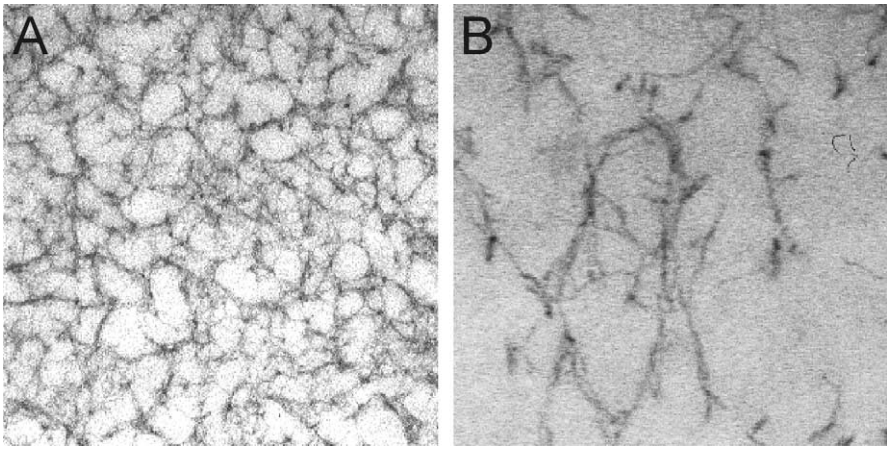

Fig. 4. p62 forms fibrils. Transmission electron microscopy of p62 aggregates reveals their fibrillar nature. Fibers of various lengths were observed. (A) 19000 mag. (B) 54000 mag.

GST-p62 completely aggregated in 3 days. In addition, after 4 days the aggregates from the 50 μM prep were so large that they became insoluble and precipitated from solution.

3.3. Native p62 protein undergoes a conformational change during aggregation

To address whether p62 aggregates exhibit a conformational change, we performed Fourier transform infrared (FTIR) spectroscopy. FTIR is a method for investigation of protein secondary structure. The FTIR spectra revealed a large change in peak intensity at 1638 cm^{-1} over time, suggesting that aggregated p62 is transformed into a partially folded conformation with a significant amount of β-structures compared to the native protein (Table 1). As a separate means of examining the β-sheet content of the aggregated preparation, ThT binding was also examined (Fig. 3). ThT is a histological dye that fluoresces when it binds to β-sheet structure. The observed increase in ThT fluorescence over time confirms a proportional increase in the β-sheet content of p62 as it aggregates. Only multimeric fibrillar forms, not multiple β-sheet domains in native proteins give significant fluorescence with ThT. This change in structure is commonly observed in amyloidous protein aggregates [14,17].

3.4. p62 aggregates form fibrils

Next, we set out to further examine whether the aggregates were amorphous or fibrillar. The p62 aggregate preparations were analyzed by transmission electron microscopy (TEM) (Fig. 4). Observation of the images revealed that the aggregates were composed of long fibrils, which is consistent with the ThT fluorescence results (Fig. 3).

3.5. Overexpression of p62 in HEK cells leads to aggregation and aggresome formation

To determine whether p62 forms aggregates in vivo, HEK cells were transfected with GFP-p62, and the cells were viewed by confocal microscopy (Fig. 5). By 12 h, small punctate aggregates began to appear. After 2–3 days, many large aggregates were visible. By 6–7 days, the aggregates appear to form large aggresomes. To examine the effects of p62 aggregation on

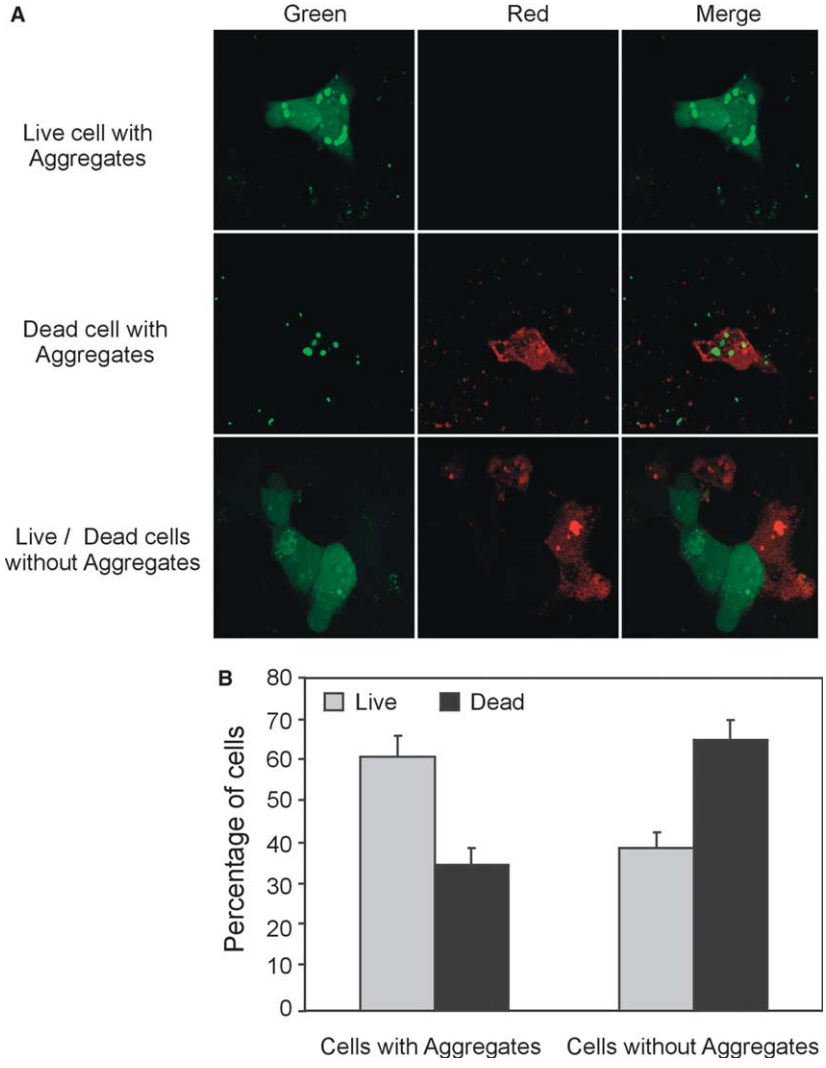

Fig. 6. p62 aggregates are associated with living cells. (A) Two days after GFP-p62 transfection, cells were treated with viability/cytotoxicity kit, and viewed with a confocal microscope. Representative live cells stained green and dead cells are red. Green dots are GFP-p62 aggregates. (B) Cells with or without aggregates ($n = 200$) were observed and counted as alive or dead from three separate experiments ($X \pm$ S.E.M).

cell viability, HEK cells transfected with GFP-p62 were incubated for 2 days, a live/dead stain was applied to the cells, and then the proportion of cells that were alive or dead were scored (Fig. 6). We observed that cells with aggregates were more likely to be alive than dead, although there were a percentage of the cells overexpressing p62 that were dead. By comparison, cells lacking p62 aggregates were more likely to be dead than alive. Therefore, the formation of p62 aggregates may serve a protective role and influence cell viability.

4. Discussion

Here, we demonstrate that p62 can spontaneously form fibrillar β-sheet aggregates with significant β-sheet secondary structure. GST-p62 formed time- and concentration-dependent aggregates in vitro. At high concentrations, these aggregates became large inclusions that precipitated from solution. Aggregation was coupled with an increase in the β-sheet content of the protein, and TEM analysis revealed that p62 aggregates are similar in structure to amyloid fibrils and other AD-type aggregates [9,10]. Although the concentrations used in the in vitro assays are non-physiological, these findings have implications for the role of p62 in neurodegenerative disease.

Under normal conditions, damaged, misfolded, or otherwise potentially neurotoxic proteins are degraded by the proteasome [3]. However, if there is a malfunction in the UPS or some other stress that may overload the UPS, such as an increase in reactive oxygen species, the cell may respond by compartmentalizing damaged proteins into aggregates [18,19]. Aggregates were originally considered to be the neurotoxic species in neurodegenerative disease-effected cells, but it may be that certain proteins can become neurotoxic unless sequestered by the cell. While the cell usually degrades these proteins via the proteasome, there might be an alternative mechanism to sequester dysfunctional proteins to prevent them from interacting with native and functional proteins if the proteasome is somehow defective. The hypothesis that aggregation is a protective mechanism continues to gain support [2,8,20]. Shin originally proposed that p62 binds to polyubiquitinated proteins in order to store them in a structure called a sequestosome, and that cellular signals cause the polyubiquitinated proteins to be released from the sequestosome for proteosomal degradation [21]. We extend this finding demonstrating that p62 recruits dysfunctional proteins destined for degradation via its UBA domain, which leads to sequestration of these proteins [2], and thereby protects the cell from potential toxic effects.

Interestingly, p62 has been associated with inclusions of not only AD but intracytoplasmic hyaline bodies [22], Mallory bodies [23], Lewy bodies [8], and expanded polyglutamine characteristic of HD [24], all of which contain detergent insoluble polyubiquitinated proteins. Given our earlier findings [2], and those herein, we propose that these diverse inclusions represent sequestration of ubiquitinated misfolded proteins, which arise as a response mechanism to protect the cell from death, and may thereby delay the progression of disease. Thus, we propose that disease progression may be accelerated in the absence of p62 due to an inability to sequester proteins in aggregates. This hypothesis is currently being tested in p62−/− mice.

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