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Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases

Jeanne M.M. Tan[1,2,3,‡], Esther S.P. Wong[1,†,‡], Donald S. Kirkpatrick[6], Olga Pletnikova[5], Han Seok Ko[2], Shiam-Peng Tay[1], Michelle W.L. Ho[1], Juan Troncoso[3,5], Steven P. Gygi[6], Michael K. Lee[5], Valina L. Dawson[2,3,4], Ted M. Dawson[2,3,4,‡] and Kah-Leong Lim[1,7,8,*,‡]

[1]Neurodegeneration Research Lab, National Neuroscience Institute, Singapore, [2]Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, USA, [3]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA, [4]Department of Neuroscience and Physiology, Johns Hopkins University School of Medicine, Baltimore, MD, USA, [5]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA, [6]Department of Cell Biology, Harvard Medical School, Boston, MA, USA, [7]Duke-NUS Graduate Medical School, National University of Singapore, Singapore and [8]Department of Biological Sciences, National University of Singapore, Singapore

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Although ubiquitin-enriched protein inclusions represent an almost invariant feature of neurodegenerative diseases, the mechanism underlying their biogenesis remains unclear. In particular, whether the topology of ubiquitin linkages influences the dynamics of inclusions is not well explored. Here, we report that lysine 48 (K48)- and lysine 63 (K63)-linked polyubiquitination, as well as monoubiquitin modification contribute to the biogenesis of inclusions. K63-linked polyubiquitin is the most consistent enhancer of inclusions formation. Under basal conditions, ectopic expression of K63 mutant ubiquitin in cultured cells promotes the accumulation of proteins and the formation of intracellular inclusions in the apparent absence of proteasome impairment. When co-expressed with disease-associated tau and SOD1 mutants, K63 ubiquitin mutant facilitates the formation of tau- and SOD-1–positive inclusions. Moreover, K63-linked ubiquitination was found to selectively facilitate the clearance of inclusions via autophagy. These data indicate that K63-linked ubiquitin chains may represent a common denominator underlying inclusions biogenesis, as well as a general cellular strategy for defining cargo destined for the autophagic system. Collectively, our results provide a novel mechanistic route that underlies the life cycle of an inclusion body. Harnessing this pathway may offer innovative approaches in the treatment of neurodegenerative disorders.

INTRODUCTION

Most, if not all, neurodegenerative diseases are marked by the presence of ubiquitin-positive protein aggregates. Indeed, the accumulation of insoluble proteinaceous deposits enriched with ubiquitin and components of the ubiquitin-proteasome system (UPS) occur in a broad spectrum of human neurodegenerative disorders, such as the neurofibrillary tangles in Alzheimer's disease (AD) and frontotemporal dementia, Lewy

*To whom correspondence should be addressed at: Neurodegeneration Research Laboratory, National Neuroscience Institute, 11 Jalan Tan Tock Seng, 308433, Singapore. Tel: 6563577520; Fax: 6562569178; Email: kah_leong_lim@nni.com.sg
†Present address: Department of Anatomy and Structural Biology, Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Bronx, NY, USA.
‡J.M.M.T., E.S.P.W., T.M.D and K.L.L contributed equally to this work.

bodies (LBs) in Parkinson's disease (PD) and Bunina bodies in amyotrophic lateral sclerosis (ALS) (1). This general phenomenon that typifies an otherwise diverse array of diseases suggests a common mechanism in the formation of protein inclusions associated with these varied disorders. Supporting this, several studies collectively point toward an intimate relationship between UPS aberrations and the biogenesis of neurodegenerative disease-linked protein inclusions (1–3).

Ubiquitin-proteasome system is an intracellular protein degradation system that is responsible for the majority of protein turnover within the cell (4). In this system, proteins destined for degradation are covalently tagged with a polymeric ubiquitin chain in which the terminal residue (G76) of one ubiquitin molecule is linked through an isopeptide bond to a lysine (K) residue (most commonly K48) within another. The G76–K48 polyubiquitinated substrate is then targeted for degradation by the 26S proteasome, a large protease complex consisting of a barrel-shaped 20S proteolytic core in association with two 19S regulatory caps, one on each side of the barrel's openings. It is important to mention that the ubiquitin sequence contains seven lysine residues (at positions 6, 11, 27, 29, 33, 48 and 63) and polyubiquitin chain assembly can occur at any of these lysine residues (5,6). In addition, proteins can also be monoubiquitinated (5,6). Notably, both K63-linked polyubiquitination and monoubiquitination of proteins are not typically associated with their degradation (6–8).

Currently, it is unclear whether different ubiquitin topologies contribute to the formation of an inclusion body. However, we have recently demonstrated that K63-linked ubiquitination facilitates the formation of LB-like inclusions in cultured cells (9). This observation, together with the recognition that ubiquitin-enriched protein aggregates are seemingly stabilized structures, prompted us to investigate the likely generic role of non-proteolytic ubiquitin modifications in the formation of inclusions associated with neurodegenerative diseases. We show here in the setting of ectopic overexpression of different ubiquitin species that lysine 48 (K48)-linked polyubiquitination, as well as non-proteolytic monoubiquitination or lysine 63 (K63)-linked ubiquitin modification contribute to the biogenesis of inclusions. K63-linked polyubiquitin is the most consistent enhancer of inclusions formation. Moreover, K63 ubiquitin-modified inclusions are preferentially cleared by autophagy. Taken together, these data point toward a potential mechanism of protein inclusion biogenesis and clearance that may be important in neurodegenerative diseases.

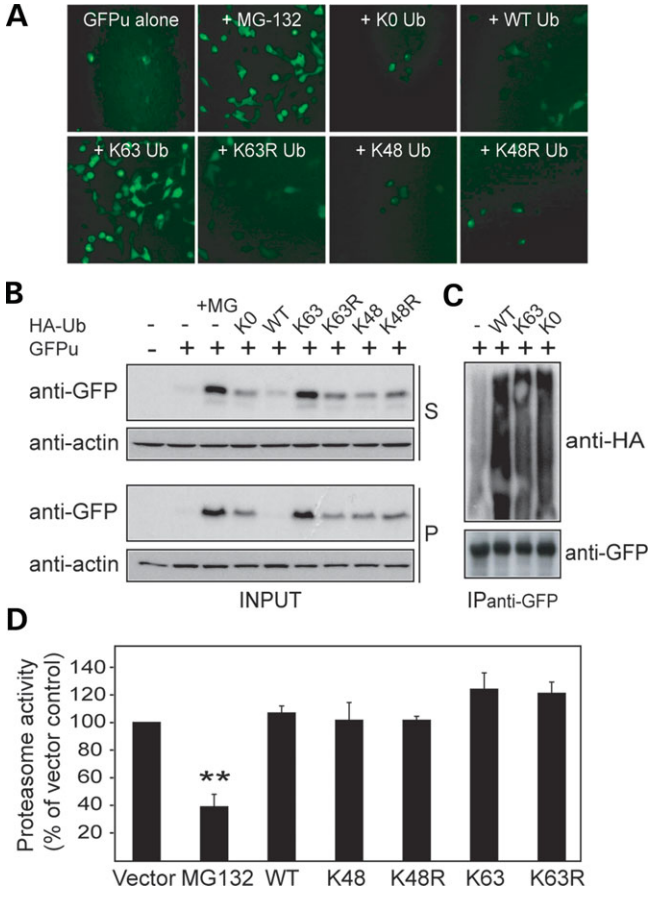

Figure 1. K63-linked ubiquitination promotes the stability of the short-lived GFPu protein. (**A**) Fluorescence microscopy of HEK 293 cells transfected with GFPu in the presence and absence of proteasome inhibitor, MG-132 or in the presence of wild-type or mutant ubiquitin co-expression. A carefully titrated amount of GFPu was used in our transfection paradigm such that GFPu expression is negligible in untreated control cells. (**B**) Anti-GFP immunoblots of cell extracts sequentially prepared with Triton X-100 (S) and SDS (P) from HEK 293 cells transfected with GFPu alone or with various HA-tagged ubiquitin species. Membranes were stripped and re-probed for β-actin to show equivalent loading of the different lysates. (**C**) Anti-GFP and anti-HA immunoblots of GFPu immunoprecipitates (IP_{GFP}) prepared from HEK 293 cells transfected with GFPu alone or with wild type K63 or K0 ubiquitin. Each of these experiments was repeated at least three times. (**D**) Bar graph showing the chymotrypsin-like proteasome activities of lysates prepared from cells transfected with various ubiquitin species, as indicated. Control cells treated with MG-132 is indicated. ($^{*}P < 0.05$, $^{**}P < 0.001$ versus untreated control group).

RESULTS

Expression of K63 ubiquitin mutant promotes the stability of the short-lived GFPu protein

To determine whether different ubiquitin species could alter the stability of proteins, we co-transfected HEK 293 cells with the unstable protein, GFPu (10), in the absence or presence of HA-tagged wild-type, K0, K48, K48R, K63 or K63R ubiquitin. K48 and K63 ubiquitin mutants contain arginine substitutions on all their lysine residues except the one at position 48 and 63, respectively, and are thus expected to promote the proteasome-linked G76–K48 and the proteasome-independent G76–K63 ubiquitin linkages, respectively. On the other hand, the K48R and K63R ubiquitin mutants contain a single lysine to arginine mutation at position 48 and 63, and are expected to disrupt G76–K48 and G76–K63 ubiquitin chain assembly, respectively. The K0 ubiquitin mutant is a lysineless ubiquitin theoretically only capable of mediating monoubiquitination. Consistent with the reported instability of GFPu (10), we observe a negligible number of GFPu-positive cells among the transfected control cells and a significant increase following treatment with proteasome inhibitors such as MG-132 (Fig. 1A) or lactacystin (data not shown). Interestingly, we also observe a dramatic enhancement in the number of GFPu-positive cells following co-expression of K63 mutant ubiquitin (Fig. 1A). This high level of enhancement is not observed when GFPu is

co-expressed with other ubiquitin species, including K48 and K0 mutant (Fig. 1A). Anti-GFP immunoblotting of Triton-X detergent-soluble (S) cell lysates reveals a prominent accumulation of GFPu in cells transfected with K63 ubiquitin and in cells treated with proteasome inhibitors (Fig. 1B). This accumulation is also observed in the Triton-X detergent-insoluble (P) fraction (Fig. 1B). In contrast, the co-expression of GFPu with ubiquitin species other than K63 ubiquitin produces substantially smaller effects, suggesting that GFPu accumulation is preferentially enhanced in the presence of K63-linked ubiquitination (Fig. 1B). Next, we examined whether GFPu is ubiquitinated following overexpression of K63 ubiquitin. GFPu precipitated from cells transfected with HA-tagged wild-type ubiquitin or K63 mutant ubiquitin shows a robust ladder of anti-HA immunoreactivity in both cases, indicating competent polyubiquitination of GFPu by both ubiquitin species (Fig. 1C). Interestingly, we also observe robust ubiquitination associated with the co-expression of GFPu and K0 ubiquitin, suggesting that GFPu can be multi-monoubiquitinated (Fig. 1C). Although GFPu appears capable of being modified by ubiquitin signals of various topologies, K63-linked polyubiquitination appears to preferentially promote its accumulation (Fig. 1B). To rule out possible impairment of the proteasome by K63 ubiquitin overexpression, we performed *in vitro* 20S proteasome activity assays with lysates prepared from cells transfected with wild-type, K48, K48R, K63 or K63R ubiquitin. Whereas the proteasome activity in MG-132-treated control cell lysates decreases by ~60% relative to control cells, we did not observe any proteasomal impairment associated with the overexpression of the various ubiquitin species examined, including K63 ubiquitin (Fig. 1C). Similar results were obtained with lysates prepared from cells stably expressing wild-type, K63 or K63R ubiquitin species (data not shown). Our results therefore suggest that overexpression of mutant ubiquitin species does not overtly impair the catalytic activity of the proteasome core particle (20S). However, these data do not exclude the possibility that different ubiquitin-linked chains, including K63-linked ubiquitin chains, may exert inhibitory effects through interactions with regulatory components present within the 19S cap.

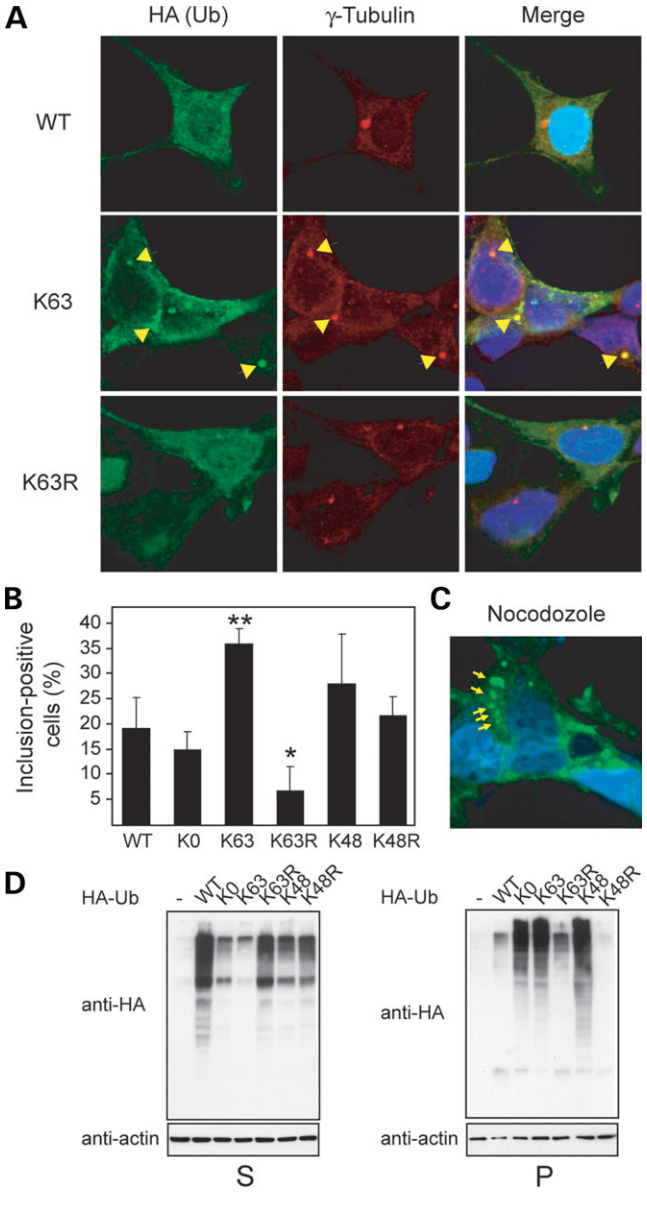

Figure 2. K63-linked ubiquitination enhances the formation of protein aggregates. (**A**) Representative confocal images of SH-SY5Y cells ectopically expressing wild-type (WT), K63 or K63R ubiquitin immunostained with anti-ubiquitin (green) or anti-γ-tubulin (red). Arrows in merge pictures show the co-localization of K63 (but not WT or K63R) ubiquitin-positive inclusions with γ-tubulin (yellow). (**B**) Bar graph showing the percentage of cells containing anti-HA-ubiquitin-positive inclusions in cells ectopically expressing various ubiquitin species, as indicated. ($^{*}P < 0.05$, $^{**}P < 0.001$ versus control group). (**C**) Dispersion of anti-FLAG (Ub)-positive inclusions (green) occurs in nocodozole-treated K63 ubiquitin-expressing cells. (**D**) Anti-HA immunoblots of cell extracts sequentially prepared with Triton X-100 (S) and SDS (P) from SH-SY5Y cells transfected with various ubiquitin species, as indicated. Each of these experiments was repeated at least three times.

Expression of K63 ubiquitin mutant promotes the formation of intracellular aggresome-like aggregates

To determine the relative contribution of various ubiquitin topologies toward inclusions formation, SH-SY5Y neuroblastoma cells were transfected with wild-type, K0, K48, K48R, K63 or K63R ubiquitin. The examination of transfected cells by confocal microscopy reveals the occasional occurrence of anti-HA-immunoreactive inclusions in ~16% of control cells transfected with wild-type ubiquitin (Fig. 2A and B). When cells were transfected with K48 mutant ubiquitin in place of wild-type ubiquitin, the number of inclusion-positive cells increases slightly but did not approach statistical significance (Fig. 2B). Similarly, the number of the inclusion-positive cells is comparable between wild-type and K48R or K0 mutant ubiquitin-expressing cells (Fig. 2B). However, when cells were transfected with K63 mutant ubiquitin in place of wild-type ubiquitin, the number of the inclusion-positive cells increases significantly by >2-fold (Fig. 2A and B). These inclusion bodies, which are often peri-nuclear in location, commonly co-localize with the centrosome marker γ-tubulin and resemble aggresome structures (Fig. 2A). Treatment with the microtubule depolymerizing agent nocodozole led to dispersion of these perinuclear aggregates into multiple

smaller aggregates scattered throughout the cytoplasm and confirmed their aggresome-like property (Fig. 2C). Substituting K63 with K63R in transfected cells resulted in a significant reduction in their propensity to generate aggresome-like inclusions (Fig. 2A and B). Interestingly, anti-HA immunoblotting of the Triton-X detergent-soluble (S) and -insoluble (P) fractions (SDS soluble) prepared from these transfected cells reveals a global accumulation of anti-HA positive, high molecular weight proteins in the P fractions prepared from cells overexpressing K0, K48 or K63, but not wild-type, K48R or K63R ubiquitin (Fig. 2D). In contrast, wild-type, ubiquitinated proteins accumulate mainly in the Triton-X detergent-soluble (S) fractions (Fig. 2D). Our results suggest that excessive monoubiquitination, as well as K48- or K63-linked polyubiquitination, could influence the cellular distribution of proteins, but only K63-linked polyubiquitination significantly promotes the formation of aggresome-like aggregates.

Both K63- and K48-linked ubiquitination promote mutant tau and SOD1 accumulation and inclusion formation

Next, we examined the effects of overexpression of different ubiquitin mutants on the propensity of a disease-associated tau mutant to form inclusions (Fig. 3 and Supplementary Material, Fig. S1). The analysis of mutant (P301L) tau-transfected SH-SY5Y cells under confocal microscopy reveals a generally uniform cytoplasmic staining of the exogenous tau protein, although a subset of cells (~19% of transfected cells) expressing the mutant tau produce inclusions (Fig. 3A and Supplementary Material, Fig. S1A). Upon MG-132 treatment, the number of inclusion-positive cells associated with mutant tau expression increases by >3-fold relative to control cells (Fig. 3A and Supplementary Material, Fig. S1A). This observation is consistent with a recent report showing that proteasomal inhibition stabilizes tau inclusions (11). Co-expression of mutant tau with K63 ubiquitin, but not wild-type or K63R ubiquitin, in the absence of MG-132 treatment resulted in a significant enhancement of tau-positive inclusion formation (Fig. 3A and Supplementary Material, Fig. S1A). The number of tau-positive inclusion also increases significantly in the presence of K48 and K48R ubiquitin overexpression, but more modestly so in the presence of K0 ubiquitin overexpression (Fig. 3A and Supplementary Material, Fig. S1A). Similar observations are made when we substituted tau mutant with ALS-associated mutant SOD1, except that in this case, K0 ubiquitin, along with wild-type or K63R ubiquitin species, have no significant effects on the propensity of mutant SOD to form inclusions when co-expressed (Fig. 3B and Supplementary Material, Fig. S1B). It thus appears that in the presence of disease-associated proteins, both K48- and K63-linked ubiquitin modifications (and to a lesser extent, monoubiquitination) could participate in the formation of inclusions mediated by the pathogenic proteins. Notably, sequential detergent fractionation of lysates prepared from these transfected cells reveals a selective accumulation of mutant P301L (Supplementary Material, Fig. S1C) and mutant A4V SOD (Supplementary Material, Fig. S1D) protein in the detergent-insoluble fraction with K63 ubiquitin co-expression.

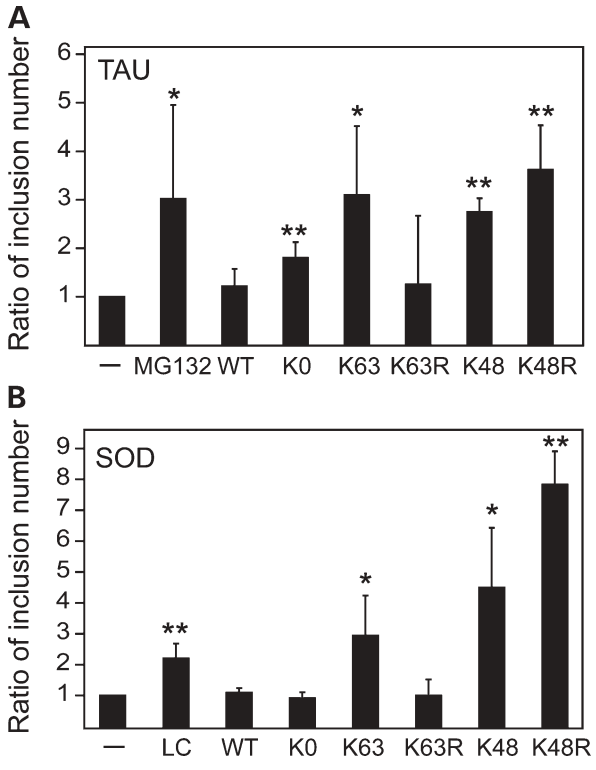

Figure 3. Both K48- and K63-linked ubiquitination promotes the formation of tau- and SOD-positive inclusions. Bar graph showing the ratio of inclusion number relative to untreated cells (−) observed in (**A**) P301L mutant tau-transfected cells or (**B**) SOD-A4V mutant-transfected cells under various conditions ($^{*}P < 0.05$, $^{**}P < 0.001$ versus control group). LC refers to lactacystin-treated cells. Each of these experiments was repeated at least three times.

Inclusions enriched with K63-linked polyubiquitin chains are preferentially cleared by autophagy

Emerging evidence implicates autophagy, a lysosome-mediated bulk degradation system, as a key regulator of inclusion dynamics (12–14). We wondered whether ubiquitin linkages on protein inclusions influenced their degradation through autophagy. Accordingly, autophagic clearance of inclusions formed under conditions of proteasomal impairment was investigated using a method recently described by Fortun *et al*. (13). SH-SY5Y cells transiently transfected with wild-type, K0, K48, K48R, K63 and K63R ubiquitin species were initially treated with lactacystin for 16 h to facilitate protein inclusion formation before a recovery period (24 h) in normal culture media supplemented either with normal (10%) or low (1%) serum. Cells recovering in low serum conditions undergo stimulated autophagy (13). Cells were initially visualized using the lysosomal stain, lysotracker. Consistent with activation of the autophagic system in response to serum starvation, we observed an apparent increase in the size and number of lysosomal vacuoles in control cells recovering from proteasomal inhibition in low

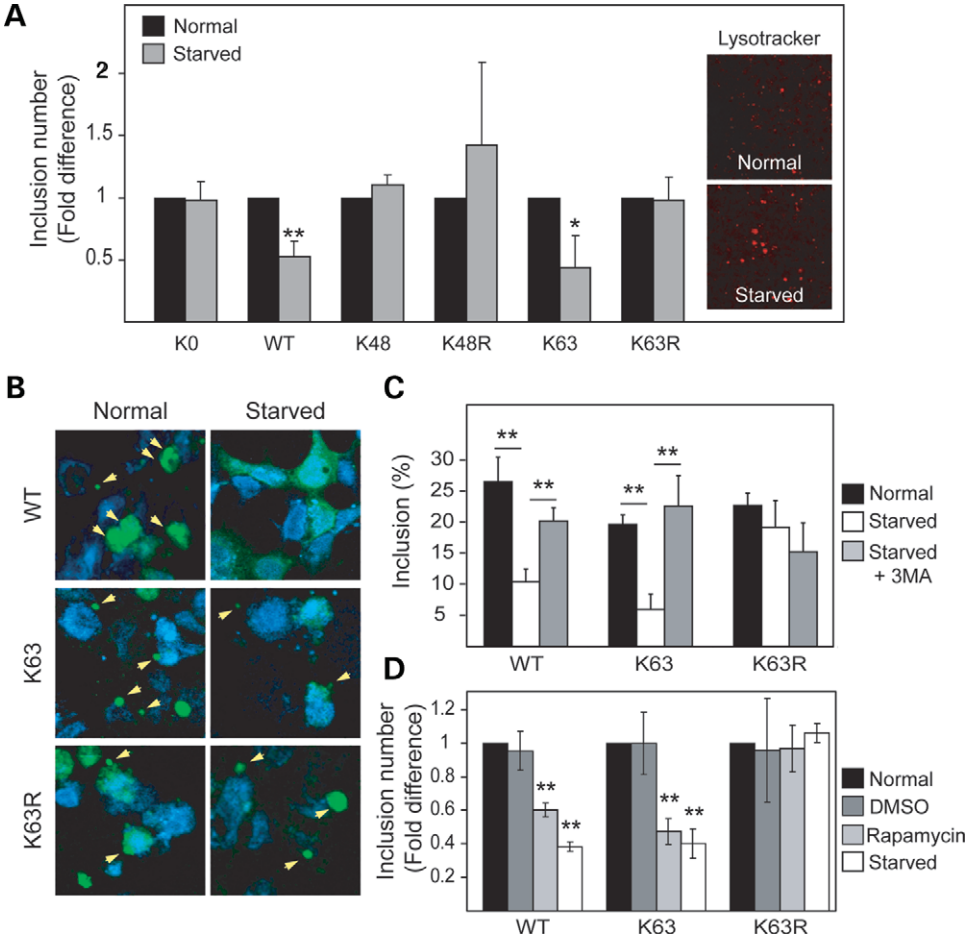

Figure 4. Inclusions enriched with K63 ubiquitin are preferentially degraded by the autophagic system. (**A**) Bar graph showing the relative fold difference in the number of inclusions observed in SH-SY5Y cells transfected with HA-tagged WT, K0, K63, K63R, K48 and K48R ubiquitin recovering in low (1%) or normal (10%) serum after treatment with lactacystin ($^{*}P < 0.05$, $^{**}P < 0.001$ versus control group). Note the increased number and size of lysotracker-stained lysosomes in starved cells (*inset*). (**B**) Representative confocal images of anti-FLAG (ubiquitin)-stained SH-SY5Y cells stably expressing WT, K63 or K63R ubiquitin that have undergone similar treatment. The relative difference in number of inclusions observed among these cells, as well as with those treated with 3-MA or rapamycin in the presence of low serum conditions, is depicted by the bar graph shown in (**C**) and (**D**), as indicated ($^{*}P < 0.05$, $^{**}P < 0.001$). Each of these experiments was repeated at least three times.

serum-containing media compared with those recovering in normal serum-containing media (Fig. 4A). In cells ectopically expressing either wild-type or K63 mutant ubiquitin, we observed a comparable reduction in the number of ubiquitin-positive inclusions when they were recovering in low serum conditions compared with those that recover in normal serum (Fig. 4A), suggesting the removal of inclusions via the autophagic pathway. However, this phenomenon is not observed in cells ectopically expressing all other forms of ubiquitin species examined, including K48 and K0 ubiquitin mutant (Fig. 4A). To extend these findings, experiments were conducted with SH-SY5Y cells stably expressing wild-type, K63 or K63R ubiquitin. Similarly, there is a reduction in the size as well as the number of ubiquitin-positive inclusions in cells stably expressing either wild-type or K63 mutant ubiquitin when these cultures recover in low serum conditions compared with cultures that recover in normal serum (Fig. 4B and C). Again, the average reduction in the number of wild-type ubiquitin- and K63-positive inclusions associated with serum-starved induced autophagy, i.e. 60 and 70%, respectively, are comparable (Fig. 4B and C). In contrast, the number of inclusions in cells recovering in low or normal serum remains essentially unchanged in the presence of K63R mutant ubiquitin overexpression (Fig. 4B and C). To exclude the possibility that the observed reduction in wild-type or K63 ubiquitin-positive inclusions in cells undergoing stimulated autophagy is because of a decrease in their formation rather than an increase in their clearance, we treated cells recovering in low serum with a widely used autophagy inhibitor, 3-methyladenine (3-MA) to block lysosomal degradation. No reduction in the number of ubiquitin-positive inclusions is observed in either wild-type or K63 ubiquitin-expressing cells recovering in low serum condition in the presence of 3-MA, suggesting that inhibition of autophagy prevents the removal of inclusions in these cell types (Fig. 4C). On the other hand, 3-MA treatment does not appear to have a significant effect on the population of inclusions generated in cells expressing K63R ubiquitin (Fig. 4C). This is consistent with an impaired clearance of inclusions associated with non-K63-linked ubiquitination

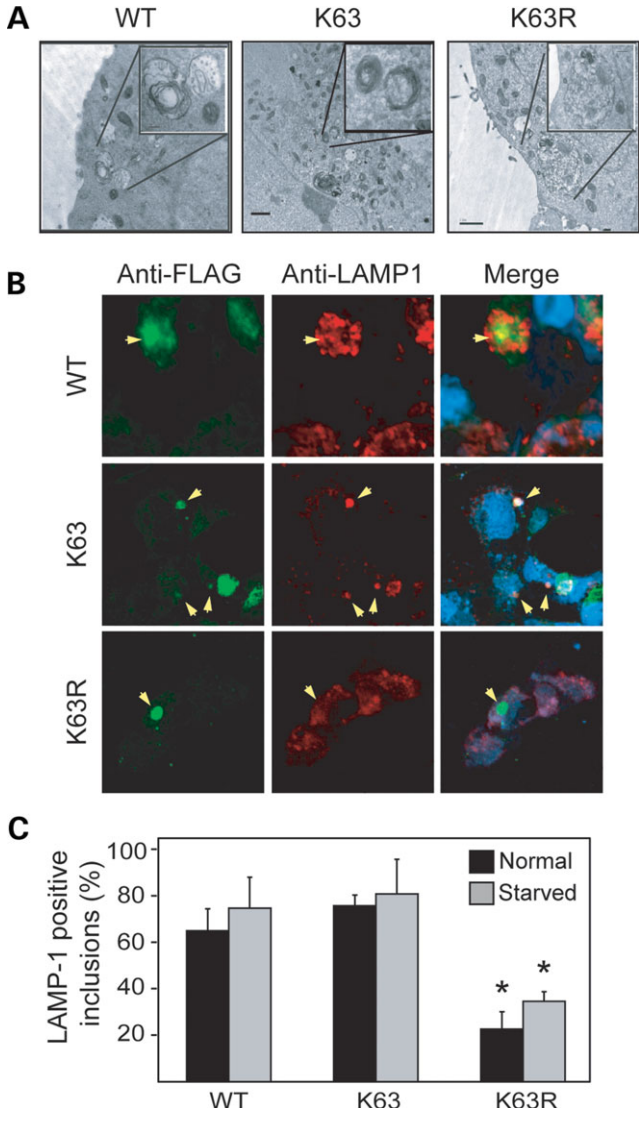

Figure 5. K63 ubiquitin-positive inclusions are enriched with LAMP1. (**A**) Representative electron micrographs showing multi-lamellar structures resembling autophagolysosome in SH-SY5Y cells stably expressing WT and K63 ubiquitin (but not K63R) that have undergone a similar treatment regime as in described in Fig. 4A. (**B**) Representative confocal images of SH-SY5Y cells stably expressing WT, K63 or K63R ubiquitin that have undergone the above treatment regime immunostained with anti-FLAG (ubiquitin) (green) or anti-LAMP1 (red). Arrows in merge pictures show the co-localization of LAMP1 with WT and K63 (but not K63R) ubiquitin-positive inclusions (yellow). (**C**) Bar graph showing the percentage of LAMP-1/ubiquitin-positive inclusions in these cells (*$P < 0.05$, K63 or K63R versus WT). Each of these experiments was repeated at least three times.

(Fig. 4C). Conversely, direct stimulation of autophagy in these cell lines via pharmacological activation of mTOR by rapamycin essentially reproduces the phenomenon brought about by serum starvation, thus supporting the role of macroautophagy in the clearance of K63 ubiquitin-enriched inclusions (Fig. 4D). Similar observations were made when we repeated our above experiments in the presence of tau P301L mutant (Supplementary Material, Fig. S2). We found that tau-positive inclusions formed in cells co-expressing mutant tau and wild-type or K63 ubiquitin are preferentially cleared under conditions of stimulated autophagy (Supplementary Material, Fig. S2). On the other hand, co-expression of mutant tau with K63R ubiquitin leads to impaired autophagic clearance of tau-positive inclusions (Supplementary Material, Fig. S2). Collectively, our results suggest that K63-linked ubiquitination facilitates the autophagic clearance of inclusions.

Lending support to the above suggestion, the analysis of the different exogenous ubiquitin-expressing cell lines under electron microscopy reveals the frequent presence of multilamellar structures resembling autophagolysosomes in cells stably expressing wild-type and K63 ubiquitin that are recovering in low serum after lactacystin treatment. Conversely, such structures are absent in cells stably expressing K63R that have undergone the same treatment paradigm (Fig. 5A). To further ascertain that inclusions ubiquitinated via wild-type or K63 ubiquitin under conditions of proteasomal impairment represent staging grounds for lysosomal degradation, we examined the association of FLAG-ubiquitin-positive inclusions with a lysosomal-specific marker, LAMP-1 (Fig. 5B). Ubiquitin-positive inclusions formed in cells stably expressing wild-type or K63 ubiquitin exhibit a predominant tendency (between 60 and 80%) to co-localize with LAMP-1, whereas only a fraction (~30%) of K63R-ubiquitin-positive inclusions stains positively with LAMP-1 (Fig. 5B and C). Although there is a reduced number of inclusions under serum-starved conditions (Fig. 5B and C), we did not observe a significant difference in the number of ubiquitin/LAMP-1-positive inclusions between the respective sets of cells recovering in normal and low serum conditions (Fig. 5B and C). Nonetheless, the association of a majority of wild-type or K63 ubiquitin-positive inclusions with LAMP1 suggests an efficient recruitment of lysosomal structures to K63 polyubiquitinated proteins, which concomitantly primes the inclusions for autophagic clearance. Similarly, mutant tau-mediated inclusions positive for wild-type or K63 ubiquitin also recruit LAMP1 efficiently, suggesting that K63 polyubiquitinated tau are intimately associated with autophagolysosomes (data not shown). Taken together, our results suggest that K63-linked ubiquitination may participate in the formation of inclusions associated with various neurodegenerative diseases, and that it acts as a signal for autophagic clearance of inclusions.

DISCUSSION

The major finding of this study is that non-classic, proteasomal-independent, K63 ubiquitin chain assembly, through its influence on the dynamics of protein inclusions, represents a novel strategy utilized by the cell to regulate protein homeostasis. By promoting the formation of protein inclusions as well as tagging them for subsequent clearance via autophagy, K63-linked polyubiquitination appears to control the life cycle of an inclusion body.

As protein inclusions are a consistent hallmark of neurodegenerative diseases, much attention is focused on their roles in disease pathogenesis and the mechanisms underlying their biogenesis. Interestingly, several reports (15–18), including an elegant live-imaging study conducted by Arrasate *et al*. (19),

suggest a neuroprotective function for inclusion formation. This is in agreement with the emerging evidence implicating a pathogenic role of soluble disease-associated protein intermediates (1,20–23). It is thus tempting to think that the cell has evolved a defence mechanism against a build up of a soluble toxic load that it cannot otherwise degrade through normal means by channelling this load to an inert location for subsequent handling by autophagy. In this way, a susceptible neuron may prolong its survival and limit neurodegeneration. It is conceivable, as we and others have recently speculated (8,24), that non-proteolytic ubiquitination of proteins could participate in this process, potentially by helping to divert proteasomal load away from an otherwise overloaded machinery. Supporting this, we have recently demonstrated that proteasome-independent K63-linked ubiquitination facilitates the formation of LB-like inclusions in cultured cells (9). Furthermore, a very recent mass spectrometry-based study demonstrated the accumulation of K63-linked polyubiquitin in a mouse model of HD (25). In this study, we observed that ectopic expression of K0, K48 or K63 ubiquitin promotes the formation of tau-positive inclusions mediated by a co-expressing pathogenic tau mutant. Our results suggest that multiple ubiquitin topologies may be involved in inclusions formation. However, these different types of ubiquitin modification appear to have varying effects on the formation of intracellular inclusions under different conditions. For example, when mutant tau is replaced by an SOD mutant, K0 ubiquitin co-expression no longer has an effect on the propensity of the SOD mutant to generate inclusions. On the other hand, when pathogenic proteins are absent altogether, only K63 ubiquitin, following its overexpression, seems to be the only one capable of significantly enhancing the formation of inclusions in cultured cells. These repeated observations, together with our previous study (9), suggest that K63 ubiquitination may be a common denominator underlying protein inclusion formation. The failure of K48R ubiquitin overexpression in mimicking faithfully the effects brought about by K63 overexpression may be because of other ubiquitin linkages promoted by the K48R mutant, such as the degradation-associated K29-linked polyubiquitination (7). Likewise, we observe dissimilar effects between K63R and K48 overexpression on inclusions formation. Notwithstanding this, the precise role of each of the above-discussed ubiquitin species in the biogenesis of disease-related protein inclusions remains to be further clarified through techniques capable of examining the relative contribution of endogenous ubiquitin species to inclusion formation.

An interesting recent development pertaining to inclusion dynamics is the suggestion that inclusion bodies act as staging areas for the disposal of protein aggregates resistant to proteasomal degradation via the autophagic route (13,26). Morphological evidence of autophagy is present in several neurodegenerative disorders, including AD, PD and HD (12,27,28). Moreover, the two recent independent reports clearly indicates the importance of autophagy in neurodegeneration and inclusion formation (29,30). However, it remains controversial whether autophagy is clearing monomeric and oligomeric precursors of aggregates, or inclusions themselves. Unlike the UPS, where the mechanism of cargo selection is well defined, an important mystery that remains to be solved is how cargo is selected for autophagic degradation. At least with inclusion bodies, we have provided a mechanism that underlies cargo recognition by the autophagic system. Given the similar positive effects elicited by both wild-type and K63, but not K63R or other forms of ubiquitin examined on the autophagic clearance of inclusions formed in the presence of proteasomal inhibition, our results suggest that K63 ubiquitin chain assembly is potentially an important form of ubiquitin modification of intracellular inclusions that is responsible for subsequently targeting them for autophagy.

In conclusion, we have provided a mechanism that underlies cargo recognition by the autophagic system as our results suggest that K63 ubiquitin chain assembly is potentially an important form of ubiquitin modification of intracellular inclusions that is responsible for subsequently targeting them for autophagy. Harnessing this pathway may offer innovative approaches in the treatment of neurodegenerative disorders.

MATERIALS AND METHODS

Plasmids, antibodies and reagents

Plasmids expressing HA-tagged ubiquitin and various ubiquitin mutants have been previously described (9). pCDNA3-FLAG-tagged wild-type and mutant ubiquitin constructs were derived from their corresponding HA-tagged counterparts by means of PCR-mediated sub-cloning methodologies. The myc-tagged tau P301L and FLAG-tagged SOD A4V plasmids were kindly provided by Dr. L. Petrucelli (Mayo Clinic, USA) and Dr. R. Takahashi (Kyoto University, Japan), respectively. The following antibodies were used: monoclonal anti-[c-myc]-peroxidase, anti-HA-peroxidase, rabbit anti-HA (all from Roche Diagnostics), monoclonal anti-FLAG-peroxidase, anti-β actin, rabbit anti-γ-tubulin (all from Sigma), rabbit anti-ubiquitin (DAKO), monoclonal anti-LAMP1 (H5G11) (Santa Cruz Biotechnology Inc.), rhodamine-conjugated anti-mouse IgG (Molecular Probes), FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.), FITC-conjugated anti-rabbit IgG (BD Pharmingen), Rhodamine-conjugated anti-rabbit IgG (Molecular Probes), TritC-conjugated anti-goat (Santa Cruz) and FITC-conjugated anti-guinea pig IgG (Sigma). Stock solutions of the following chemicals were prepared and stored at -20°C: MG-132 (1 M in DMSO), 3-methyladenine (10 mM in DMEM), rapamycin (0.2 mg/ml in DMSO) and nocodozole (2 mg/ml in DMSO) (all from Sigma), clasto-lactacystin-β-lactone (Affiniti Research) (5 mM in dH_2O) and LysoTracker® Red DND-99 (Molecular Probes) (1 mM in DMSO).

Cell culture and transfection

SH-SY5Y cells were grown in DMEM containing 10% FBS in 5% CO_2 atmosphere. Cells were transiently transfected with various expression vectors using the LipofectAMINE 2000 reagent (Invitrogen) according to manufacturer's instructions. 2 days later, sequential fractionation of transfected cell lysates into Triton-X-soluble (S) and SDS-soluble (P) fractions was performed and analyzed as described previously (31). To facilitate our study on inclusions clearance, we generated

SH-SY5Y cells stably expressing WT, K63 and K63R ubiquitin using previously described methods (31). All positive stable cell lines used for the experiments described here were maintained in serum-containing DMEM supplemented with 200 μg/ml Geneticin (Invitrogen) to prevent extrusion of the integrated constructs.

Immunocytochemistry, proteasome assay and lysotracking

Cellular localization and confocal microscopy was carried out as described previously (9,31). A minimum of 50 cells were counted from each of the three independent wells per experiment. Cells were counted in a blinded manner and quantitative results reported are an average of at least three experiments. Transfected cells are scored as positive when they contain ubiquitin-positive spherical bodies that are visible at ×40 magnification. More often than not, a single perinuclear inclusion body per cell is observed. Proteasome activities were determined by incubating lysates (10 μg of protein) with substrates Suc-LLVY-AMC for 1.5 h at 37°C. The relative amount of AMC released was measured using a fluorometer equipped with a 380/460 nm filter set (TECAN). Lysosome tracking was carried by incubating cells with 50 nM LysoTracker for 30 min at 37 °C followed by three washes in PBS before fixing in 4% paraformaldehyde and mounting with FluorSave reagent (Calbiochem) before viewing.

Inclusion formation and autophagic removal

The autophagic clearance of inclusions formed under conditions of proteasomal impairment was investigated using a method recently described by Fortun *et al*. (13). Cells were first treated with 5 μM lactacystin to facilitate inclusion formation. After 16 h incubation, the treated cells were allowed to recover in normal media for 24 h. Concurrently, a parallel set of treated cells were incubated with starvation media (1% serum) to stimulate autophagy either in the presence or in the absence of 10 mM 3-methyladenine, an autophagy inhibitor. Thereafter, cells were processed for immunocytochemical staining for blinded evaluation of inclusions.

Ultrastructural analysis

Cells were washed with PBS prior to fixing in 2.5% glutaldehyde at 4°C for 4 h. After which, cells were washed twice with PBS before treatment with 2% osmium tetroxide for 2 h at room temperature. Cells were then packed in 5% w/v gelatin and fixed with 2.5% glutaldehyde for 10 min before sequential dehydration and subsequent embedding in aradite resin. Semithin and ultrathin sections were cut in an ultrotome (Leica UCT) and post-stained with lead citrate prior to viewing Images were acquired with Ultrathin sections (<100 nm) viewed with Phillips EM208 100 kV transmission electron microscope.

Statistical analysis

Statistical significance for all the quantitative data obtained was analyzed using Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.001$) unless otherwise stated.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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