

ANALYSIS OF P62-UBA INTERACTING PROTEINS

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THESIS ABSTRACT

ANALYSIS OF P62-UBA INTERACTING PROTEINS

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Sequestosome 1/p62 interacts with and traffics K63-polyubiquitinated proteins through its ubiquitin associating domain (UBA). Herein we demonstrate that the hyperaccumulation of K63-polyubiquitinated proteins occurs in the absence of p62 making knock-out mice a rich source of K63-polyubiquitinated proteins for proteomic analysis. Formic acid fraction of wild-type and knock-out mice brain were subjected to p62 GST-UBA pull down, then shotgun LC-MS/MS was employed to identify those p62 UBA-interacting proteins. Using this approach, we identified 30 proteins consisting of nine classes: cytoskeleton / structural protein, energy / metabolism, membrane transport / ion channel, signaling, chaperon, intracellular trafficking, nuclear, neurogenesis, and unknown / unassigned proteins. The results of Western-blotting and immunoprecipitation of a subset reveal that those p62-interacting proteins are accumulated in p62 knock-out

mice brains and are K63-polyubiquitinated. Our results support a model whereby p62 shuttles K63-polyubiquitinated proteins for proteasomal degradation.

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TABLE OF CONTENTS

LIST OF FIGURES.....	xi
LIST OF TABLES.....	xiii
LIST OF ABBREVIATIONS.....	xiv
I. LITERATURE REVIEW.....	1
1.1. The fate of misfolded proteins.....	1
1.2. Ubiquitin proteasome pathway.....	3
1.2.1. Ubiquitination.....	3
1.2.2. 26S proteasome.....	7
1.2.3. Shuttling proteins and the UBA domains.....	10
1.3. Protein aggregation, aggresome and neurodegeneration.....	13
1.4. Sequestosome 1/ p62 and aggregation.....	18
1.5. Chaperones and protein aggregation	21
II. ANALYSIS OF P62-UBA INTERACTING PROTEINS.....	30
2.1. Introduction.....	30
2.2. Materials and Methods.....	33
2.3. Results.....	41
2.4. Discussion.....	61
2.5. Conclusions.....	68

2.6. References.....69

LIST OF FIGURES

Fig.1	The pathway of ubiquitin-linked degradation of proteins and the 26S proteasome	2
Fig.2	The ubiquitylation pathway.....	4
Fig.3	Monoubiquitination, polyubiquitination and seven lysine residues	5
Fig.4	Structure of the 26S proteasome complex.....	8
Fig.5	Structure of the 20S core and 19S regulatory particle.....	8
Fig.6	Presentation of ubiquitinated substrates to the 26S proteasome.....	11
Fig.7	The proteins containing UBA domains have diverse structure and function.....	12
Fig.8	The UPS and pathogenesis of neurodegeneration.....	16
Fig.9	A schematic diagram showing the domain organization of the p62 protein.....	20
Fig.10	Domain structure of CHIP and schematic presentation of the CHIP multi-subunit ubiquitin ligase.....	22
Fig.11	K63 polyubiquitinated proteins accumulate in formic acid fraction of p62 knock-out mice brain	43
Fig.12	Polyubiquitin chain competition in GST-UBA pull down assay	46
Fig.13	Analysis of accumulated proteins by LC-MS/MS	47

Fig.14	Accumulation of proteins in formic acid fraction of p62 knock-out mice brain and proteasome activity in KO mice	54
Fig.15	Analysis of Hsp70 protein	59
Fig.16	Model of p62 proteasomal shuttling.....	65

LIST OF TABLES

Table I. Protein aggregates and inclusion bodies are hallmarks of most neurodegenerative diseases.....	17
Table II. Proteins identified employing p62 GST-UBA pull-down and LC-MS/MS.....	49
Table III. Subset of proteins selected for further analysis.....	63

LIST OF ABBREVIATIONS

Acidic interaction domain	AID
Alzheimer's disease	AD
Amyloid β peptide	A β
Amyloid precursor peptide	APP
Carboxy terminus of Hsp70-interacting protein	CHIP
Coomassie Brilliant Blue	CBB
Enhanced chemiluminescence	ECL
Familial amyotrophic lateral sclerosis	fALS
Formic acid	FA
Homologous to E6AP carboxy terminus	HECT
Human embryonic kidney cells	HEK
Huntington's disease	HD
Knock-out	KO
Liquid-chromatography and tandem mass spectrometer	LC-MS/MS
Low molecular weight protein 7	LMP7
Mass spectrometry	MS
Microtubule organizing center	MTOC
Nuclear factor - kappa B	NF-kB

Nuclear magnetic resonance	NMR
Parkinson's disease	PD
Phox and Bem1p	PB1
Proteasome maturation protein	POMP
Really Interesting New Gene	RING
Relative standard deviation	RSD
Src homology 2	SH2
Superoxide dismutase	SOD
Transmission electron microscope	TEM
Tetratricopeptide repeat domain	TPR
Ring-finger protein tumor necrosis factor receptor-associated factor 6	TRAF6
Ubiquitin	Ub
Ubiquitin associate domain	UBA
Ubiquitin interacting motif	UIM
Ubiquitin-like domain	UbL
Ubiquitin proteasome pathway	UPS
Wild-type	WT

LITERATURE REVIEW

The fate of misfolded proteins

Mother nature makes mistakes. In normal cells, large amounts of newly synthesized proteins are defective “off-pathway” products. Even with abundant molecular chaperones, nearly 30% of nascent proteins are misfolded due to mutations or inefficient assembly (1). The cell must cope with these mistakes. Misfolded proteins can either be degraded via ubiquitin proteasome pathway (UPS) shortly after their synthesis or aggregate into high molecular weight oligomers (2). The ultimate fate of misfolded proteins depends on kinetic partitioning between these two competitive pathways (3). Because the aggregates are more stable than the intermediate conformers, to degrade misfolded substrates effectively the proteasome must win the competition for substrates before they aggregate.

Under normal condition, defective proteins are removed promptly before any damage can be caused to the cells. However under certain situations in nerve cells, proteins accumulate to form the pathological inclusion bodies which eventually contribute to neurodegenerative diseases and other protein-aggregate diseases (4, 5).

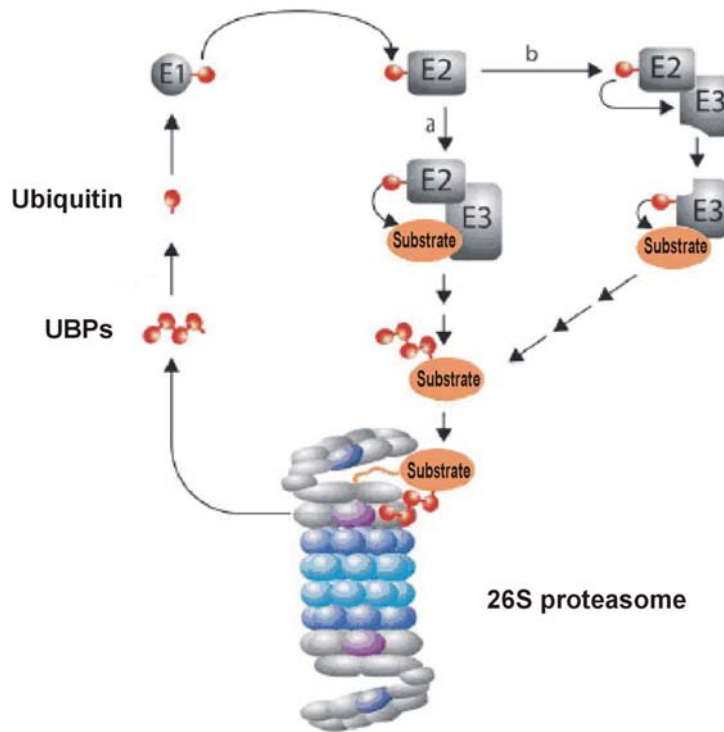


FIG. 1. The pathway of ubiquitin-linked degradation of proteins and the 26S proteasome (Wolf, 2004 *Cell. Mol. Life Sci.* 61: 1601-1614).

Ubiquitin proteasome pathway

The UPS pathway is a cellular quality control system that tags misfolded proteins with ubiquitin for degradation by the 26S proteasome. Protein degradation via UPS involves two steps: 1) covalent attachment of polyubiquitin chains to target proteins, and 2) degradation of the tagged proteins by 26S proteasome complex with release of free and reusable ubiquitin (Fig.1).

I. Ubiquitination

Ubiquitin is a family of proteins which may have variable sequences but display structural similarity (6). Mature ubiquitins contain a signature diglycine sequence exposed only after proteolytic processing (7). Ubiquitin acts as a signal through covalent attachment to other proteins, which enables those proteins to be recognized and processed by specific cellular machineries.

Three enzymes are involved in ubiquitination of substrates, eventually resulting in formation of a bond between the C-terminus of ubiquitin (Gly76) and the ϵ -amino group of a substrate lysine residue. Ubiquitin-activating enzyme (E1) forms a thiol ester with the carboxyl group of Gly76, activating C-terminus of ubiquitin. The activated ubiquitin molecule thus is carried by ubiquitin-conjugating enzyme (E2) and transferred to the substrate lysine residue by ubiquitin-ligases (E3) (Fig.2) (8). More ubiquitins can be added to form polyubiquitin chains. The terminal carboxyl of each ubiquitin is linked to the ϵ -amino group of a lysine residue of the adjacent ubiquitin in the chain. Ubiquitin

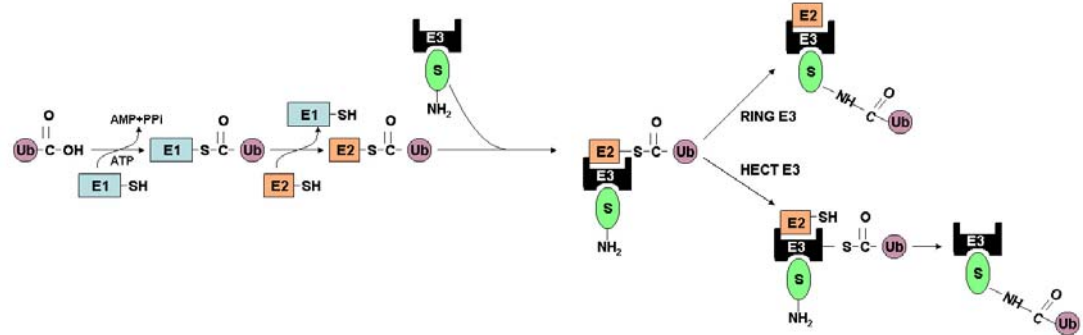


FIG. 2. A schematic representation of substrate ubiquitination (Weissman, 2001 *Nat. Rev. Mol. Cell Biol.* 2: 169-178).

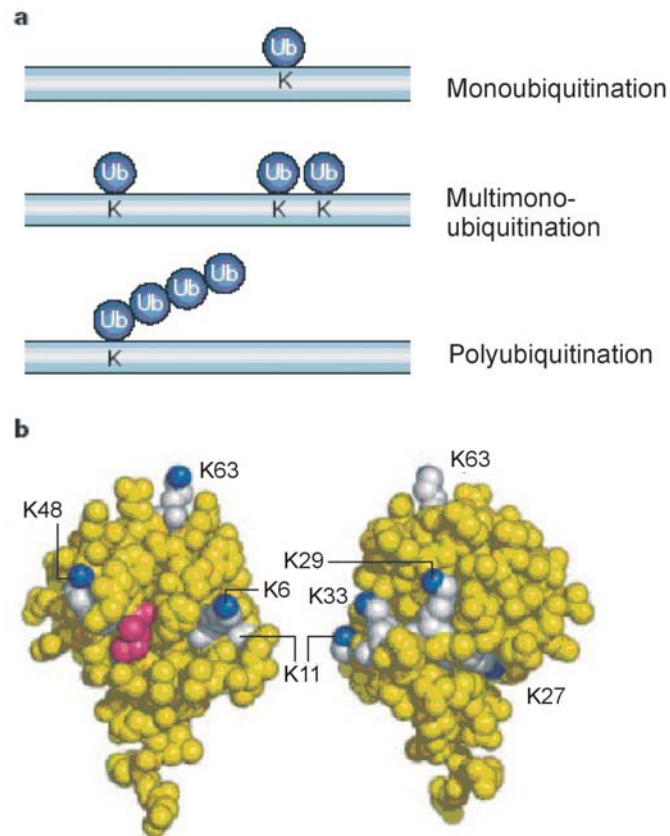


FIG. 3. a) Ubiquitin can be added as a single molecule at one or more sites. Alternatively, branched chains of polyubiquitin may be formed. b) Space filled model of ubiquitin indicating the seven lysine residues (Hicke et al, 2005 *Mol. Cell Biol.* 6: 610-621).

itself can be modified at all seven lysine residues (K6, K11, K27, K33, K29, K48, K63) *in vivo* (Fig.3) (9). Polyubiquitin chains linked through K48 are the primary signal for protein degradation. K63-linked chains have been shown to be an important signal in DNA repair, ribosome function, mitochondrial DNA inheritance, stress response and targeting proteins for endocytosis (8). It is also reported that a model substrate tagged with K63-linked tetra-ubiquitin effectively signal substrate degradation (10). At least four ubiquitin moieties are required for substrate recognition by the 26S proteasome (11).

The ubiquitin conjugation cascade contains a large family of E2s and an even larger set of E3s. For example, in budding yeast there is one E1, eleven E2s and more than twenty E3s (7). The large number of E3 enzymes may reflect the extraordinary diversity of the ubiquitinated substrates in eukaryotes. All E3 enzymes belong to three protein families, Homologous to E6AP Carboxy Terminus (HECT), Really Interesting New Gene (RING), and UFD2 homology (U-box) proteins. Those E3s share a common E2-binding domain and a substrate-interacting domain. One remarkable feature of the ubiquitin conjugation pathway is the modulation of target protein selection. The substrate specificity depends mainly on the identity of E3 (7). On the other hand, biochemical studies have shown that the identity of E2 can influence the recognition of specific structures of a polymeric ubiquitin modification (12), indicating that the specificity of the E2/E3 interaction may determine the final destination of ubiquitins.

II. 26S proteasome

Proteasome is a multimeric protease complex which plays a central role in protein degradation through both ubiquitin-dependent and ubiquitin-independent mechanisms. The 26S proteasome complex consists of a 20S core particle which is proteolytically active, and 1 or 2 19S regulatory caps which are responsible for recognition, unfolding, de-ubiquitylation and translocation of substrate proteins into the lumen of the core particle (Fig. 4) (13).

The proteolytic core 20S proteasome consists of four stacked rings with two outer rings embracing two central head-to-head rings. The outer rings are each composed of seven different alpha subunits and the inner rings of seven different beta subunits (Fig. 5). The overall structure of the 20S core resembles a barrel with dimensions of 15nm in length and 11nm in diameter. A central proteolytic chamber is formed by two face-to-face β -rings and is separated by 3nm wide β -annuli. Access to the chamber requires reorganization of the N-terminal H0 helixes of the α -subunits which normally form a seal by the interaction of side chains. The N-terminal of α 3-subunit plays a critical role in the seal formation. Binding of 19S cap can induce the channel opening. The 19S ATPase Rpt2 is the key element in this process. Three subunits β 1, β 2 and β 5 form the catalytic site. β -subunits gain the proteolytic activity by autolytic processing of the N-terminal propeptides and exposure of the critical threonine residue.

The components of the 19S regulator are responsible for recognition, unfolding, de-ubiquitylation and translocation of substrate proteins into the lumen of the core particle,

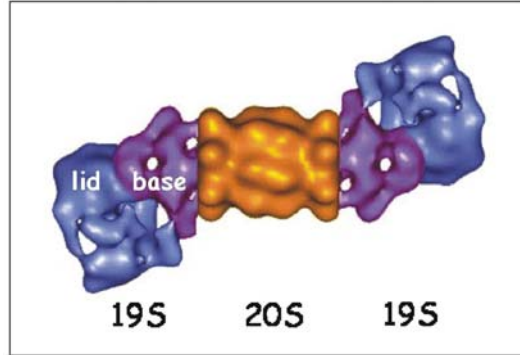


FIG. 4. Structure of the 26S proteasome complex. (<http://www.biw.kuleuven.be/dtp/cmpg/pgprb.htm>)

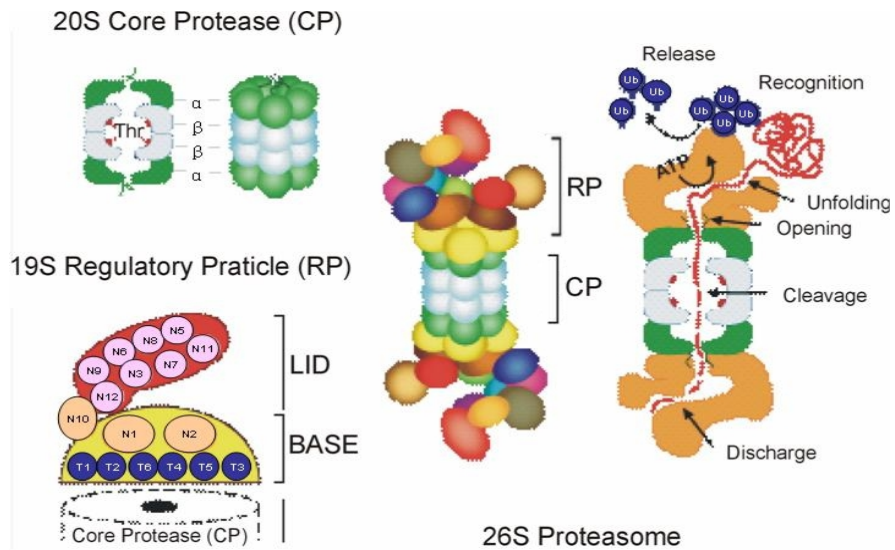


FIG. 5. Structure of the 20S core and 19S regulatory particle. (<http://plantsubq.genomics.purdue.edu/plantsubq/html/guide.html>)

where the substrate chains are degraded (13). The 19S regulator is composed of 17 or 18 subunits. In high salt concentrations, it breaks down into two sub-complexes, including the lid and the base. The base consists of 6 ATPases (Rpt1 to Rpt6) which show a high level of similarity to one another. These ATPases can form a six-membered ring and interact directly with the α -ring of 20S proteasome. Protein substrates need to pass through the center of this ring in order to enter the catalytic chamber of 20S proteasome. In addition, the ATPase ring is involved in the anti-chaperon activity required to unfold the protein substrates. The base also includes non-ATPase subunits. One of them is Rpn10 (S5a), which contains an ubiquitin interacting motif (UIM) domain. Other subunits include Rpn1 and Rpn2. The lid sub-complex consists of eight non-ATPase subunits, in which only the function of Rpn11 is known. It plays a key role in the recycling of ubiquitin by cleaving the ubiquitin chain from the protein substrate.

In addition to standard proteasomes, cells are able to produce immunoproteasomes as a transient response to cytokines IFN- γ or TNF- α (14). IFN- γ induces biosynthesis of proteasome maturation protein (POMP) and proteasomal β 5i subunit low molecular weight protein 7 (LMP7), accelerating the assembly of immunoproteasome in which three catalytic subunits are replaced by homologous subunits (β 1i, β 2i, β 5i) (15). Immunoproteasome may participate in generating antigenic peptides displayed on MHC-class I molecules (16) but are not limited to this function. Recent observations reveal that in familial amyotrophic lateral sclerosis (fALS) patients, impaired degradation of mutant SOD1 is associated with a decrease in constitutive proteasomes and an increase in the immunoproteasome level, resulting in selective motor neuron degeneration (17). In

Huntington's disease (HD), high level of immunoproteasome subunits (LMP2 and LMP7) was also observed, associated with the sign of neurodegeneration, indicating immunoproteasome plays a role in the pathogenesis of neurodegenerative diseases (18).

III. Shuttling proteins and UBA domains

Polyubiquitin chains signal target proteins for degradation by the proteasome complex. Recognition of poly-ubiquitinated proteins by 26S proteasome plays a critical role in protein degradation. Presentation of a ubiquitinated substrate to 26S proteasome needs an ubiquitin interacting protein, such as S5a, Dsk2, Rad23 and p62 (19,20) (Fig.6).

Ubiquitin-interacting proteins involved in ubiquitination / deubiquitination generally have ubiquitin-association (UBA) domains which can directly binds to ubiquitin (Fig.7). Previous studies have shown that most UBA domains prefer to bind polyubiquitin chains rather than monoubiquitin. Some UBA domains even prefer to bind K63-linked polyubiquitin chains than K48-linked chains (21). In addition, the interaction between ubiquitin and UBA domain is a low-affinity interaction. NMR chemical shift mapping shows that Ub specifically but weakly binds to a conserved hydrophobic epitope on UBA domains, while the UBA domains can bind to the hydrophobic patch on the surface of the five-stranded β -sheet of Ub with different orientations (22). The weak interaction may enable rapid assembly and disassembly between transient associated proteins. UBA-containing proteins might contribute to the regulated capture and transient stabilization of proteins that are otherwise constitutively degraded (23). A recent finding has shown that the functional Ub-binding motifs are required for the localization of Ub-binding proteins

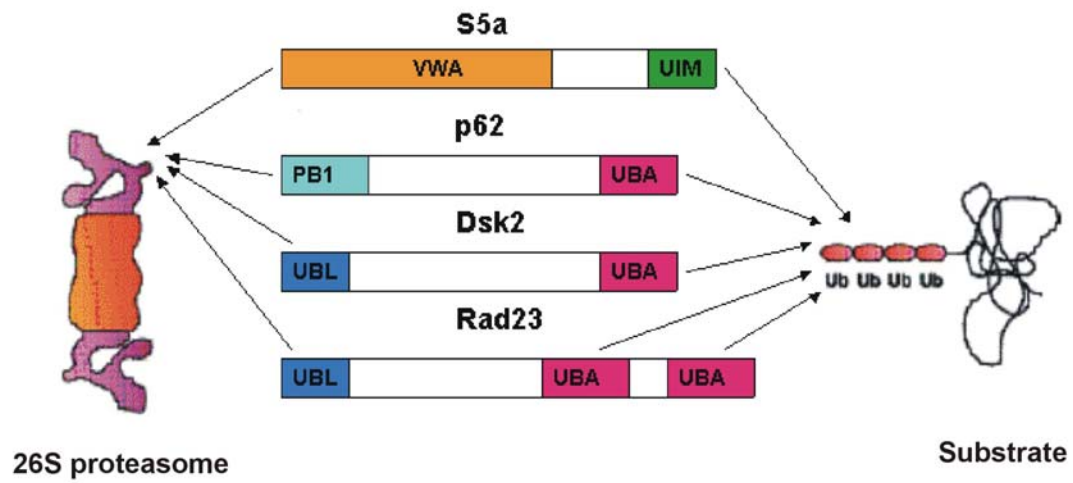


FIG. 6. Presentation of ubiquitinated substrates to the 26S proteasome (Hartmann-Petersen and Gordon, 2004 *Cell. Mol. Life Sci.* 61:1589–1595, modified).

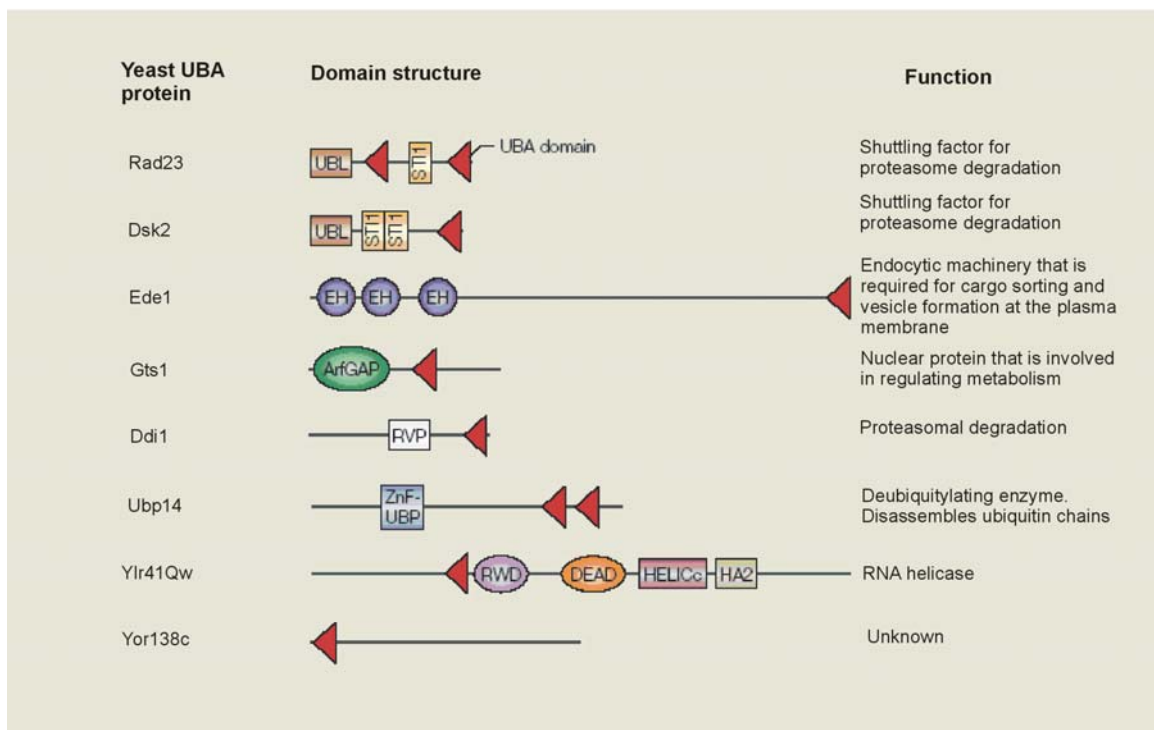


FIG. 7. Proteins containing UBA domains have diverse structure and function (Hicke et al, 2005 *Mol. Cell Biol.* 6: 610-621).

into protein aggregates (24), suggesting a common mechanism of Ub-mediated sequestration of essential Ub-binding proteins into aggregates.

Except UBA domains that bind polyUb chains, those shuttling proteins commonly contain a ubiquitin-like domain (UbL) that binds the proteasome (25,26,27). Thus these proteins are able to shuttle the poly-ubiquitinated substrates to the 26S proteasome for degradation (28). Recently, Madura et al. reported that ataxin-3, a proteasome-associated factor, associates with the shuttling protein Rad23 to mediate the degradation of ubiquitinated substrates, suggesting an important role for shuttling proteins in ubiquitin proteasome pathway (29). Since each type of polyUb chain forms a different conformation (30), it has been suggested that unique UBA domains may recognize specific types of polyUb chains. Therefore each shuttling protein may present chain-specific poly-ubiquitinated substrates to the proteasome for degradation. Rassi et. al. recently examined the polyubiquitin interaction properties of 30 UBA domains. Their results support the model that the UBA domains bind polyUb chains in a linkage-selective manner (31).

Protein aggregates, aggresome and neurpdegeneration

Protein turnover is related to functional ubiquitin proteasome system. Failure to remove the ubiquitinated proteins may lead to accumulation of those proteins and aggregate formation (32). The capacity of ubiquitin proteasome pathway can be exceeded either by overexpression of substrates or due to a decrease in proteasome activity. In

cultured cells, proteasomal inhibitors can cause the aggregation of an overexpressing disease-associated protein (33), indicating that dysfunction of proteasome might be an initiating factor to the formation of aggregates. Recent studies have shown that a wide-range of non-disease-associated proteins are found in aggregates when cells overexpressing mutant SOD-1 are treated with proteasomal inhibitor, including ubiquitinated or nitrated α -tubulin, SOD-1, α -synuclein and 68K neurofilaments (34). Previous research reveals that transient expression of two unrelated aggregation-prone proteins caused nearly complete inhibition of the ubiquitin proteasome system, indicating that protein aggregation can directly impair the UPS function (35). A positive-feedback mechanism is proposed to explain the turnover point of protein aggregation. Impaired proteasome function may result in increased protein aggregates, which lead to a further decline of proteasome activity. On the other hand, that UPS impairment is not the result of steady-state sequestration of UPS components, or simple substrate competition (36). Direct physical interaction between proteasome and aggregates is not required for UPS impairment, indicating the protein aggregates influence the activity of proteasome in a currently unknown manner.

Small protein aggregates can form large aggregates called aggresomes, in which molecular chaperones, proteasome subunits, ubiquitins and intermediate filament proteins are co-localized (3). The formation of aggresomes occurs at the microtubule organizing center (MTOC) and is considered to be a process distinct from protein aggregation (37,38). The formation of cytoplasmic inclusion bodies requires active transport of

misfolded proteins on microtubules, with redistribution of the intermediate filament proteins to form a cage surrounding the core of aggregated, ubiquitinated protein (32).

Recent evidence has shown that early protein aggregates may be toxic to neuronal cells. Soluble dimers and trimers, protofibrils or fibrils of A β peptide are cytotoxic (39). On the other hand, although the early pre-fibrillar disease-associated protein aggregates are harmful to cells, the mature fibrils are relatively harmless (40). In addition, previous studies reveal that UPS impairment is independent of inclusion body formation (36). Altogether those observations suggest that aggresome pathway could be cytoprotective by recruiting toxic proteins and forming small aggregates (41)

The accumulation of protein aggregates and formation of inclusion bodies associated with many age-related neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease etc., suggest that there is a direct link between protein aggregation and the resulting pathology (4) (Fig. 8). The accumulation of ubiquitin conjugates may reflect the failed attempt of UPS to remove the damaged protein (42).

An important player linking aberrations in the UPS to the pathogenesis of Parkinson's disease is Parkin, an ubiquitin ligase E3 (43). Previous report reveals that Parkin can associate with Rpn10 (S5a), a subunit of 26S proteasome, indicating that Parkin may be able to transfer ubiquitin conjugates for proteasomal degradation (44).

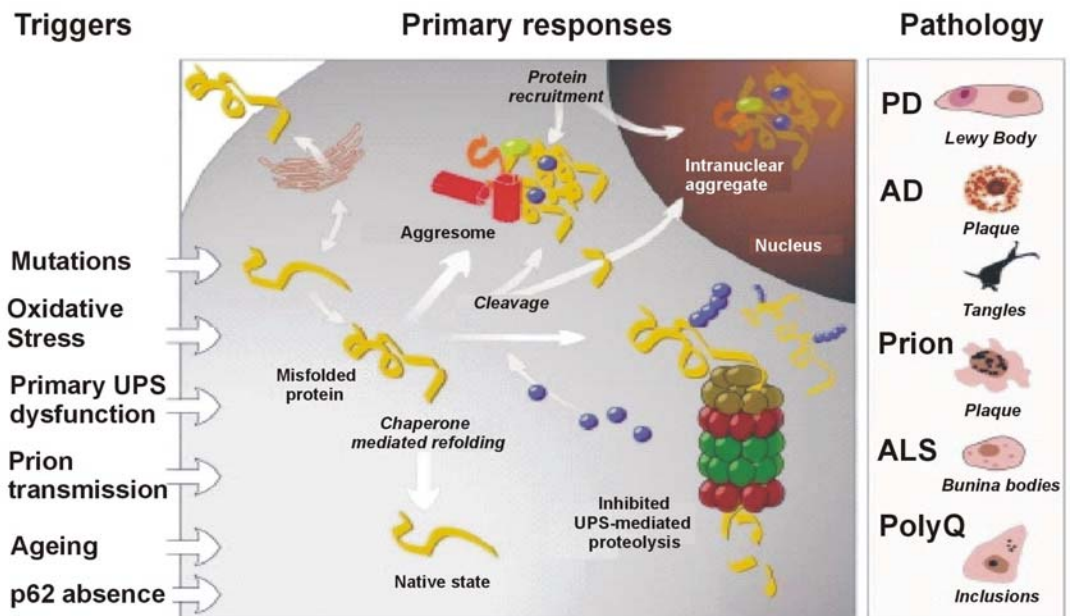


FIG. 8. The UPS and pathogenesis of neurodegeneration (Ciechanover and Brundin, 2003 *Neuron*, 40: 427–446, modified).

TABLE I

Protein aggregates and inclusion bodies are hallmarks of most neurodegenerative diseases (Sarah et al, 2003 *Curr. Opin. Genet. Dev.* 13: 253-261).

Disease	Aggregate type	UPP components	Location
Alzheimer disease	Plaques	Ubiquitin Proteasome	Extracellular
	Tangles	Ubiquitin Proteasome	Cytoplasmic
Parkinson disease	Lewy bodies	Proteasome Ubiquitin Hsps E3 ligase – Parkin DUB – UCH-L1	Cytoplasmic
Multiple system atrophy	Glial inclusions	Ubiquitin	Cytoplasmic
Polyglutamine disease	Inclusions	Proteasome Ubiquitin Hsps	Cytoplasmic / nuclear
Prion diseases	Aggresome - like	Ubiquitin Hsps	Cytoplasmic

Defect in Parkin may result in the accumulation of its substrates, such as α -synuclein, contributing to the pathogenesis of PD.

Some other aggregate-prone proteins participate in neurodegenerative diseases include Alzheimer's disease (Table I). There are two types of protein deposits in AD: extracellular amyloid plaques rich in amyloid β peptides ($A\beta$), and intracellular neurofibrillary tangles containing tau (45). Previous studies in our lab reveal that p62 can shuttle ubiquitinated tau for proteasomal degradation. Disturbing tau trafficking may result in the accumulation of insoluble / aggregated tau in the brain, contributing to AD (46). Amyloid β peptides are produced by proteolytic cleavage of the amyloid precursor peptide (APP). In solution, $A\beta$ peptides may undergo self-assembly leading to the transient appearance of soluble protofibrils and eventually to insoluble fibrils (47). Recent proteomic study of plaques in AD patients revealed that a total of 488 proteins co-isolated with plaques. Moreover 26 proteins are enriched in plaques by comparison with surrounding non-plaque tissues, including proteins involved in cell adhesion, cytoskeleton and membrane trafficking, chaperones, kinase/phosphatase and regulators (48).

Sequestosome 1/ p62 and aggregation

Sequestosome 1/p62 is a novel cellular protein that was initially identified as a phosphotyrosine independent ligand of the src homology 2 (SH2) domain of p56^{lck} (12). p62 contains an ubiquitin associated (UBA) domain at its C-terminus, which selectively

binds K63-linked polyubiquitin chains (19,25,49). The UBA domain of the human p62 protein forms a compact three-helix bundle. A Pro³⁹² → Leu substitution mutation can modify the UBA domain by extending the N terminus of helix 1. This modification does not affect interaction of the UBA domain with multitubiquitin chain binding, but somehow can cause Paget's disease of bone (50). Recent studies reveal that p62 protein lacking a UBA domain fails to form aggregates in HEK cells, indicating that the UBA domain is critical for sequestering ubiquitinated proteins (25).

p62 also contains an SH2 binding domain, an acidic interaction domain (AID) that binds aPKC, a ZZ domain, a binding site for the RING finger protein TRAF6 and two PEST sequences (Fig. 9) (49). By comparison, the N-terminus of p62 protein can directly interact with the proteasome subunit component (25), and the localization of protein substrates to the proteasome is sufficient for degradation (51). Thus p62 is viewed as a shuttling protein, playing an important role in sequestering polyubiquitinated substrates, interacting with ubiquitinated substrates through its UBA domain and the proteasome through its N-terminus. In support of this role for p62, two K63-polyubiquitinated substrates have been identified, tau and TrkA, that are shuttled by p62 for proteasomal degradation (46, 52).

The N-terminal PB1 domain of p62 is involved in p62 self-interaction, indicating p62 may also play a role in aggregate formation. p62 can form oligomers when the basic cluster domain in one molecule interacts with the OPCA loop in the other (53). Overexpression of p62 resulted in large aggregates, while depletion of p62 retarded

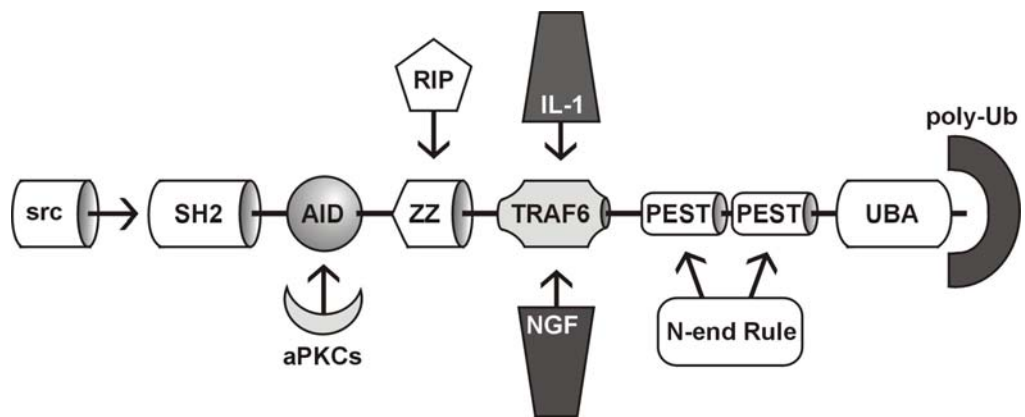


FIG. 9. A schematic diagram showing the domain organization of the p62 protein.

protein degradation and led to accumulation of proteins (25). Recent observations in our lab reveal that the p62 can spontaneously form fibril aggregates with significant β -sheet secondary structure in a time- and concentration- dependent manner *in vitro* (54). TEM analysis revealed that p62 aggregates share similar structures with amyloid fibrils and other AD-type aggregates. Moreover, p62 aggregates can form large insoluble inclusions at high concentrations, suggesting a role of p62 in neurodegenerative diseases (54). p62 may also act as a scaffold of TRAF6 through the TRAF6 binding site (49). TRAF6 is a K63-linked chain specific ubiquitin ligase whose recruitment is required for selective activation of transcription factor NF- κ B (55). TRAF6 co-localizes in protein aggregates. Recent study has shown that p62-UBA domain is required for TRAF6 polyubiquitination. The N-terminal dimerization domain or the TRAF6 binding site also affects both polyubiquitination and oligomerization of TRAF6 (56). When the interaction between p62 and TRAF6 is disrupted by competitive TRAF6 peptide, the formation of protein aggregates in cultured cells can be suppressed and cell's capability of survival enhanced (46). Therefore it is possible that p62 may regulate activation of NF- κ B through recognition of TRAF6-catalyzed polyUb chains and/or recruitment of TRAF6 to a microenvironment to enhance the protein ubiquitination.

Chaperons and protein aggregation

Hsp70 proteins are central components of the chaperone system which assists non-native intermediates to fold to the native state (57). Under normal conditions, the potentially toxic early aggregates might be unfolded or restructured by molecular

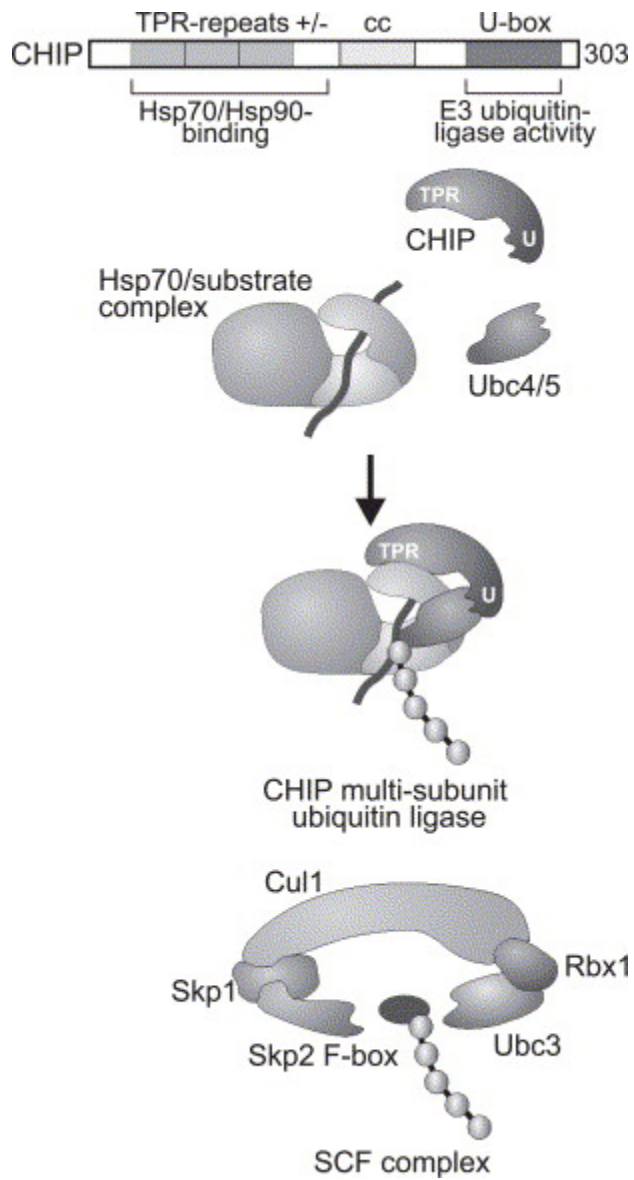


FIG.10. Domain structure of CHIP and schematic presentation of the CHIP multi-subunit ubiquitin ligase (Esser et al, 2004 *Biochim. Biophys. Acta* 1695: 171-188).

chaperons (58). It is reported that HSP70 can interact with the polyglutamine aggregates through rapid association and disassociation (59), capable of suppressing polyQ-induced cytotoxicity along with HSP40 (60). Other researchers show that molecular chaperones might be able to modulate the aggregation process, suppressing the formation of larger, SDS-insoluble fibrillar aggregates (61, 62). The induction or overexpressing HSP70 can significantly reduce detergent insoluble tau levels *in vitro* and *in vivo* (63). Altogether, molecular chaperons can be viewed as regulators of protein aggregation and a possible therapy to neurodegenerative diseases.

In addition, molecular chaperones and energy-dependent proteases may cooperate closely in protein quality control. Hsp70 proteins are involved in ubiquitin proteasome system by activating CHIP, carboxy terminus of Hsp70-interacting protein (Fig. 10). CHIP is a chaperone-dependent E3 ligase which interacts with Hsp70 through N-terminal tetratricopeptide repeat (TPR) domain, whereas its C-terminal U-box domain contains E3 ubiquitin ligase activity (64). Numerous non-native proteins are bound by Hsp70 before being presented to CHIP for poly-ubiquitination and further proteasomal degradation (65). Interestingly, AD-related protein tau is one of the substrates of the CHIP E3 ligase (46). Petrucelli et al. reported that CHIP ubiquitinates tau and promote tau aggregation (63). Shin et al. observed that CHIP mediates degradation of α -synuclein, a major component of Lewy bodies in Parkinson's disease (66). Given the observations that Hsp70 and CHIP are present in aggresome/ inclusion bodies with p62, there are some possible questions. Does p62 sequester ubiquitinated Hsp70 into the aggresome? Is Hsp70 deubiquitinated afterward and function as chaperones or contribute to proteasomal degradation together

with CHIP? Is that the reason that aggresomes play a protective role in the cell? Further research is needed to explore these questions.

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INTRODUCTION

Proteins destined to be degraded by the ubiquitin proteasome system are marked by covalently attached poly-ubiquitin chains (1). Ubiquitin (Ub) is a small peptide of 76 amino acids where the C terminus can be linked to the α amino group of a lysine residue in the substrate. Three enzymes are involved in substrate ubiquitination: ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin-ligase E3. Following the linkage of the first ubiquitin, more ubiquitin moieties can be attached to the previous Ub via different lysine residue to form homogenous polyUb chains. There are seven lysine residues in Ub: K6, K11, K27, K29, K33, K48 and K63. All seven lysines can be used for polyUb chain assembly *in vivo* (2). K48-linked polyUb chains are the principle signal for proteolysis by 26S proteasome (3), while K63-linked polyUb chains can also be degraded by proteasome (4). The substrates tagged by K63-chains have yet to be identified and the specific role in proteasome degradation remains controversial.

Recognition of poly-ubiquitinated proteins by 26S proteasome plays a critical role in protein degradation. Proteins that mediate this process commonly contain a ubiquitin-like domain (UBL) that binds the proteasome and ubiquitin associate domains (UBA) that

bind polyUb chains (5,6,7). Thus these proteins are able to shuttle the poly-ubiquitinated substrates to the 26S proteasome for degradation (8). Recently, Madura et. al. reported that ataxin-3, a proteasome-associated factor, associates with the shuttling protein Rad23 to mediate the degradation of ubiquitinated substrates, suggesting an important role for shuttling proteins in ubiquitin proteasome pathway (9). Since each type of polyUb chain forms a different conformation (10), it has been suggested that unique UBA domains may recognize specific types of polyUb chains. Therefore each shuttling protein may present chain-specific poly-ubiquitinated substrates to the proteasome for degradation. Rassi et. al. recently examined the polyubiquitin interaction properties of 30 UBA domains. Their results support the model that the UBA domains bind polyUb chains in a linkage-selective manner (11).

Sequestosome 1/p62 is a novel cellular protein that was initially identified as a phosphotyrosine independent ligand of the src homology 2 (SH2) domain of p56^{lck} (12). p62 contains a binding site for the RING finger E3, TRAF6, and a UBA domain that specifically associates with K63-polyubiquitinated proteins (5). In addition, the N-terminal PB1 motif of p62 enables its interaction with the proteasome, suggesting p62 is a novel shuttling protein. Recently, p62 was shown to shuttle tau for proteasomal degradation (13). In the absence of p62, the trafficking of K63-polyubiquitinated tau to the 26S proteasome was affected and the accumulation of insoluble / aggregated tau occurred in the brain, contributing to neurodegeneration (13). Thus we propose that p62 knock-out (KO) mouse may hyperaccumulate K63-polyubiquitinated substrates, being a rich source for proteomic study.

In this study, we address the following questions: What type of proteins bind p62's UBA domain? Which proteins can accumulate in the absence of p62 in the brain of p62 knock-out mice? Are the proteins K63 poly-ubiquitinated? Herein we report the findings of a proteomic study to address these questions. We demonstrate that p62 knock-out mice hyperaccumulate K63-polyUb chains. Poly-ubiquitinated proteins were isolated from KO mouse brain by p62 GST-UBA pull-down assay and UBA-associated proteins were identified using shotgun LC-MS/MS.

MATERIALS AND METHODS

Reagents and Antibodies — Monoclonal mouse anti-ubiquitin, anti-HSP70, anti-neurofilament triplet M, anti-histone H4, anti-ankyrin, anti-CaM kinase II, anti-adaptin, rabbit anti-TrkA and goat anti-septin were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Mouse anti-ubiquinol-cytochrome *c* reductase core protein 1 and anti-ATP synthase β subunit were from Molecular Probe (Eugene, OR). Mouse anti-spectrin and anti-drebrin were obtained from GeneTex Inc (San Antonio, TX). Mouse anti-Ras-GAP was from Transduction Laboratory (Lexington, KY). Rabbit anti-tau was purchased from DAKO (Carpinteria, CA). Mouse anti-tubulin was purchased from Sigma (St. Louis, MO). Seven types of human polyUb linkages were synthesized and quantified by Cell Signaling Technology, Inc (Beverly, MA). K48 or K63 polyUb chains are a gift from Cecile Pickart, Johns Hopkins University.

Mice — Six month old p62 knock-out and wild-type control mice are generated from an original pair obtained from Jorge Moscat, Madrid, Spain (14). All mice were kept under pathogen-free conditions and handled according to Auburn University IACUC which abides by NIH guide lines.

Preparation of Insoluble Proteins — Half of a mouse brain was homogenized with 1 ml ice-cold 1 M sucrose in RAB buffer (0.5 mM MgSO₄, 1 mM EGTA, 0.1 M MES, pH 7.0). The homogenate was centrifuged at 50,000 x g for 20 min at 4 °C. The pellet was extracted with 1 ml RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% DOC, 0.1% SDS, pH 8.0), vortex and centrifuged at 50,000 x g for 20 min at 4 °C to obtain RIPA soluble fraction. The pellet was sonicated with 500 µl 70% formic acid to obtain the FA fraction, which includes highly insoluble aggregated proteins (15). Protein concentrations were detected using DC assay (Bio-Rad Laboratories, Hercules, CA). Formic acid fraction was lyophilized to remove formic acid, resuspended in 500 µl RIPA buffer, and stored in -80 °C freezer.

Western Blotting — Protein (50 µg) isolated by 70% formic acid from 6 month mouse brain was resuspended in RIPA buffer and boiled with SDS-PAGE sample buffer (50% glycerol, 0.3 M Tris, 10% SDS, 25% β-mercaptoethanol, a pinch of bromphenol blue), followed by analysis employing 7.5% SDS-PAGE. After being transferred to a nitrocellulose membrane, the proteins were stained with Ponceau S (0.5% Ponceau stain, 5% TCA). The blots were blocked with 7% milk in TBS buffer (20 mM Tris, pH 7.5, 0.2 M NaCl, 0.1% Tween 20). Anti-ubiquitin antibody (1:1000, Santa Cruz) was added and the blot was incubated at 4 °C for overnight. After addition of anti-rabbit-mouse IgG antibody (1:3000, Amersham Biosciences Corp.), detection was performed using the ECL reagent kit (Amersham Biosciences Corp., Piscataway, NJ).

GST Pull-Down Assay — The glutathione beads coupled with p62 UBA domain were generated as previous described (5). GST-UBA beads were washed with binding buffer (20 mM Tris pH 7.6, 50 mM NaCl, 0.1% NP40, 0.5 mM DTT, 1 mM PMSF) containing 25 µg/ml of BSA. Five micrograms of prepared beads were added to 750 µg mouse brain lysate or 500 µg formic acid fraction (prepared as described before) and rotated for 2 h at room temperature. After being washed three times with binding buffer, the beads were boiled in SDS-PAGE sample buffer for 2 min, followed by separation employing 7.5% SDS-PAGE gel and staining with Coomassie Brilliant Blue (CBB).

Chain Analysis — Heavy-isotope labeled internal peptides corresponding to all seven types of human polyUb linkages were synthesized and quantified by the amino acid analysis (Cell Signaling Technology, Inc, Beverly, MA), including di-glycine tagged signature peptides at Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63 (2). The synthetic peptides were used to optimize the selection of parent ions, fragmentation condition in a selective reaction monitoring (SRM) on an LCQ-DECA XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The quantification of the linkage was carried out essentially as previously described (16,17). GST-UBA pull down samples (50%) were separated on a 10% SDS gel and stained with Coomassie blue G-250. Proteins larger than the immunoglobulin G heavy chain (~55 kDa) were excised from the gel and subjected to in-gel digestion (18). A mixture of seven isotopically labeled signature peptides (1 pm of each) was added to the in-gel digestion solution prior to incubation. After proteolysis, a peptide mixture containing the endogenous peptides and internal standards was separated by reverse-phase chromatography in a 20-min gradient

of 5-35% solvent B (Solvent A, 0.4% acetic acid, 0.005% heptafluorobutyric acid, 95% water; flow rate: ~0.3 μ l/min). An endogenous peptide was eluted at the exact retention time as the heavy labeled internal standard. Both peptides were selected, fragmented and analyzed in a selective reaction monitoring mode. The ratio between the intensities of the fragment ion pairs allowed an accurate measure of the relative abundance of the endogenous versus the internal standard with known quantity. All seven linkages were quantified simultaneously in the same run. Each sample was quantified three times to obtain the relative standard deviation (RSD).

Chain Competition — Glutathione beads coupled with p62 UBA domain were washed with binding buffer (20 mM Tris pH 7.6, 50 mM NaCl, 0.1% NP40, 0.5 mM DTT, 1 mM PMSF) containing 25 μ g/ml of BSA. Five micrograms of GST-UBA beads and 2 μ g K48 or K63 polyUb chains were added to 750 μ g brain lysate or formic acid extracted material. After rotation for 2 h at room temperature, the beads were washed three times with binding buffer and boiled in SDS-PAGE sample buffer, then analyzed by 7.5% SDS-PAGE followed by immunoblotting with ubiquitin antibody.

Protein Analysis by Mass Spectrometry — Protein samples were separated on a 6 - 12% SDS gel (0.75 mm thick) and stained with Coomassie Blue G-250. The entire lane was cut into 15 pieces followed by in-gel trypsin digestion (18). The resulting peptides from each gel piece were dissolved in buffer A (0.4% acetic acid, 0.005% heptafluorobutyric acid, 5% acetonitrile). A pressure cell was used to load each sample onto a 50- μ m inner diameter \times 12-cm self-packed, fused silica C18 capillary column as

described (19). Peptides were eluted during a 2-h gradient from 10 to 30% buffer B (0.4% acetic acid, 0.005% heptafluorobutyric acid, 95% acetonitrile; flow rate, ~300 nl/min). Eluted peptides were ionized under high voltage (1.8-2 kV) and detected in an MS survey scan from 400 to 1700 atomic mass units with 2 microscans followed by three data-dependent MS/MS scans (3 microscans each, isolation width 3 atomic mass units, 35% normalized collision energy, dynamic range 3 min) in a completely automated fashion on an LCQ-DECA XP-Plus ion trap mass spectrometer (Thermo Finnigan, San Jose, CA).

Database Searching for Protein Identification — The Sequest algorithm was utilized for searching all MS/MS spectra against the human reference data base (<ftp.ncbi.nih.gov/genbank>, July, 2003). The parameters were set to allow parent ion mass tolerance to be three and to consider only the b and y ion series. Modifications were permitted to allow the detection of the following (mass shift shown in daltons): oxidized methionine (+16), carboxymethylated cysteine (+57), and phosphorylated serine, threonine, and tyrosine (+80). We used more stringent Sequest criteria than described previously (20, 2). 1) Only fully tryptic peptides were considered; 2) ΔCn score is at least 0.08; and 3) Xcorr should be larger than 2.0, 1.7, or 3.3 for charge states of +1, +2, +3, respectively. To reduce false-positives further, we manually verified proteins matched by less than three peptides, because no false-positives were found among proteins identified by at least three distinct peptides (2). Therefore, all peptides were accepted with high confidence. The conversion from the identified peptides to proteins was complicated by the presence of different names for the same protein and/or by the sharing of peptides within several proteins (*e.g.* protein paralogs) (21). Thus we manually verified all proteins

and removed the redundancy. Typically, we accepted proteins identified by at least one "unique peptide." Obvious contaminant such as trypsin was removed.

Protein Quantification by Mass Spectrometry — Quantitative protein comparison was shown by the number of peptides identified for a specific protein by mass spectrometry (22). Protein abundance in wild-type and knock-out mice samples were determined as number of peptides larger than ten.

Western Blotting Analysis for Selected Proteins — Formic acid samples were lyophilized and resuspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% DOC, 0.1% SDS, pH 8.0). The sample was analyzed by 10% SDS-PAGE gel and transferred to nitrocellulose membrane. Protein bands were stained with Ponceau S. All blots except TrkA were blocked with 7% milk in TBS buffer (20 mM Tris, pH 7.5, 0.2 M NaCl, 0.1% Tween20). TrkA was blocked with 7% milk in Western blotting wash buffer (PBS, 0.1% Triton-100, 0.1% Tween-20). After washing three times, primary antibodies were added (anti-HSP70, 1:1000; anti-histone H4, 1:500; anti-tubulin α subunit, 1:3000; anti-drebrin 1, 1:750; anti-CaM kinase II α subunit, 1:10000; anti-septin 5, 1:750; anti-adaptin α subunit, 1:1000; anti-TrK C-14, 1:1000). Secondary antibodies (anti-rabbit IgG, 1:3000, anti-mouse IgG, 1:3000, Amersham Biosciences Corp.; anti-goat IgG, 1:3000, Santa Cruz) were added next. Detection was performed using ECL reagent kit.

Statistical Analysis — The results of Western blots were scanned by an EPSON TWAIN 1200U scanner. The signal density was integrated into a numeric number and the mean of numbers for two samples from wild-type or knock-out mice was used for the statistical analysis. Paired Student's *t*-test was performed and $P < 0.05$ was considered to have a significant difference.

Proteasome Activity in p62 knock-out mice — Whole brain from p62 wild-type ($n = 3$) and knock-out ($n = 9$) mice was homogenized in RAB buffer (0.1 M MES, pH 7.0, 1 mM EGTA, 0.5 mM $MgSO_4$) using a mortar and pestle and the supernatant was used to examine both trypsin and chymotrypsin activity of the fraction as described (23). Briefly, equal concentration (100 μ g) of lysate was incubated with 40 μ M of either trypsin specific (Boc-Leu-Ser-Thr-Arg-7-Amido-4-Methylcoumarin) or chymotrypsin specific (N-succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin) fluorescent substrate at 37 °C for 30 min. Fluorescence was measured at 370 nm excitation and 430 nm emission. Duplicate samples from each mouse were performed for each assay. The data was analyzed using Student's *t*-test.

Immuno-staining — All the staining was performed employing Histostain DS kit from Zymed laboratories (San Francisco, CA) according to the manufacturer's instruction. Briefly, all the procedures were done at room temperature unless otherwise mentioned. Sections are deparaffinized with xylene and rehydrated in a graded series of ethanol. The sections were incubated for 10 min in peroxidase quenching solution (1 part of 30% hydrogen peroxide to 9 parts of absolute methanol) and washed 3 times with Phosphate

Buffered Saline (PBS, pH 7.5), then blocked for 3 h using serum blocking solution. After 3 h, the sections were incubated with primary antibody (1:25) diluted in PBS overnight at 4 °C, followed by rinsing of the sections with wash buffer (PBS containing 0.05% Tween 20) for 3 times, then biotinylated secondary antibody was added for 30 min. The slides were then washed three times with wash buffer and alkaline phosphatase conjugate was added to the sections and incubated for 10 min. The sections were washed and incubated with the substrate chromogen mixture for 10 min. Then finally the sections were rinsed with distilled water. The section was mounted in permanent mounting media (Vecta mount, Vector Laboratories, Burlingame, CA), covered with a cover slip and heated at 65 °C for 30 min to seal the section.

Immunoprecipitation — Brain lysate (750 µg) was diluted with Triton lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na₃VO₄, 1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin) to 500 µl and incubated with 3 µl of primary antibody at 4 °C for 3 h, then with 50 µl of 50% IgG agarose beads for 2 h at 4 °C. The beads were washed three times with triton lysis buffer and boiled in SDS sample buffer. The eluted proteins were separated on 10% SDS-PAGE gel and subjected to immunoblotting with antibodies to HSP70 and Rpt1. Detection was performed using ECL reagent kit.

RESULTS

p62 knock-out mice hyperaccumulate K63-polyubiquitinated proteins —

Sequestosome 1/ p62 has been reported to serve as a shuttling protein involved in degradation of K63 polyUb proteins (5). Through its ubiquitin associating (UBA) domain, p62 delivers substrates to 26S proteasome for degradation. Thus we proposed that, in the absence of p62, poly-ubiquitinated substrates cannot be shuttled to the proteasome, which will eventually lead to the accumulation of these proteins. We set up a proteomic study to test this hypothesis. Brains of wild-type and knock-out mice were used as our model system. Insoluble proteins were isolated from mouse brain by differential extraction employing RAB, RIPA, and FA. We observed differential accumulation of ubiquitinated proteins in the FA fraction of KO mice compared to samples isolated from wild-type mice (Fig. 11A), indicating that an increase in polyubiquitination and accumulation of those proteins in the FA fraction are related to the absence of p62.

LC-MS/MS was employed to determine the type of polyubiquitin chains associated with the insoluble proteins. All seven types of polyUb linkages were synthesized and quantified. Those synthetic peptides were used as standards in the quantification of polyUb chains associated with GST-UBA pull down conducted on the

FA fraction isolated from wild-type and knock-out mouse brain. The results reveal that the predominate polyUb chains in the FA fraction of wild-type mouse brain is a K48 linkage, while the predominate chains in the p62 knock-out mice sample is a K63 linkage (Fig. 11B). In addition, there is an increased amount of polyUb chains in knock-out mice compared to wild-type, which is compatible with hyperaccumulation of polyUb observed in the FA sample from knock-out mice (Fig. 11A). Moreover there are fewer K48 linkages detected compared to K63 linkages in the FA fraction from knock-out mice (Fig. 11B), suggesting that accumulation of K63-polyUb proteins may suppress the synthesis of K48 chains.

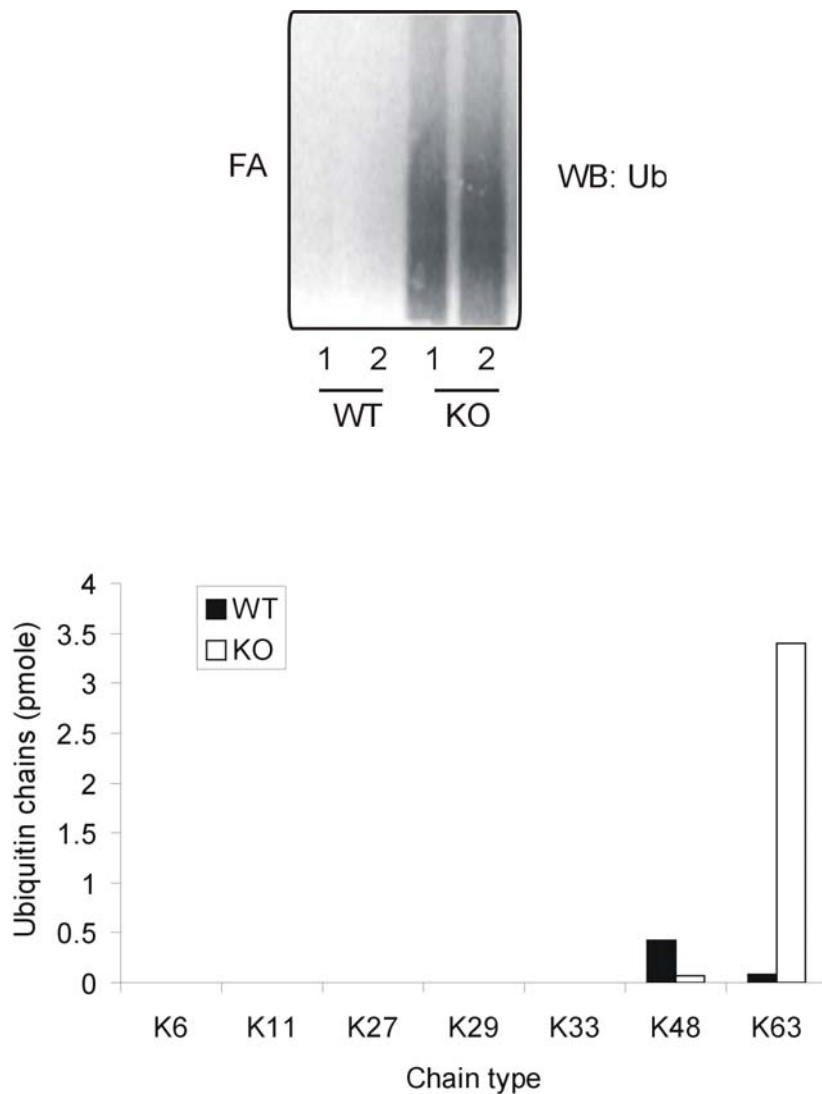


FIG. 11. K63 polyubiquitinated proteins accumulate in formic acid fraction of p62 knock-out mice brain. *A*, Formic acid samples from wild-type (WT) and p62 knock-out (KO) mice brain were prepared as described in Materials and Methods. Fractions were analyzed by SDS-PAGE and immunoblotting with anti-ubiquitin antibody. *B*, Heavy-isotope labeled internal peptides containing seven types of polyUb chains were synthesized (see Methods) and used as standards for chain analysis. Formic acid samples were analyzed by SDS-PAGE. After in-gel digestion, a mixture of FA sample and heavy labeled internal standard were analyzed by LCQ-DECA XP ion mass spectrometer. The ubiquitin chains were quantified by the ratio between endogenous peptide versus the internal standard.

Analysis of UBA-interacting proteins by LC-MS/MS — Since the FA accumulated proteins are enriched in polyUb proteins, we set up a pull-down assay to characterize the identity of the proteins in the FA fraction. p62 contains a UBA domain that can bind K63 polyUb proteins (5). In a pull-down assay, the GST-tagged UBA domain was linked to glutathione beads and used to isolate the interacting proteins from the FA fraction of mouse brain.

Those interacting proteins were then subjected to SDS-PAGE separation and stained with Coomassie Brilliant Blue (CBB). Employing MALDI-TOF analysis, we identified the 60 kD protein as keratin and the 42 kD protein as actin. We observed numerous specific UBA-interacting proteins in the pull down, some appeared to increase in the sample from p62 knock-out brain compared to wild-type sample (Fig. 12A). A ubiquitin chain competition assay was carried out to examine the type of polyUb chains contained in those UBA-interacting proteins. K63 chain input was able to affect the pull-down of ubiquitinated proteins recovered from knock-out mice, indicating the predominate polyUb chains in KO mice are K63-linkages. On the other hand, K48 chains competition with wild-type mice sample indicates that K48-linkages are the predominant polyUb chains (Fig. 12B). These findings are consistent with previous observation in MS analysis (Fig. 11B). Collectively our data reveal that the UBA pull down from p62 knock-out mice brain contain predominantly K63-polyUb proteins.

Shotgun LC-MS/MS was employed to identify these p62 UBA-interacting proteins. Proteomic analysis traditionally employs two-dimensional gel electrophoresis

for separation of protein mixtures into single detectable spots. However the technical limitation of the 2D gel method, such as limited mass range, incompatible with proteins of extreme PI or hydrophobicity, present a challenge for sensitive detection in proteomic studies (24). Therefore liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) has been used to overcome the disadvantage of 2D gels, which can analyze hundreds to thousands of proteins directly from a complex protein mixture with great sensitivity.

K63-polyUb proteins were isolated by p62 GST-UBA beads from the FA fraction of wild-type or p62 knock-out mice brain (Fig. 13A). Our results reveal there are a total of 30 unique proteins present in the p62 UBA pull down (Table II), consisting of nine functional categories (Fig. 13B). In addition, we compared the relative abundance of peptides isolated from wild-type and knock-out FA fraction. The results showed most cytoskeleton proteins and almost all membrane transport, signaling, intracellular trafficking and nuclear proteins increased in knock-out mice brain (Table II).

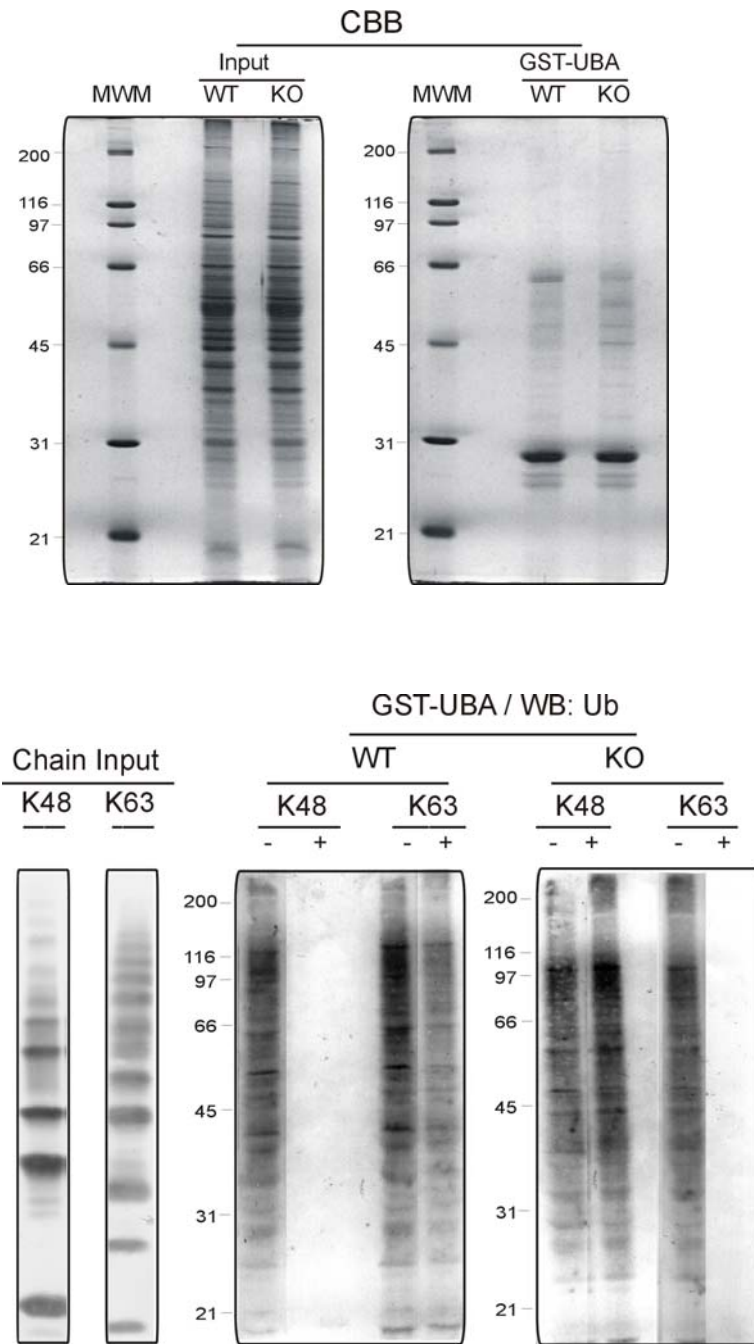


FIG. 12. Polyubiquitin chain competition in GST-UBA pull down assay. *A*, Brain lysates (750 μ g, wild-type or knock-out) were interacted with GST-UBA beads. The interacted proteins were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue G-250. A fraction of 750 μ g brain lysates was input as control. *B*, Two micrograms of K48 and K63 polyUb chains were added into 750 μ g of brain lysates and subjected to a GST-UBA pull down assay. The interaction of polyUb chains and UBA construct were analyzed by immunoblotting with anti-ubiquitin antibody.

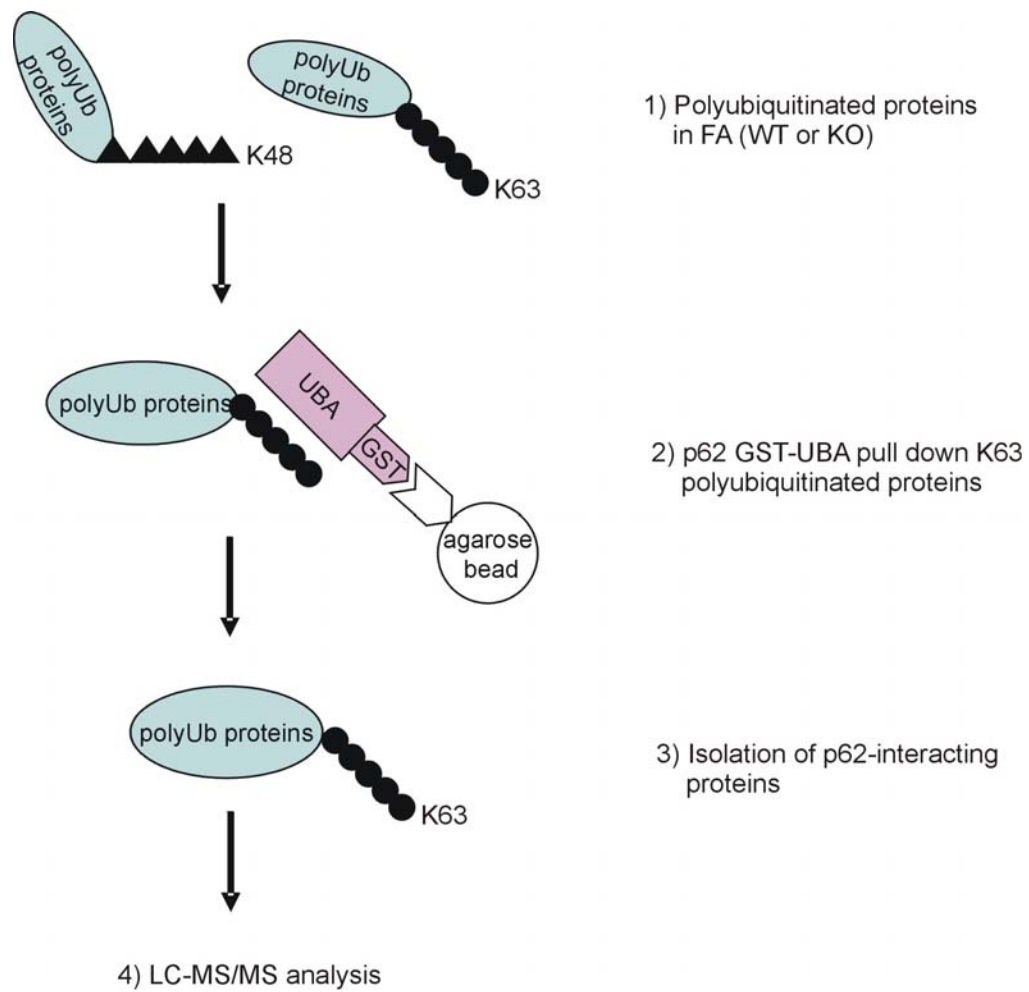


FIG. 13. **Analysis of accumulated proteins by LC-MS/MS.** A, Flow Diagram for the GST-UBA pull down approach. The glutathione beads coupled with p62 UBA domain were added to mouse brain lysates (1). GST-UBA interacts specifically with K63-polyubiquitinated proteins (2). After washing, GST-UBA interacting proteins were isolated (3), and subjected to LC-MS/MS analysis (4).

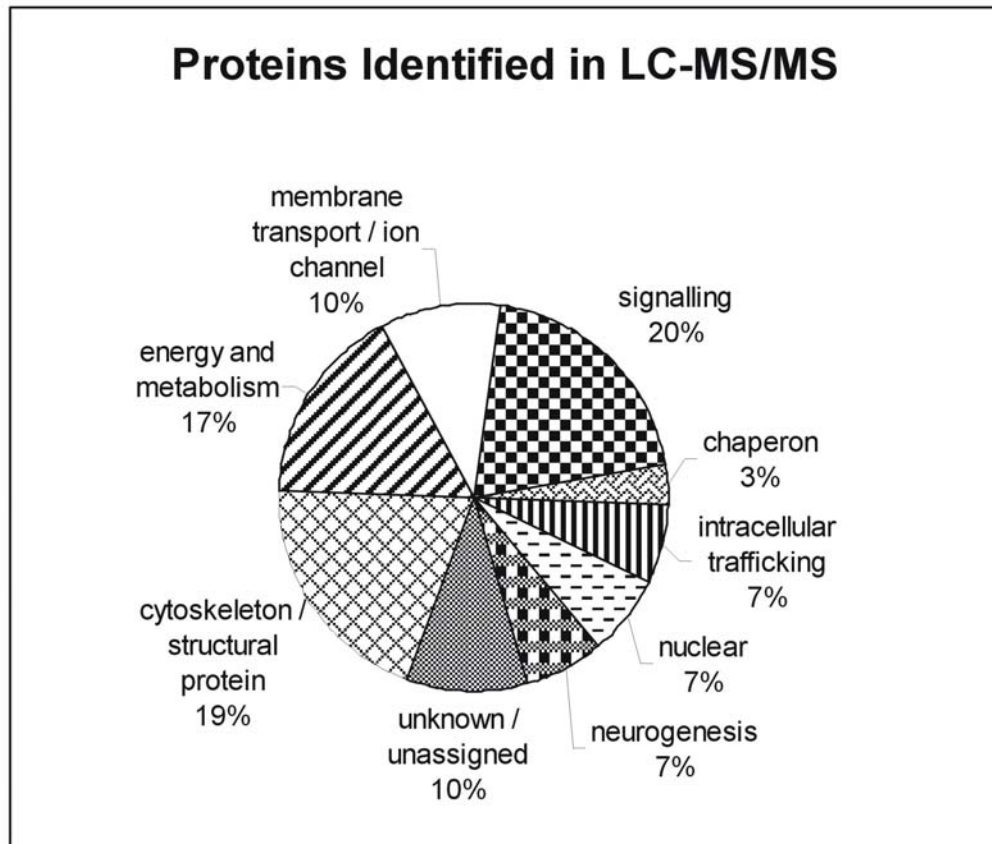


FIG. 13. **Analysis of accumulated proteins by LC-MS/MS.** B, Pie chart that summarizes the known functions of proteins identified by LC-MS/MS.

TABLE II
Proteins identified employing p62 GST-UBA pull-down and LC-MS/MS

Accession no.	Protein Name	# of peptide	
		WT	KO
cytoskeleton / structural proteins			
29788785	tubulin, beta polypeptide	10	17
5174737	tubulin, beta, 4	8	16
21361322	tubulin, beta, 5	12	19
14210536	tubulin beta 6	7	10
4507195	Spectrin, beta, non-erythrocytic 1 isoform 1	23	25
4507191	spectrin, alpha, non-erythrocytic 1	17	22
12667788	myosin, heavy polypeptide 9, non-muscle	24	14
40354192	keratin 10	11	11
4507729	tubulin, beta 2	12	23
29788768	tubulin, beta polypeptide paralog	11	22
5174735	tubulin, beta 2	11	19
30315658	spectrin, beta, non-erythrocytic 1 isoform 2	22	23
4501885	beta actin	16	23
4501887	actin, gamma 1 propeptide	16	23
4501881	alpha 1 actin precursor	12	12
4885049	cardiac muscle alpha actin proprotein	12	12
4501883	alpha 2 actin	11	11
4501889	actin, gamma 2 propeptide	11	11
41149240	similar to beta-tubulin 4Q	14	17
5902122	spectrin, beta, non-erythrocytic 2	0	2
10947052	ankyrin 2 isoform 1	2	9
10835119	myosin VA(heavy polypeptide 12, myosin)	4	6
17921989	tubulin alpha 1	3	5
9507215	tubulin, alpha 8	1	3
42558279	tubulin, beta 8	6	8
17921991	tubulin, alpha 2 isoform 2	4	5
20535366	similar to Tubulin alpha-3/alpha-7 chain	4	6
17921993	tubulin, alpha 2 isoform 1	4	6
41125726	alpha-tubulin isotype H1-alpha	4	6
10947054	ankyrin 2 isoform 2	2	8
41202625	similar to tubulin , beta 5	7	6
17986283	tubulin, alpha 3	5	8
5174477	tubulin, alpha, ubiquitous	5	8
14389309	tubulin, alpha 6	5	8
32967601	ankyrin-3 isoform 1	0	2
13562114	beta tubulin 1, class VI	3	3
28416946	myosin 18A isoform a	1	2
13124879	smooth muscle myosin heavy chain 11 isoform SM1	6	2
13124875	smooth muscle myosin heavy chain 11 isoform SM2	6	2
29788766	tubulin, beta polypeptide 4, membrane Q	5	6
energy and metabolism			

4504183	glutathione transferase	2	3
4885281	glutamate dehydrogenase 1	4	3
11079228	N-ethylmaleimide-sensitive factor	3	2
5031777	isocitrate dehydrogenase 3(NAD+) alpha precursor	0	2
23065563	glutathione S-transferase M5	1	2
4503571	enolase 1	4	3
23065552	glutathione S-transferase M3	2	2
31377775	glutamine dehydrogenase 2	3	2
membrane transport / ion channel			
4757808	plasma membrane calcium ATPase 2 isoform b	0	2
16418379	syntaxin 1B2	1	2
21361181	Na+/K+ -ATPase alpha 1 subunit isoform a proprotein	2	3
signalling			
5032139	synaptotagmin I	0	2
9945439	septin 5 isoform 1	0	3
4502695	cell division cycle 10 isoform 1	1	6
4507297	syntaxin binding protein 1	5	6
25952118	calcium/calmodulin-dependent protein kinase IIA isoform 2	2	9
25952114	calcium/calmodulin-dependent protein kinase IIA isoform 1	2	9
26667206	calcium/calmodulin-dependent protein kinase II gamma isoform 5	1	3
26667196	calcium/calmodulin-dependent protein kinase II gamma isoform 2	1	3
26667211	calcium/calmodulin-dependent protein kinase II gamma isoform 6	1	3
26667199	calcium/calmodulin-dependent protein kinase II gamma isoform 3	1	3
26667191	calcium/calmodulin-dependent protein kinase II gamma isoform 4	1	3
26667203	calcium/calmodulin-dependent protein kinase II gamma isoform 1	1	3
26051206	calcium/calmodulin-dependent protein kinase IIB isoform 2	1	5
26051204	calcium/calmodulin-dependent protein kinase IIB isoform 1	1	5
26667189	calcium/calmodulin-dependent protein kinase II delta isoform 2	1	3
26667180	calcium/calmodulin-dependent protein kinase II delta isoform 3	1	3
26667183	calcium/calmodulin-dependent protein kinase II delta isoform 1	2	6
8922712	septin 11	0	3
19924101	synapsin II isoform IIb	2	2
chaperon			
13676857	heat shock 70D protein 2	2	2
24234686	heat shock 70kDa protein 8 isoform 2	3	3
5729877	heat shock 70kDa protein 8 isoform 1	3	3
intracellular trafficking			
14917109	adaptor-related protein complex 2, mu 1 subunit	0	2
4758182	dynamain 1 isoform 1	2	3
19913416	adaptor-related protein complex 2, alpha 1 subunit isoform 2	0	2
19913414	adaptor-related protein complex 2, alpha 1 subunit isoform 1	0	2
22027651	adaptor-related protein complex 1 beta 1 subunit isoform a	0	2
22027653	adaptor-related protein complex 1 beta 1 subunit isoform b	0	2
4557469	adaptor-related protein complex 2, beta 1 subunit	0	2
nuclear			
5032007	purine-rich element binding protein A	0	3
4503475	eukaryotic translation elongation factor 1 alpha 2	1	3
42656938	similar to elongation factor 1 alpha	1	2
4503471	eukaryotic translation elongation factor 1 alpha 1	2	3

neurogenesis			
4505123	myelin basic protein	3	3
18426915	drebrin 1 isoform a	4	4
18426913	drebrin 1 isoform b	3	3
unknown / unassigned			
41125739	similar to FKSG30	13	10
29736622	similar to RIKEN cDNA 47324959G21 gene	6	7
42656473	similar to pote protein	4	3

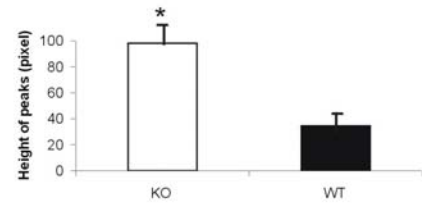
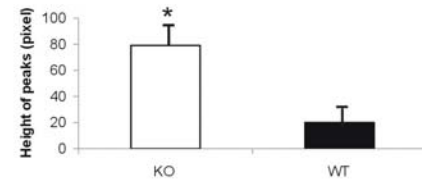
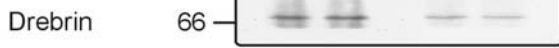
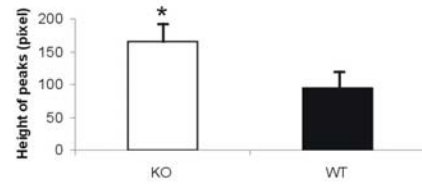
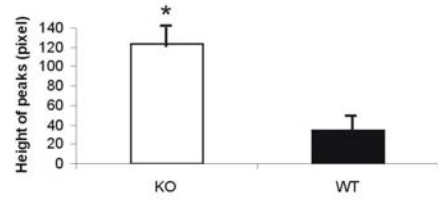
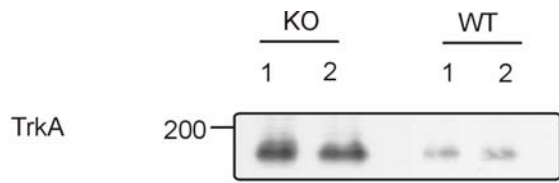
p62 UBA-interacting proteins accumulate in knock-out mice brain — In order to examine if the p62 UBA-interacting proteins accumulate in the absence of p62, we selected six representative p62-UBA interacting proteins from the total pool for further study including adaptin, drebrin, HSP70, tubulin, CaMK II and septin. In addition, we included TrkA as the positive control since we have observed that TrkA accumulates in the brain of knock-out mice as a K63 polyUb protein¹. We also included histone H4, which was not identified in the LC-MS/MS analysis but has been reported to be polyubiquitinated, as a negative control for the analysis.

Formic acid fractions recovered from wild-type and knock-out mouse brains were subjected to SDS-PAGE and Western blotted. The results reveal that all of the representative p62 UBA-interacting proteins except tubulin accumulated in knock-out mouse FA sample compared to wild-type (Fig. 14A). On the other hand, the negative control histone H4 failed to accumulate. These findings indicate that the turnover of those proteins may be interrupted by an absence of p62.

Protein turnover is related to a functional ubiquitin proteasome system. Failure to remove the ubiquitinated proteins may lead to accumulation of those proteins (26). The capacity of the ubiquitin proteasome pathway can be exceeded either by overexpression of substrates or due to a decrease in proteasome activity. Transient expression of two unrelated aggregation-prone proteins caused nearly complete inhibition of the ubiquitin proteasome system, indicating that accumulated proteins can directly impair the UPS

¹ T. Geetha, J.R. Babu, J. Peng, and M.W. Wooten, manuscript in preparation ⁽²⁵⁾

function (27). Thus we examined the activity of the enzymes that are a component of the proteasome in wild-type and p62 knock-out mice. Interestingly, we observed a slight increase of the chymotrypsin-like activity of the 26S proteasome in knock-out mouse brain, but no difference in trypsin activity (Fig. 14*B*). These findings indicated that the delayed turnover of p62- interacting protein are not due to the dysfunction of the proteasome, but because those proteins may not be shuttled to the proteasome for degradation in the absence of p62 (5).



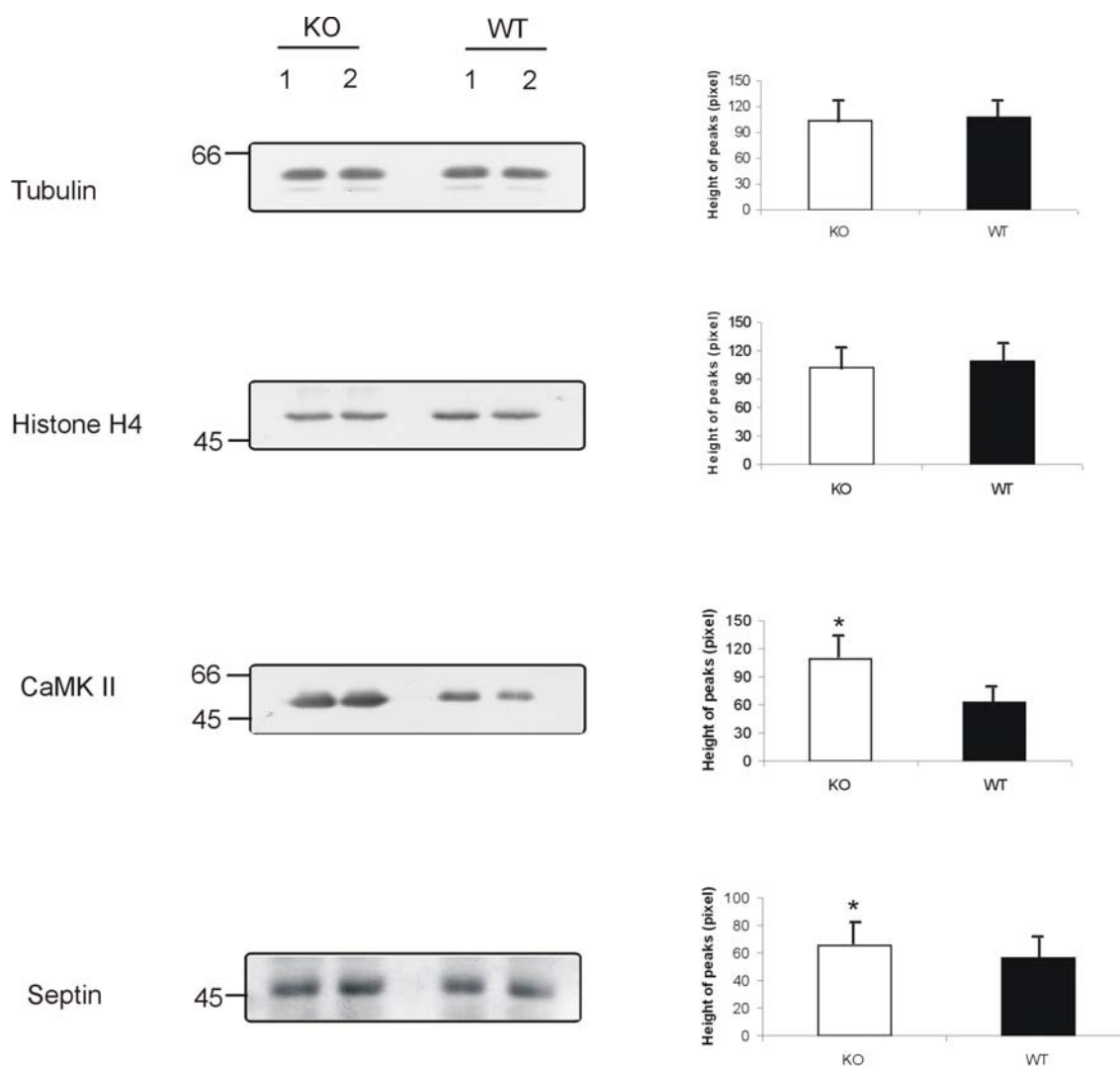


FIG. 14. **Accumulation of proteins in formic acid fraction of p62 knock-out mice brain and proteasome activity in KO mice.** A, Formic acid (FA) fractions of wild-type (WT) and p62 knock-out (KO) mice brain (n = 2 each) were prepared as described in Materials and Methods. FA fractions were Western blotted with antibodies to TrkA, adaptin, drebrin, HSP70, tubulin, CaMK II, histone H4 and septin. The blots were scanned by an EPSON TAIWAN scanner and the intensity of the protein band was used to generate the bar chart. Student *t*-test was employed to compare the differences of the means between WT and KO groups (* KO > WT).

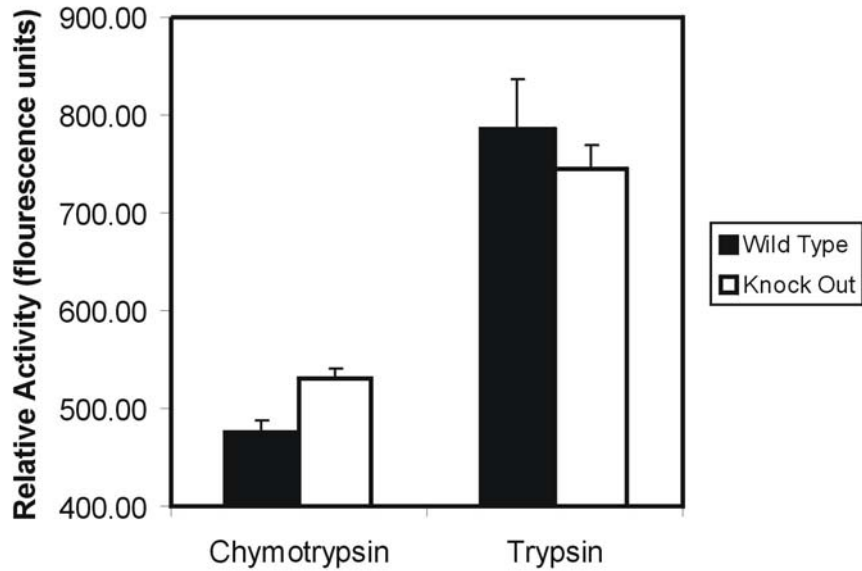


FIG. 14. **Accumulation of proteins in formic acid fraction of p62 knock-out mice brain and proteasome activity in KO mice.** *B*, Lysates prepared from wild-type (WT) and p62 knock-out (KO) mice brain were used to examine both trypsin and chymotrypsin activity. Brain lysate (100 μ g) was incubated with 40 μ M of either trypsin specific or chymotrypsin specific fluorescent substrate. Fluorescence was measured at 370 nm excitation and 430 nm emission. Data are shown in mean \pm SEM. Asterisks indicate significant difference between means.

Hsp70 is degraded in a p62-dependent manner — We have previously reported that Hsp70 interacts with p62 UBA domain (28), suggesting shuttling protein p62 play a role in accumulation of Hsp70. Therefore we selected Hsp70 as a specific protein to further explore the possible mechanism for the accumulation of K63-polyUb proteins in the absence of p62.

A p62 GST-UBA pull down was set up employing PC12 cells which were treated with NGF, a trophic factor previously shown to stimulate polyUb (29). In response to NGF treatment, various polyUb proteins were captured in the pull-down (Fig. 15A, top). The UBA pulldowns were blotted with antibody to Hsp70, and shown to accumulate Hsp70 post-treatment (Fig. 15A, bottom).

To examine the turnover of Hsp70 in the cell, lysosomal inhibitors chloroquine and NH₄Cl, as well as proteasome inhibitor MG132 treatments were utilized in these experiments. Western blotting results reveal that the turnover of Hsp70 is affected dramatically by proteasomal inhibitor MG132, but not by lysosomal inhibitors (Fig. 15B), indicating that Hsp70 is degraded via the Ubiquitin Proteasome Pathway (UPS). Thus, we set up an immuno-precipitation assay for Hsp70 in wild-type and p62 knock-out mice brain lysates to explore the interaction between Hsp70 and proteasome. These results reveal that the absence of p62 impairs the interaction between Hsp70 and proteasome subunit Rpt1, suggesting that p62 may act as a shuttling protein to deliver Hsp70 to the proteasome for degradation (Fig. 15C).

In addition, we set up an immuno-staining analysis to examine the accumulation of HSP70 *in vivo*. The results reveal that there are more HSP70 staining neurons in knock-out mice brain than in wild-type mice brain (Fig. 15D). Altogether, our findings reveal that Hsp70 interacts with the UBA-domain of p62.

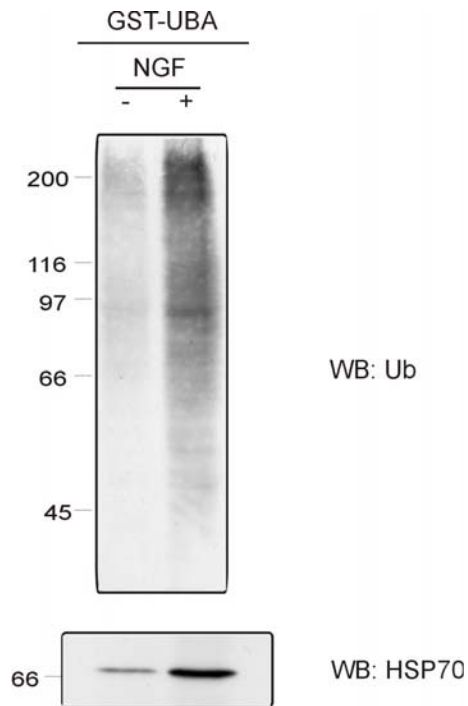


FIG. 15. *A*, PC12 cells were treated with 50 ng/ml NGF for 15 min. PC12 lysates (1 mg) were added to 5 μ g GST-UBA beads. The interacting proteins were analyzed by immunoblotting with antibodies to ubiquitin (top) and HSP70 (bottom).

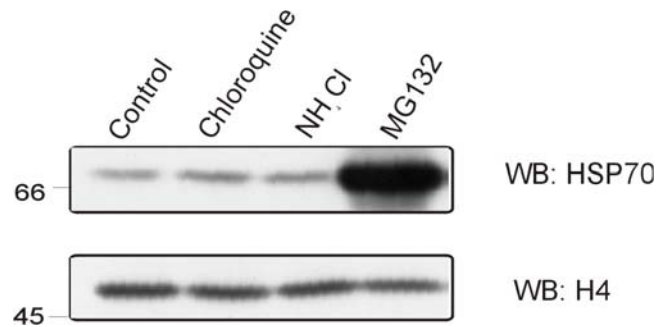


FIG. 15. *B*, PC12 cells were treated with lysosomal inhibitor chloroquine or NH₄Cl, or proteasomal inhibitor MG132 for 24 h, followed by 15 min NGF treatment. Cell lysates were analyzed by immunoblotting with antibodies to HSP70 and Histone H4.

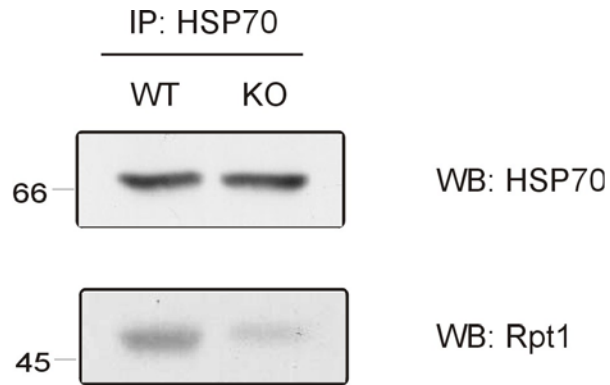


FIG. 15. C, Wild-type or knock-out brain lysates (750 μ g) were immunoprecipitated with anti-HSP70 antibody. The interacting proteins were subjected to Western blotting with antibodies to HSP70 and Rpt1.

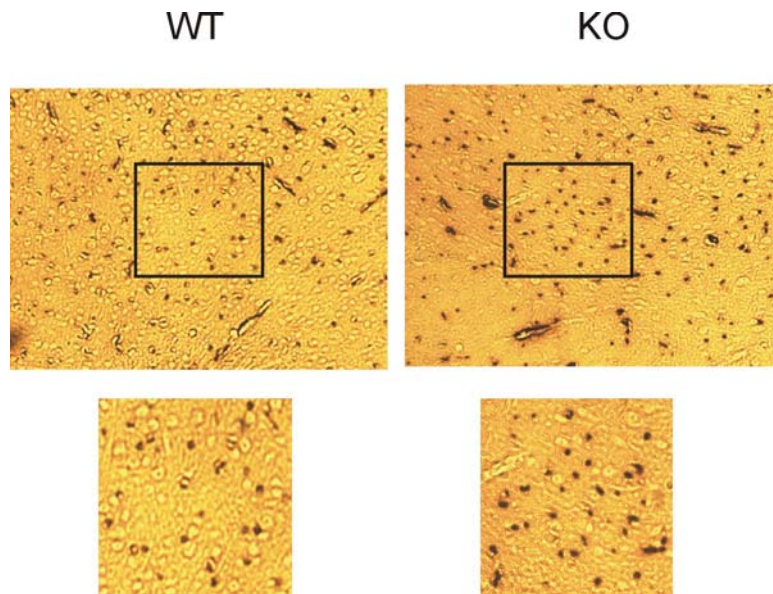


FIG. 15. D, Matched Cortical sections from wild-type (WT) and p62 knock-out (KO) paraffin embedded brains were stained with antibody against HSP70. Boxed regions were magnified to illustrate that increased number of HSP70 immunoreactive neurons in the KO brain sections.

DISCUSSION

Mass spectrometry (MS) is widely used in proteomic analysis and is well-known for its increased sensitivity. Herein we describe an approach to isolate and identify K63-polyubiquitinated proteins employing GST-UBA pull down coupled with LC-MS/MS shotgun analysis. This approach enabled us to characterize p62 UBA-interacting proteins. However two proteins previously reported to interact with p62, tau (12) and TrkA (22), were not recovered in this protein pool. Possible reasons include the variation in analysis. Mayor et. al. observed a modest variation (~17%) in duplicate MS analysis of a single sample in their study (30). Also, these proteins may represent a minor fraction in MS pool. Another reason comes from the nature of the protein ubiquitination process. Both tau and TrkA are ubiquitinated by TRAF6, an ubiquitin ligase activated by protein p62 itself (13, 25). In the absence of p62 in knock-out mice brain, both tau and TrkA may not be polyubiquitinated due to the decreased TRAF6 activity (31). Therefore TRAF6•K63 polyubiquitinated substrates may be excluded from GST-UBA pulldown. However, it is possible that tau and TrkA may be the substrates of some other E3 ligases, since we have recovered K63-polyub tau² and TrkA³ in the brain of p62 knock-out mice.

² J.R. Babu, M.L. Seibenhener, T. Geetha, M.C. Wooten, D.A. Boyett, N. Cox, J. Peng, M.T. Diaz-Meco, J. Moscat, M.W. Wooten. Accumulation of Tau and Amyloid Beta in p62 Deficient Mice: Implications for Alzheimer's Disease

³ T. Geetha, J.R. Babu, J. Peng, and M.W. Wooten, manuscript in preparation

Among those 30 proteins which we found to interact with p62, several are previously reported to be K63-polyubiquitinated (Table III). The signaling protein septin is a substrate of parkin, a K63 chain-specific E3 ligase (32). Tubulin, an abundant structural protein, is also reported to be ubiquitinated by parkin (33). Moreover, previous studies by our lab showed that Hsp70 and CaMKII may interact with p62 (28). There are other interesting proteins that are K63-polyubiquitinated, such as AD-related protein drebrin and intracellular trafficking protein adaptin (28, 32, 33). According to our model (Fig. 16), K63-polyUb proteins are predicted to accumulate in the absence of p62. Our findings reveal that all five representative proteins except tubulin accumulated in knock-out mice brain, consistent with our hypothesis. Interestingly, Yin et al. reported that the K63 dimer may selectively inhibit deubiquitination while K48 dimer showed no inhibitory effect (34), suggesting another contributing factor to hyperaccumulation of K63-polyUb chains.

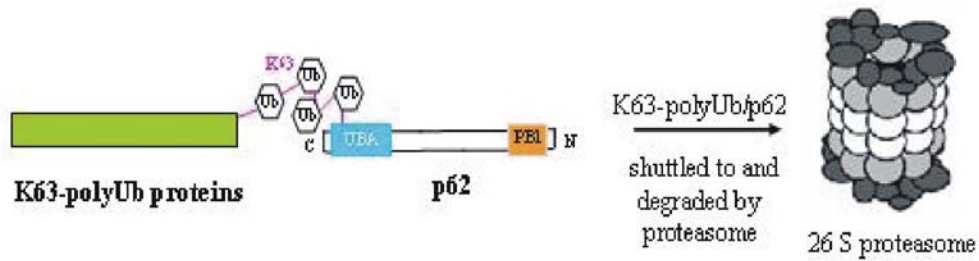
TABLE III
Subset of proteins selected for further analysis

Protein Name	MW (kD)	polyUb chain specificity	E3	Accumulation
Septin	40.2	K63	Parkin ⁽³²⁾	↑ in KO
CaM kinase II	50	*	*	↑ in KO
Tubulin	50	K63	Parkin ⁽³³⁾	KO = WT
HSP 70	70	K63	TRAF6 ⁽²⁸⁾	↑ in KO
Drebrin	77.3	*	*	↑ in KO
Adaptin	100	*	*	↑ in KO
Positive control				
TrkA	140	K63	TRAF6 ⁽²⁵⁾	↑ in KO
Negative control				
Histone H4	35	*	E3histone ⁽⁴³⁾	KO = WT

* Indicates there is no previous report in the literature.
25,28,32,33,43 listed in references

Given that K63 polyUb proteins accumulate in p62 KO mice brain, our data supports the model where p62 shuttles K63-polyUb proteins to the 26S proteasome for degradation (Fig. 16). Although the K48 chain is the main signal for proteasomal degradation, K63 may serve as a competent proteolytic signal as well (4). The significant increase of polyUb and K63 chains in knock-out mice suggest that p62 is a major shuttling protein for K63 polyUb substrates. Trafficking of those substrates would be interrupted in the absence of p62, leading to protein accumulation (Fig. 16). Previous studies suggest that accumulated proteins may directly impair the 26S proteasome function by the overloading of substrates (27, 35). However our data reveal there is no significant decrease in proteasome activity in the presence of protein accumulation, instead a slight increase of chymotrypsin-like activity of the 26S proteasome was observed in knock-out mice brain (Fig. 14B). Our observation is compatible with the previous findings in which the total level of the proteasome did not change during the impaired degradation of mutant SOD1 (44). On the other hand, a decrease in constitutive proteasomes and an increase in the immuno-proteasome level were observed, suggesting that the immunoproteasome may associate with protein accumulation. More detailed examinations are needed to reveal the exact role of the 26S proteasome in the pathogenesis of neurodegenerative diseases.

Wild-Type mice:



p62 knockout mice:

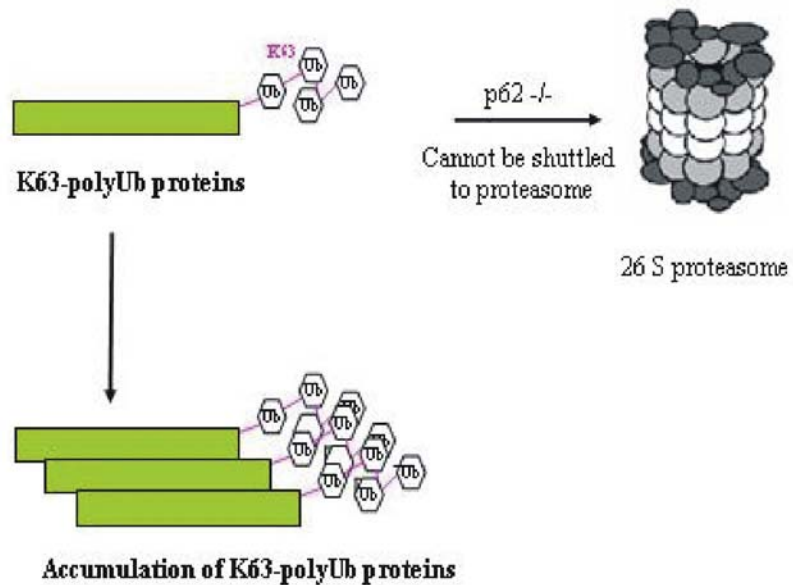


FIG. 16. Model of p62 proteasomal shuttling. K63-polyubiquitinated proteins interact with p62's UBA domain and are shuttled by p62 for proteasomal degradation. In the absence of p62, the shuttling process is blocked, resulting in hyperaccumulation of K63-polyubiquitinated proteins in the cell.

Sequestosome 1/ p62 may also play a role in protein aggregation. Protein aggregation and the formation of inclusion bodies are the hallmarks of a number of protein-aggregation diseases, including, but not limited to neurodegenerative diseases (36, 37). Accumulated / aggregated proteins are sequestered into non-membrane-bound electron-dense areas to form a sequestosome / inclusion bodies (38). Employing 2D gel with MALDI-TOF mass spectrometry, p62 has been identified as a common component of Mallory bodies (MBs) which are the cytoplasmic inclusions in hepatocytes in alcoholic hepatitis or nonalcoholic liver disorder (39). Furthermore, p62 has been detected in other disease-associate cytoplasmic inclusions, such as neurofibrillary tangles in Alzheimer's disease and Lewy bodies in Parkinson's disease (39). p62 is rapidly induced in hepatocytes preceding MB formation (40) and may promote aggregation and sequestration of abnormal proteins. In this regard, MBs contain keratin, HSPs that are recruited to these inclusions by interaction with the UBA domain of p62 (41). This finding is consistent with our results.

We have previously shown that overexpression of p62 resulted in large aggregates (5), dependent upon its UBA domain. We also observed that mouse embryo fibroblasts treated with proteasome inhibitors fail to form aggresomes in the absence of p62 (data not shown). Sequestosome 1/ p62 aggresomes are needed for cell survival (42). Thus, p62 containing sequestosomes appear to localize proteins and triage those proteins for degradation. Since p62 is a stress-regulated protein, MBs, and Lewy bodies containing p62 (36-40) are likely to represent sites where misfolded aggregated proteins accumulate as a defense mechanism for cell survival (42). Thus, in the absence of p62 the failure of

aggresomes to form leads to an overload of the aggregated protein thereby contributing to apoptosis. We predict that p62 knock-out mice fail to form MBs during chronic hepatic stress. Likewise, brains of p62 knock-out mice should contain excessive, non-degraded K63-polyub proteins in the absence of inclusions, thereby contributing to neurodegeneration. Our findings herein support this model and contribute to the identification of proteins regulated by this pathway.

CONCLUSIONS

1. Shuttling protein p62 interacts with K63-polyubiquitinated proteins.
2. p62 shuttles K63-polyUb proteins to 26S proteasome for degradation.
3. K63-polyUb proteins are hyperaccumulated in p62 knock-out mice brain.
4. p62 may contribute to aggresome formation in neurodegenerative diseases.

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