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Sequestosome 1/p62 shuttles polyubiquitinated tau for proteasomal degradation

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Abstract

Inclusions isolated from several neurodegenerative diseases, including Alzheimer's disease (AD), are characterized by ubiquitin-positive proteinaceous aggregates. Employing confocal and immunoelectron microscopy, we find that the ubiquitin-associating protein sequestosome1/p62, co-localizes to aggregates isolated from AD but not control brain, along with the E3 ubiquitin ligase, TRAF6. This interaction could be recapitulated by co-transfection in HEK293 cells. Employing both *in vitro* and *in vivo* approaches, tau was found to be a substrate of the TRAF6, possessing lysine 63 polyubiquitin chains. Moreover, tau recovered from brain of TRAF6 knockout mice, compared with wild type, was not ubiquitinated. Tau degradation took place through the ubiquitin–proteasome pathway and was dependent upon either the K63-polyubiquitin chains or upon p62. In brain lysates of p62 knockout mice, tau fails to co-interact with Rpt1, a proteasomal subunit, thereby indicating a requirement for p62 shuttling of tau to the proteasome. Our results demonstrate that p62 interacts with K63-polyubiquitinated tau through its UBA domain and serves a novel role in regulating tau proteasomal degradation. We propose a model whereby either a decline in p62 expression or a decrease in proteasome activity may contribute to accumulation of insoluble/aggregated K63-polyubiquitinated tau.

Keywords: neurodegeneration, proteasome, tau, ubiquitin.
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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 $p56^{lck}$ (Joung *et al*. 1996). The protein was cloned by two independent groups as a co-interacting protein of the homologous atypical PKC isoforms, zeta/iota, and is also named ZIP: zeta PKC interacting protein (Puls *et al*. 1997; Sanchez *et al*. 1998). p62 was shown to bind ubiquitin non-covalently (Vadlamudi *et al*. 1996) and sequester into cytoplasmic aggregates (Shin 1998). At its C-terminus, p62 possesses a ubiquitin-associating domain (UBA), amino acids 386–434, that binds polyubiquitin chains (Schultz *et al*. 1998; Geetha and Wooten 2002; Ciani *et al*. 2003). In addition, p62 possesses several other structural motifs that suggest it might participate in the formation of multimeric signaling complexes. At its N-terminus, p62 possesses an acidic interaction domain (AID/PB1) that binds the atypical protein kinase C's zeta/iota, followed by a binding site for the RING finger protein, TRAF6, and two PEST sequences (reviewed in Geetha and Wooten 2002). The N-terminal PB1 motif of p62 shares considerable structural homology with the Ubl domain (Yoshinaga *et al*. 2003), enabling p62 interaction with the proteasome (Seibenhener *et al*. 2004). Thus, p62 is characterized as a polyubiquitin shuttling factor, interacting with K63-polyubiquitinated substrates through its C-terminal UBA domain, and delivering these substrates for degradation by interaction of its N-terminal PB1 domain with the proteasome (Seibenhener *et al*. 2004). It stands to reason that aggregates of polyubiquitinated proteins may accumulate in the cytoplasm over time if shuttling proteins, such as p62, fail to target proteins for degradation or if the activity of the proteasome is compromised.

Accumulation of polyubiquitinated proteins into insoluble aggregates is a hallmark of many neurodegenerative diseases

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Abbreviations used: Aβ, amyloid beta peptide; AD, Alzheimer's disease; ALLN, *N*-acetyl-Leu-Leu-Norleu-AL; BSA, bovine serum albumin; EM, electron microscopy; GST, glutathione-s-transferase; NFTs, neurofibrillary tangles; PBS, phosphate-buffered saline; PHF, paired helical filament; p-Tau, abnormally hyperphosphorylated tau; TRAF6, tumor necrosis factor receptor associated factor 6; Ub, ubiquitin; UBA, ubiquitin binding domain; WB, western blot.

(Lang-Rollin *et al*. 2003). The molecular mechanism by which these aggregates form is poorly understood. Multiple factors may contribute to aggregate formation in brain of Alzheimer's disease (AD) individuals; however, disturbances in the ubiquitin–proteasome pathway have been linked as an underlying factor. Furthermore, the activity of the proteasome is decreased during the course of AD (Keller *et al*. 2000), and the neurotoxic amyloid beta peptide ($A\beta_{1-42}$) has been shown to bind the proteasome and block its activity as well (Gregori *et al*. 1995; Lam *et al*. 2000). Employing an *in vitro* expression cloning approach, we have recently shown that the UBA domain of p62 can interact with several proteins that are implicated in the pathophysiology of AD (Pridgeon *et al*. 2003). In addition, p62 has been co-localized to ubiquitin/tau-containing aggregates in hippocampus and cortex in individuals with AD, but is sparse or absent from normal age-matched brain free from tangles (Kuusisto *et al*. 2001, 2002). The appearance of p62 in AD brain is particularly intriguing as p62 accumulates in neurofibrillary tangles and co-localizes with hyperphosphorylated tau (Kuusisto *et al*. 2002). Therefore, the presence of p62 within neurofibrillary tangles (NFTs) raises the possibility that p62's inability to shuttle polyubiquitinated tau contributes to formation of tau aggregates and may play a contributing factor in the pathogenesis of AD.

Herein, we have undertaken a study to characterize p62 aggregates obtained from AD brain to examine their composition and understand their relationship to the pathophysiology of AD. We find that tau is a K63-polyubiquitinated substrate of TRAF6 that interacts with the UBA domain of p62 and is targeted for proteasomal degradation. Our results reveal that p62 plays a novel regulatory role in delivery of substrates to the proteasome. Based on our observations, we suggest that formation of p62-tau aggregates in the brain of individuals with AD may arise in response to clearance of tau under proteasome impaired conditions (Gregori *et al*. 1995; Lam *et al*. 2000). Alternatively, a decline in p62 expression could lead to accumulation of aggregated tau, thereby contributing to neurodegeneration.

Materials and methods

Reagents

Mouse anti-ubiquitin, anti-TRAF6 and goat anti-tau (clone C-17) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). 12E8 (p-tau) that recognizes phosphorylated S-262 and S356 was a generous gift from Dr Gail Johnson, University of Alabama, Birmingham. Rabbit anti-UbcH7, ubiquitin K63 only and ubiquitin aldehyde Ub-H was from Boston Biochem (Cambridge, MA, USA), and rabbit anti-tau was purchased from DAKO (Carpinteria, CA, USA). Mouse anti-Rpt1, 26 S proteasome and ubiquitin-activating enzyme E1 (mammalian) were purchased from Affiniti Research Products (Exeter, UK). Cycloheximide, ubiquitin and the inhibitors *N*-acetyl-Leu-Leu-Norleu-AL (ALLN), and chloroquine were from Sigma Aldrich (St Louis, MO, USA). Z-Leu-Leu-Leu-CHO (MG132) was obtained from Biomol Research Laboraories,. The live/dead viability/cytotoxicity kit was purchased from Molecular Probes (Eugene, OR, USA), horseradish peroxidase conjugated secondary antibody from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) reagents from Bio-Rad Laboratories (Hercules, CA, USA). All p62 constructs used in this study have been previously described (Sanchez *et al*. 1998; Wooten *et al*. 2001).

Human brain samples

Post-mortem adult human brain specimens (hippocampus) were obtained from the Harvard Brain Tissue Resource Center, McLean Hospital, Massachusetts, in accordance with the Institutional Review Board-approved guidelines. The samples were clinicopathologically verified for definite Alzheimer's disease (AD, Braak 6). Control hippocampus tissue was obtained from University of Alabama Birmingham, Alzheimer's Disease Research Center. Brain tissues ($n = 6$) were from both sexes with a mean age of 79 for the AD samples and a mean age of 77 for the control samples and were obtained with a mean post-mortem delay of 11 h for AD and 10 h for the control.

Cells culture and transfection

Human embryonic kidney 293 cells (HEK293) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 humidified incubator. PC12 cells were grown on plates coated with rat-tail collagen in DMEM containing 10% horse serum and 5% calf serum and antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin). Subconfluent HEK293 cells were transfected by the calcium phosphate method using a commercially available transfection kit (Specialty Media, Phillipsburg, PA, USA) and PC12 cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). After 12-h transfection, the medium was removed and cells were rinsed once with phosphate-buffered saline (PBS) and incubated with fresh medium.

Isolation of inclusion bodies

A previously described procedure was employed to isolate inclusions from human brain (Jensen *et al*. 2000; Lee and Lee 2002; Kuazi *et al*. 2003). Procedures were carried out at 4°C unless otherwise stated. The hippocampus was homogenized using homogenization buffer (HB: 0.32 M sucrose, 50 mM Tris HCl at pH 7.4, 5 mM EDTA, 1 μg/mL leupeptin, 1 μg/mL pepstatin and 17.4 μg/mL phenylmethylsulfonyl fluoride), filtered through glass wool, followed by washing three times with HB and centrifuged at 1000 *g* for 10 min to obtain a pellet. The pellet was dissolved in HB and Percoll was added to a concentration of 14% (v/v). The sample was overlaid on 35% percoll (v/v in HB) and centrifuged for 30 min at 35 000 *g*. The material at the interface was collected and washed three times at 4000 *g* for 10 min in 50 mM Tris-HCl-buffered saline at pH 7.4 containing 1 μg/mL leupeptin, 1 μg/mL pepstatin and 17.4 μg/mL phenylmethylsulfonyl fluoride. The pellet was suspended in TBS buffer containing 5 mM $MgCl_2$, 2 mM EGTA, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 17.4 μg/mL phenylmethylsulfonyl fluoride and 1 μg/mL DNase I. The

suspension was vigorously shaken and rotated overnight at 37°C followed by washing three times in sucrose Tris buffer (STB: 0.32 M sucrose, 50 mM Tris-HCl at pH 7.4, 1 μg/mL leupeptin, 1 μg/mL pepstatin and 17.4 μg/mL phenylmethylsulfonyl fluoride) at 4000 *g* for 10 min. The resulting pellet was dissolved in 12% Percoll (v/v in STB) and overlaid on 35% Percoll (v/v in the STB). After centrifugation at 35 000 *g* for 30 min, the material banding just below the sample/35% Percoll interface, was collected and filtered through 20-μm nylon mesh (Small Parts, Miami Lakes, FL, USA). The protein amount was determined using Bradford reagent (Bio-Rad Laboratories) and bovine serum albumin (BSA) as a standard.

Immunofluorescence staining

All steps were conducted at 22–25°C unless stated otherwise. The inclusion bodies were placed on poly-L-lysine coated 12-well slides (Cel-line/Erie Scientific Co., Portmouth, NH, USA) and allowed to partially dry. Inclusion bodies on the slides were fixed for 10 min in 3% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) and permeabilized with 0.1% Triton X-100 in PBS for 15 min. The inclusion bodies were incubated in blocking buffer (3% non-fat dry milk in PBS) for 1 h, prior to incubation with primary antibodies at 4°C overnight. As control, primary antibody was omitted. After washing with PBS, the secondary antibody (Molecular Probes) in blocking buffer was applied for 1 h. After extensive washing in PBS, the samples were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA). Alternatively, HEK cells were fixed with 3% paraformaldehyde and processed in a similar fashion through primary and secondary antibodies.

For Thioflavin S staining, the inclusions were fixed and incubated with 0.05% thioflavin S (Sigma) for 10 min, then washed three times with 80% ethanol (Gotz *et al*. 2001). Cells were analyzed using a Bio-Rad MRC 1024 Laser Scanning Confocal Microscope using 100 × objective. The sizes of the inclusion bodies were measured using the software Metamorph (Universal Imaging Corporation, Dowington, PA, USA).

Immunoelectron microscopy

Inclusions were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 2 h at 4°C and post-fixed for 2 h in 1% OsO_4, dehydrated in cold graded ethanol and embedded in LR White resin (Ted Pella, Redding, CA, USA). Ultra thin sections (70 nm) on grids were blocked with 2% BSA in PBS for 1 h at room temperature and incubated with primary antibody at 4°C overnight. The grids were then washed three times with PBS for 10 min, and incubated with 20 nm colloidal gold labeled protein A (Sigma) for 1 h at room temperature. In addition, controls were incubated with non-related primary antibody and gold-labeled protein A or in the absence of primary antibody. The grids were washed extensively with PBS, stained with aqueous 1% uranyl acetate and 0.4% lead citrate, air dried and examined on a Philips 301 electron microscope.

Immunoprecipitation and western blotting

Each milligram of the inclusion preparation was solubilized in buffer containing 1% SDS. To conduct immunoprecipitation, the solubilized inclusion preparation was diluted with Triton-lysis buffer. Each milligram of the preparation was incubated with 4 mg of primary antibody at 4°C for 3 h and then with 50 μL of 50% agarose-coupled secondary antibody for two more hours at 4°C. Alternatively, in co-transfection experiments, the cells were lysed with SDS lysis buffer to detect covalent interaction (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1% SDS, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin and aprotinin) or Triton lysis buffer (SDS lysis buffer minus SDS) for non-covalent interaction (Seibenhener *et al*. 2004). Protein concentration was determined using DC assay (Bio-Rad Laboratories) for samples containing SDS lysis buffer or by Bradford procedure (Bio-Rad Laboratories) for all samples without SDS, using bovine serum albumin (BSA) as a standard. The beads were washed three times with lysis buffer, eluted by boiling the beads in SDS–PAGE sample buffer, and subjected to SDS–PAGE in 7.5–12% acrylamide gels. SDS–PAGE molecular weight standards included: 200 kDa myosin, 116 kDa β-galactosidase, 97 kDa phosphorylase β, 66 kDa bovine serum albumin, 31 kDa carbonic anhydrase, 21 kDa trypsin inhibitor, 14 kDa lysozyme and 6.5 kDa aprotinin. Immunoblotting was performed on nitrocellulose membranes and analyzed by western blotting with ECL detection reagents (Amersham Pharmacia Biotech).

GST pull-down assay

Glutathione beads coupled with p62 UBA domain were washed three times with binding buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.1% Nonidet P-40, 0.5 M dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing 25 μg/mL of BSA. For each milligram of lysate, 2.5 μg of GST-UBA domain was added and rotated for 2 h at room temperature. The beads were washed three times with binding buffer and boiled in SDS–PAGE sample buffer, followed by separation employing a 7.5% SDS gel, and western blotted with appropriate antibody.

In vitro ubiquitination and deubiquitination assay

His-tagged tau construct was expressed in HEK293 cells by the calcium-phosphate method. The cells were collected, washed with PBS and lysed with SDS lysis buffer. The lysate was immunoprecipitated with tau antibody and bound to secondary antibody coupled to agarose beads. Post-immunoprecipitation the beads were washed by vigorously vortexing in 500 μL of wash buffer (50 mM Tris pH 7.5, 50 mM NaCl, 2.5 mM $MgCl_2$). To the tau captured on the beads, 50 μL of reaction buffer (50 mM Tris pH 7.5, 2.5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP) containing 100 ng E1, 200 ng UbcH7 (E2), 100 μg TRAF6 (E3) and 5 μg of GST-WT Ub, or its mutants K29R, K48R and K63R Ub was added (Liani *et al*. 2004; Nishikawa *et al*. 2004). The beads were incubated at 37°C for 2 h by continuous shaking, and then washed three times with reaction buffer. The proteins were released by boiling for 2 min in SDS–PAGE sample buffer, electrophoresed on 7.5% SDS–PAGE and western blotted with antibody to ubiquitin and tau. Control reactions were also conducted in the absence of ATP. For the *in vitro* deubiquitination assay, the ubiquitinated tau was treated with 26 S proteasome (40 or 80 nM) in 30 μL of buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM ATP for 2 h at 37°C. The deubiquitination of tau by the 26 S proteasome was blocked by the addition of ubiquitin aldehyde (Ub-H), an inhibitor of ubiquitin isopeptidases (1 or 10 μM) (Nishikawa *et al*. 2004). The samples

were boiled in sample buffer, resolved by 7.5% SDS–PAGE and western blotted with anti-ubiquitin or anti-tau.

Triton solubility of tau

This assay was carried out as described previously (Shimura *et al*. 2004). Briefly, cells were lysed using lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin and aprotinin) which contains 1% Triton X-100, then centrifuged at 20 000 *g* for 1 h at 4°C. The Triton soluble supernatants were collected and the pellets were further resolved in lysis buffer containing 4% SDS and centrifuged at 20 000 *g* for 1 h at 22°C. Supernatant was referred to as 1% Triton insoluble fractions. Both fractions were immunoprecipitated with tau antibody, separated by SDS polyacrylamide gel electrophoresis and analyzed by western blotting.

Turnover of tau

HEK cells were co-transfected either with antisense (AS) p62, myc-tagged p62 or K63R ubiquitin mutant along with tau. Twenty-four hours post-transfection, the cells were treated with 20 μg/mL cycloheximide for 6 h, 12 h, 24 h and 36 h. After treatment, the cells were lysed with Triton lysis buffer and protein was measured. Equal concentrations of protein (30 μg) were separated by SDS–PAGE and western blotted to assess the turnover of tau.

Cell death and cell survival assays

Live and dead cells were identified with LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes) according to the manufacturer's instruction. Cell survival was measured by MTS assay (Tanaka *et al*. 2004).

Results

Inclusions isolated from AD brain contain p62 and TRAF6

As p62 has been reported to co-localize with tau in NFTs from individuals with AD (Kuusisto *et al*. 2001, 2002), we set out to characterize inclusions isolated from AD brain and examine their size and composition. Dual fluorescence labeling of the inclusion preparation revealed the presence of amorphous aggregates that double label with both p62 and ubiquitin antibodies (Fig. 1a). In addition, these aggregates were positive for thioflavin S-staining, which detects proteins possessing beta-pleated sheet structure. Using Metamorph, the heterogeneous nature of the preparation was quantitated (Fig. 1b). The inclusion preparation was composed of three classes of aggregates ranging in size from small: 2.5–4 μm; medium: 7–9 μm; large: 12–15 μm. The inclusion preparation of samples obtained from normal age-matched brain did not possess any large/medium size aggregates, thus suggesting these may be a characteristic feature of AD brain. To further analyze the aggregates, their protein composition was analyzed by SDS–PAGE and western blotting (Fig. 1c) with various antibodies to proteins known to co-localize with inclusions by immunohistochemistry (e.g. ubiquitin, tau, phosphorylated tau), or proteins known to interact with p62

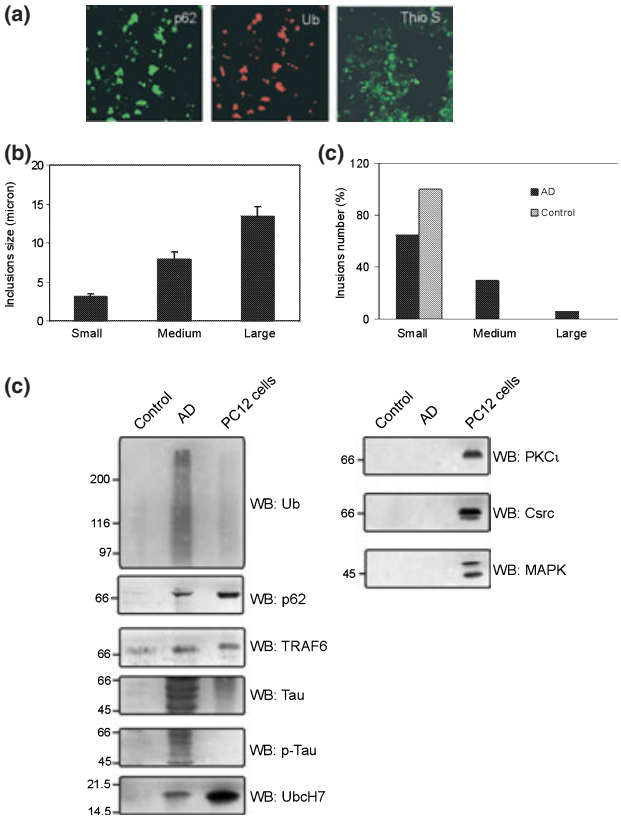

Fig. 1 Characterization of isolated inclusion bodies. (a) Confocal immunofluorescence images of AD inclusion bodies stained with antisera to p62, Ub and thioflavin S. Both p62 and Ub were visualized by secondary antibody to Texas red or Oregon green. (b) Quantitative analysis of the inclusion bodies from both control and AD brain. Left-hand graph, three different sizes of inclusions were observed in AD samples. The sizes of the inclusions (μm) observed in AD brain were measured using the software Metamorph. Presented here is the average size of the inclusion population. Right-hand graph, to examine the distribution of inclusions, 25 random fields were selected for counting for both AD and control inclusions. (c) Immunoblot analysis of the inclusion bodies from control individual, AD or PC12 cell lysates (antibody control). Equal concentrations of protein (50 μg) from inclusions or PC12 cell lysates were loaded and separated by 12% SDS–PAGE and western blotted with antibody to ubiquitin, p62, TRAF6, tau, phospho- tau (12E8), UbcH7, PKC iota, c-src or MAPK as shown.

directly (e.g. TRAF6, PKC iota), or with p62 indirectly [e.g. UbcH7 through interaction with TRAF6, c–src through interaction with PKC iota, or MAP kinase through interaction with PKC iota (Seibenhener *et al*. 1999)]. Confirming the *in situ* immunofluorescence results, the inclusion preparation from AD brain contained both p62 and ubiquitin. In addition, TRAF6, tau, phosphorylated tau and UbcH7 were also present, whereas PKC iota, c-src and MAPK were absent. These findings reveal that specific proteins are found in the inclusions along with p62.

To further analyze the ultrastructural features of the aggregates, the preparation was examined by immunoelectron microscopy (Fig. 2a). The aggregates were irregular, fibrillar, and reacted with antibodies to p62, tau, ubiquitin, TRAF6 and UbcH7, whereas control sections without antibody did not exhibit any gold particles. To investigate whether these proteins directly co-localized with the aggregates, confocal immunofluorescent microscopy was undertaken as well (Fig. 2b). We observed co-localization of p62-tau-Ub, p62-tau-TRAF6, and UbcH7-tau-TRAF6. These findings confirmed the biochemical assessement undertaken earlier (Fig. 1c), as well as, the data obtained by immunoelectron microscopy (Fig. 2a).

To further determine if the co-localization of tau-p62-TRAF6 and UbcH7 could be recapitulated *in vitro,* a co-transfection approach was undertaken. Transfection of GFP-p62 in HEK cells along with HA-ubiquitin, Flag-TRAF6, His-tau and myc-UbcH7 followed by confocal immunofluorescent microscopy revealed that the proteins co-localize to discrete spherical aggregates (Fig. 2c). Inhibition of protein degradation by treatment with ALLN or MG132 (not shown) revealed that these aggregates increased in size, and the proteins retained their property of co-localization.

Tau is a K63-polyubiquitinated substrate of TRAF6 interacting with the p62-UBA domain

Because tau was ubiquitinated in samples recovered from AD brain (Fig. 1c), we sought to determine the type of ubiquitin-linked chains employed in tau polyubiquitination by conducting complementary *in vivo* and *in vitro* assays. HEK cells were transfected with His-tau along with wild-type and K/R mutants of ubiquitin at 29, 48 and 63. We observed that tau was polyubiquitinated employing K63 polyubiquitin chains, as the K63R mutant completely abrogated polyubiquitination of tau (Fig. 3a). A small fraction of p62 interacted with co-transfected tau in the absence of ubiquitin. This is likely because of endogenous Ub resulting in tau polyubiquitination and therefore mediating interaction with p62. When tau polyubiquitination was impaired by transfection of K63R mutant Ub-construct, tau failed to co-associate with p62. Because TRAF6 exhibits E3-ubiquitin ligase activity (Joazeiro and Weissman 2000), we immunoprecipitated tau from HEK cells and included it as substrate in an *in vitro* ubiquitination assay (Fig. 3b), along with TRAF6 (E3), UbcH7 (E2) and various bacterially expressed GST ubiquitin constructs, wild-type as well as K29R, K48R, and K63R mutants. When K63 was mutated to R, *in vitro* ubiquitination was completely abrogated (Fig. 3b). Alternatively, when tau was included in the ubiquitination assay along with wild-type ubiquitin, or ubiquitin which can only generate K63 chains. Polyubiquitin chain synthesis with tau as a substrate only took place with wild-type ubiquitin and K63 chains (not shown). To map p62 interaction with tau, full-length wild-type (WT, amino acids 1–440) p62 or an N-terminal p62 deletion constructs (amino acids 1–229) or a C-terminal deletion construct (amino acids 385–440) was co-transfected into HEK cells along with HA-tagged ubiquitin. All constructs were expressed (Fig. 3c). Tau interaction with p62 was mapped to the UBA domain, as deletion of this region of p62 abrogated interaction with tau. Lysates recovered from the transfected cells (Fig. 3a) were employed in a GST-UBA pull-down assay to determine if polyubiquitinated tau could interact with the UBA domain of p62. Indeed, polyubiquitinated tau effectively interacted with p62's UBA domain (Fig. 3d), and inhibition of ubiquitination prevented tau interaction with p62, thus confirming the co-immunoprecipitation results (Fig. 3a).

To provide further evidence that tau is a TRAF6 substrate, we examined tau ubiquitination in brain lysates from TRAF6 knockout mice (Fig. 3e). Therein we observed that tau, while recovered as a polyubiquitinated protein in lysates from wild-type mice, was not polyubiquitinated in lysates recovered from the TRAF6 knockout mice. Altogether, these results reveal that tau is a K63 polyubiquitinated substrate of TRAF6 that interacts with the UBA domain of p62.

Polyubiquitinated tau is insoluble and fails to interact with microtubules

To examine the effects of tau ubiquitination on its solubility properties, HEK cells were co-transfected with tau, ubiquitin or the K63R ubiquitin mutant, followed by treatment with ALLN or not (Fig. 4a). Cells were lysed and the Triton soluble and insoluble fractions collected. Tau was observed to be polyubiquitinated, sensitive to ALLN treatment and localized to the insoluble fraction. Expression of K63R mutant ubiquitin abrogated tau polyubiquitination and its recovery in the insoluble fraction. Polyubiquitination of tau increased its association with p62, consistent with previous observations (Fig. 3a), with a concomitant decrease in tau's ability to interact with tubulin. These findings also suggested that tau may be degraded by the proteasome, as treatment with ALLN increased the amount of polyubquitianted tau captured in the immunoprecipitate. To confirm this possibility, HEK cells co-transfected with His-tau and ubiquitin were treated with either ALLN or MG132 to block proteasomal degradation, or chloroquine, a lysosomal inhibitor, and the accumulation of polyubiquitinated tau was examined (Fig. 4b). Treatment with either ALLN or MG132 blocked degradation and led to the accumulation of polyubiquitinated tau, whereas treatment with chloroquine was without effect. Longer exposure of the lysate blots revealed that tau was expressed in both the control and chloroquine transfected cells. However, both ALLN and MG132 treatment resulted in dramatic accumulation of tau protein, as well as, polyubiquitinated tau. These results thus demonstrate that tau is degraded through the ubiquitin–proteasome pathway.

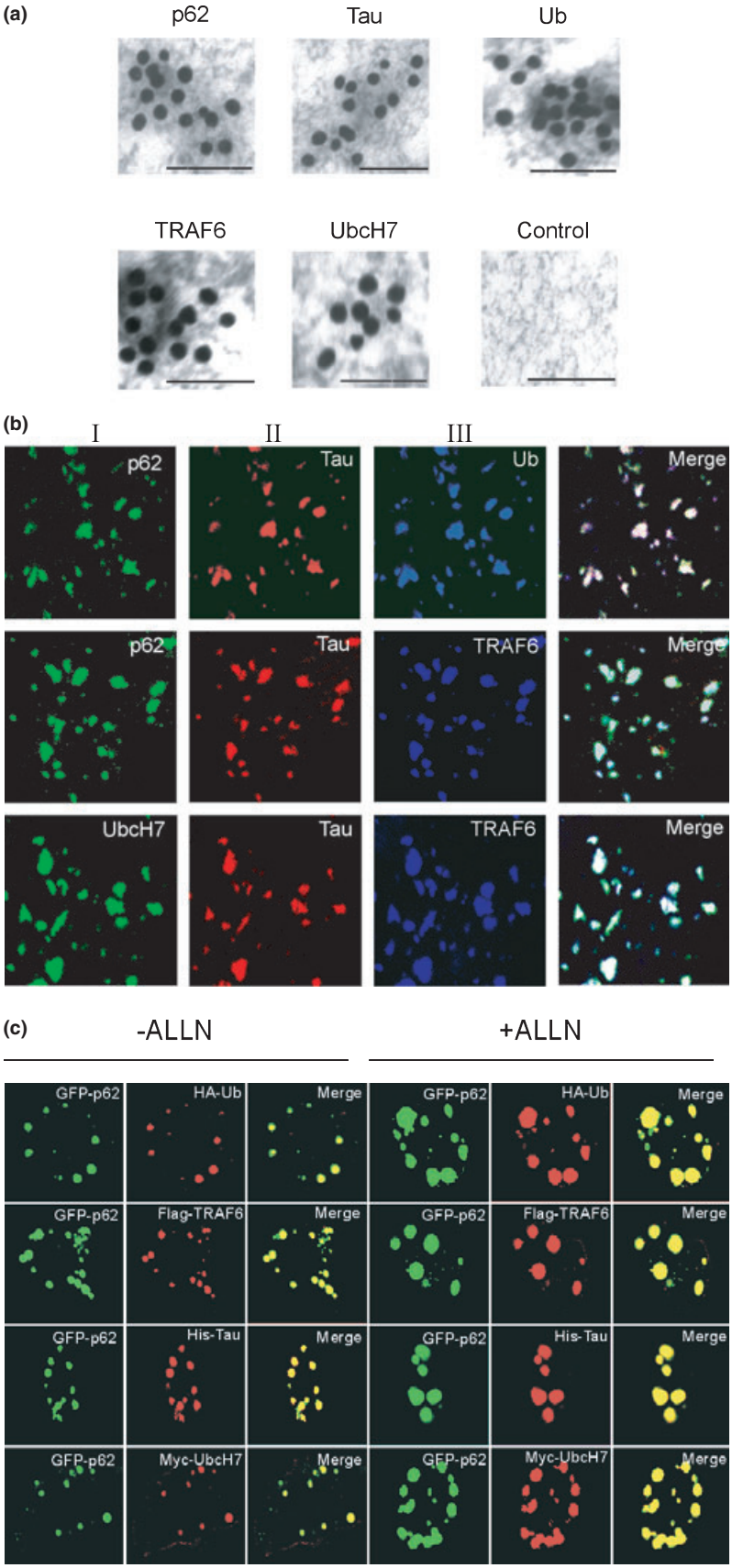

Fig. 2 Ultrastructural analysis of inclusion bodies isolated from AD brain. Immunoreactivity was analyzed by specific antibodies as shown and protein A coupled to 20 nm gold particles. Immunoreactivities are represented by solid particles. Controls were processed in the same manner, but with only protein A coupled to gold omitting the primary antibody. Scale bars =100 nm. (b) Co-localization of individual proteins in the inclusion preparation isolated from AD brain was determined by triple immunofluorescence staining and analyzed by using confocal microscopy. The inclusions were observed and photographed employing Texas Red filter (i), FITC filter (ii) or CY5 filter (iii) as shown. The merged image is shown in white. Inclusions were analyzed with specific antibodies as indicated. (c) HEK cells were co-transfected with GFP-p62 and tagged fusion constructs of TRAF6, tau, or UbcH7 as shown, followed by treatment or not with 50 μM ALLN for 12 h. Images were visualized by confocal microscopy after immunostaining with specific antibodies as shown. The merged image is shown in yellow.

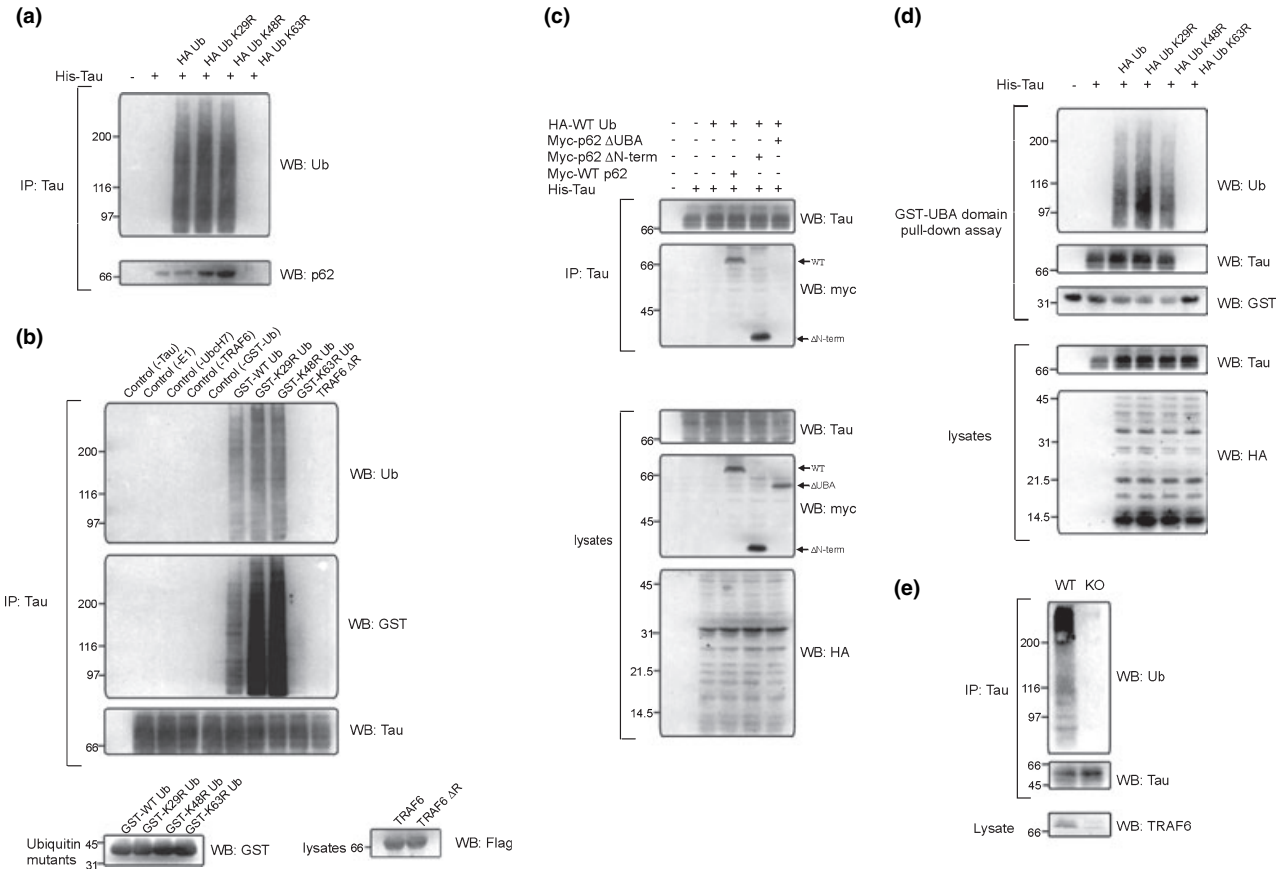

Fig. 3 Tau is K63-polyubiquinated by UBA domain of p62 (a) HEK cells were co-transfected with HA-tagged wild-type Ub, K29R, K48R or K63R point mutants of Ub along with His-tau. Cells were lysed in SDS lysis buffer and an equal concentration of lysate (750 μg) was immunoprecitated with tau antibody. The interaction was determined by western blot analysis with Ub and p62. Control expression blots are shown in (d). (b) To immunoprecipitated tau, an *in vitro* ubiquitination assay was carried out in the presence or absence of E1, UbcH7 (E2), TRAF6 (E3) along with GST-tagged wild-type Ub or K29R, K48R, K63R point-mutants of Ub. In the last lane, TRAF6ΔR (absence of RING domain) was used along with the essential components of ubiquitination machinery. The samples were subjected to western blot (WB) analysis with anti-Ub or anti-GST along with antibody for tau. The expression of GST-tagged Ub and TRAF6 were verified by blotting with GST and Flag-tag antibody. (c) HEK cells were transfected with His-tau, myc tagged wild type (WT) p62, p62-ΔN-term or p62-ΔUBA along with HA-Ub. The cells were lysed with triton lysis buffer and immunoprecipitated with tau and western blotted with myc tag and tau to map the interaction site of p62 with tau. The lysates were also western blotted with anti-tau, myc and HA to verify the expression of the constructs. (d) The lysates from (a) were also subjected to p62 GST-UBA domain pull-down assay. The interaction and ubiquitination of tau was analyzed by immunoblotting with anti-Ub, anti-tau or anti-GST antibody. The lysates were probed with tau or HA-tag antibody to quantitate the expression of the constructs. Each experiment was replicated three independent times with similar results. (e) Wild-type and TRAF6 knockout brain lysates were homogenized in SDS lysis buffer and tau was immunoprecipitated and western blotted with ubiquitin and tau antibody.

Tau is targeted for degradation by the proteasome

As tau is degraded by the ubiquitin–proteasome pathway (Fig. 4b) and p62 has previously been shown to serve as a shuttling factor for the delivery of K63-polyubiquitinated substrates to the proteasome (Seibenhener *et al*. 2004), we set out to assess the rate of tau turnover in control cells or cells with reduced p62 levels or enhanced p62 levels. This experimental paradigm would thus enable us to examine the necessity of p62 in regulation of tau turnover. Depletion of p62 levels by transfection of antisense (AS) p62 (Wooten *et al*. 2001; Seibenhener *et al*. 2004) retarded the turnover of tau (Fig. 5a). By comparison, p62 overexpression slightly enhanced the rate of turnover. Collectively these findings reveal that tau turnover is regulated by the expression of p62. The best-known function of protein ubiquitination is to target proteins for degradation by the 26 S proteasome (Madura 2002). However, K63 chains have been hypothesized to play a role aside from targeting proteins to the proteasome (Pickart 2001). To test whether the K63 polyubiquitin signal was necessary for tau degradation, the

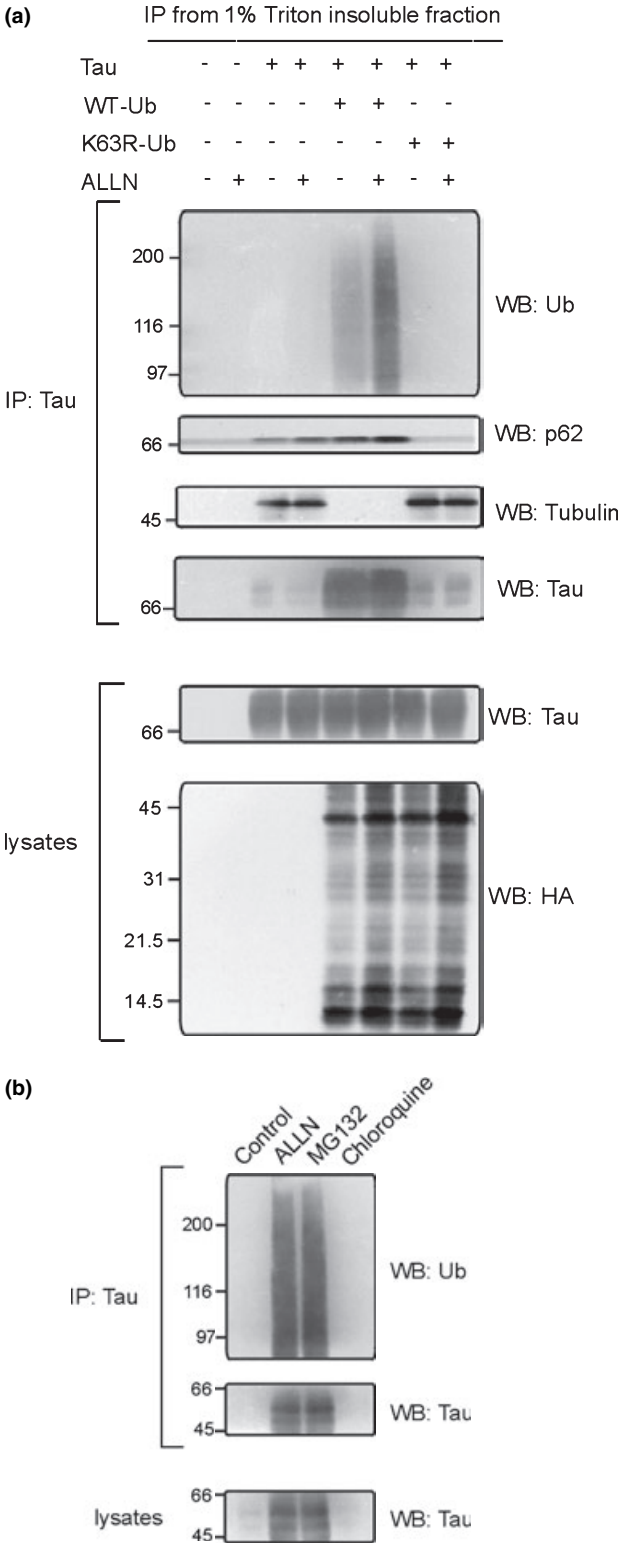

Fig. 4 Polyubiquitinated tau is insoluble and degraded by the proteasome. (a) HEK cells were transfected with His-tau, along with WT-Ub or K63R ubiquitin mutant. The transfected cells were incubated with or without ALLN (50 μM) for 12 h. The cells were lysed with triton lysis buffer containing 1% Triton X-100 and the supernatant and pellets are separated by centrifugation. The supernatant was referred to as 1% Triton X-100 soluble fraction and the pellet was referred as to 1% Triton X-100 insoluble fraction. The insoluble fraction was re-suspended in lysis buffer and immunoprecipitated with tau antibody, separated by SDS–PAGE and blotted with the Ub, p62 or tubulin antibodies. The transfected cell lysate (50 μg) was blotted with tau or HA antibody to check the expression of the constructs. (b) HEK cells were transfected with His-tau and treated with ALLN (50 μM), MG132 (5 μM), or chloroquine (150 μM) for 24 h prior to harvest. The cells were lysed with SDS lysis buffer and 750 μg protein was immunoprecipitated with tau antibody followed by western blotting with ubiquitin as shown. The lysates (30 μg) were also blotted with antibody to tau. Shown are the findings from one representative experiment, which was conducted in triplicate with similar results.

cells were transfected with K63R-ubiquitin and the turnover of tau was assessed. Expression of the mutant ubiquitin construct impaired the turnover of tau (Fig. 5a), thereby demonstrating a requirement for the chains in directing proteasomal degradation of tau. A complementary *in vitro* approach was undertaken to confirm this observation (Fig. 5b). Tau was first ubiquitinylated in an *in vitro* reaction with K63 ubiquitin. The protein complex containing polyubiquitinated tau captured on beads was washed extensively to remove trace SDS and then incubated with 26 S proteasome supplemented with $MgCl_2$ and ATP. The polyubiquitinated chains disappeared after incubation with purified 26 S proteasome and tau levels likewise diminished to the level of control untransfected cells (Fig. 5b). Omission of ATP in the reaction mix blocked the degrading activity of the 26 S proteasome (not shown). To verify that the observed disappearance of polyubiquitinated chains was as a result of degradation, a deubiquitination inhibitor, Ub-H (Nishikawa *et al*. 2004), was added to the reaction. Polyubiquitin degradation was inhibited by the presence of Ub-H. These results confirm that K63 polyubiquitinated tau is de-ubiquitinated by the 26 S proteasome. To test whether tau directly associated with the proteasome *in vitro*, tau was immunoprecipitated and western blotted with antibody to Rpt1, an ATPase component of the 19 S proteasome regulatory subunit (Elsasser *et al*. 2002); tau was observed to co-interact with Rpt1 (Fig. 5b, right panel). Recently, the p62 gene has been disrupted in mice (Duran *et al*. 2004). Moreover, we have shown that p62 co-localizes with, and co-interacts with, the proteasomal subunit Rpt1 (Seibenhener *et al*. 2004). Brain lysates prepared from wild-type or p62 knockout mice were immunoprecipitated for tau and western blotted for Rpt1 to examine p62's requirement for tau interaction with the proteasome. In immunoprecipitates from brain lysates of wild-type mice we could detect robust interaction of tau with the proteasome, whereas in immunoprecipitates conducted from lysates recovered from the p62 knockout mice we failed to detect interaction of tau with the proteasome (Fig. 5c). Altogether, these findings

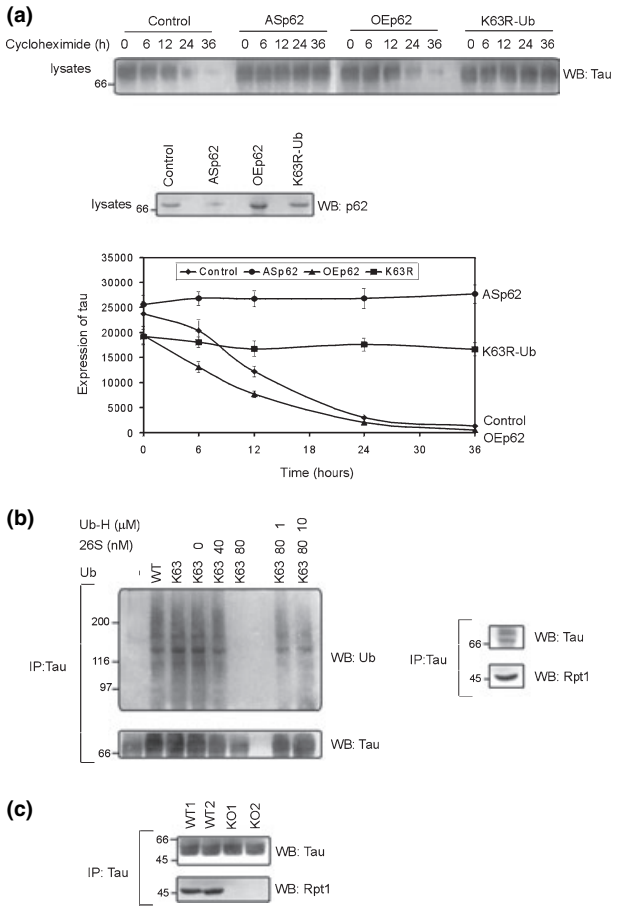

Fig. 5 Requirement for p62 in targeting tau for proteasomal degradation. (a) HEK cells were transfected with His-tau, antisense (AS) p62, myc-p62 (overexpressed, OE) or K63R Ub mutant. After 24 h transfection, the cells were treated with 20 μg/mL of cycloheximide for different times as shown at 37°C. The cells were lysed with Triton lysis buffer and equal concentrations of protein (30 μg) were western blotted with tau antibody. The whole cell lysates (50 μg) were also immunoblotted to check the expression of p62. The blot was scanned and quantitated for tau expression. The experiment was replicated three times, shown are the mean ± SEM for each blot. (b) HEK293 cells were transfected with His-tau construct and lysed in SDS lysis buffer. Tau was immunoprecipitated and an *in vitro* ubiquitination assay was conducted with E1, UbcH7 (E2), and TRAF6 (E3) along with wild-type or K63 ubiquitin. To this polyubiquitinated tau complex, 26 S proteasome or Ub-H was added for 2 h at 37°C. The samples were western blotted with anti-ubiquitin and anti-tau. Right – the *in vitro* K63-polyubiquitinated tau was incubated with 10 nM of 26 S proteasome for 30 min at 4°C. The interaction of tau with proteasome was analyzed by western blotting with tau and Rpt1 antibody. (c) Lysates (1 mg) prepared from wild-type (WT) or p62 knockout (KO) mouse brain were immunoprecipitated with antibody to tau and western blotted with antibody to Rpt1 or tau as shown.

demonstrate that K63-polyubiquitinated tau is degraded by the ubiquitin–proteasome pathway and that p62 is necessary for shuttling of tau for proteasomal degradation.

To further examine tau co-interaction with the proteasome, we took advantage of NGF's ability to stimulate tau polyubiquitination. In the course of these studies we conducted a time course to examine NGF-stimulated polyubiquitination of tau (not shown). We reasoned whether p62 were necessary for targeting of tau interaction with the proteasome that diminished p62 levels, or if inhibition of K63 polyubiquitination might impair this process. To test this idea, PC12 cells were first transfected with nothing, antisense (AS) p62 (Wooten *et al*. 2001), K63R or K48R ubiquitin mutants followed by NGF-stimulation and immunoprecipitation of tau and western blot for Rpt1. We observed NGF-stimulated polyubiquitination of tau, along with robust interaction of tau with Rpt1 (Fig. 6a). Decreased expression of p62 completely abrogated tau interaction with the proteasomal subunit Rpt1. In addition, impaired tau polyubiquitination by expression of the K63R mutant of ubiquitin, but not K48R, likewise prevented tau interaction with the proteasome (Fig. 6a). To test the effects of proteasomal inhibition on tau aggregation and cell survival, PC12 cells were stimulated with NGF followed by treatment with MG132 to block proteasome activity (Fig. 6b). Inhibition of proteasomal activity correlated with the presence of tau aggregates that also stained positive for ubiquitin (not shown) along with decreased cell survival.

Discussion

We demonstrate herein that the ubiquitin binding protein p62 associates with polyubiquitinated tau both in aggregates obtained from AD brain and in two cellular models: HEK cells transfected with p62 and NGF-treated PC12 cells. There has been considerable debate as to whether aggregates are toxic (Ciechanover and Brundin 2003), or whether aggregates represent the cell's attempt to rid itself of misfolded proteins and are hence beneficial. We observed that tau–ubiquitin aggregates are cytotoxic under proteasome-impaired conditions. Thus, a critical factor that determines the toxicity of an aggregate is the functional state of the proteasome. In this regard, the rate of polyubiquitin substrate turnover is dependent upon efficient functioning of the proteasome. Once small aggregates form they may contribute further to decreased proteasome function, as aggregates have also been shown to negatively affect the activity and function of the proteasome (Bence *et al*. 2001). Alternatively, protein aggregates may lead to global impairment of UPS function independent of sequestration of aggregates into inclusion bodies (Bennett *et al*. 2005). Since p62 plays a critical role in the delivery of substrates to the proteasome and targeting of proteins for degradation (Seibenhener *et al*. 2004), we speculate that decline in p62 expression might increase a cell's susceptibility to cell death and could promote accumulation of aggregated polyubiquitinated tau. With the generation of p62 knockout mice

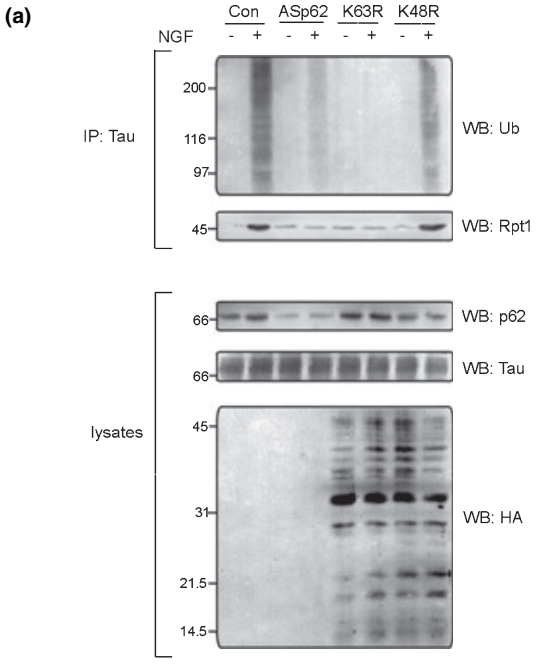

Fig. 6 Removal of p62 blocks interaction with the proteasome. (a) PC12 cells were transfected with ASp62, HA-K63R or HA-K48R mutant ubiquitin constructs. The cells were treated with NGF (50 ng/mL) for 30 min or not and lysed with Triton lysis buffer. The lysates (750 μg) were immunoprecipitated with tau antibody and western blotted with ubiquitin and Rpt1 antibody. The expression of p62 and ubiquitin constructs was determined by western blotting with p62, tau and HA tag antibody. Shown are the results of one experiment, which was conducted three independent times. (b) Cells were treated with 50 ng/mL of NGF. After 24 h MG132 (25 μM) was added or not to the cells for 24 h. Left-hand graph, stained for tau and 300 cells were scored for the presence of tau aggregates or not. Right-hand graph, survival was measured by MTS assay.

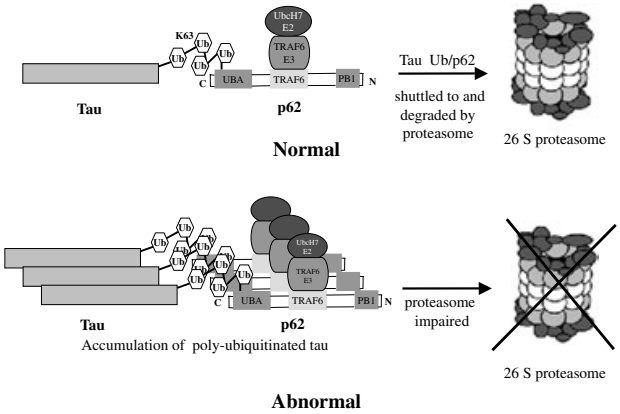

Fig. 7 Schematic representation of p62/tau proteasomal shuttling. K63-polyubiquitinated tau interacts with p62's UBA domain and is targeted by the N-terminus of p62 for proteasomal degradation. Inhibition of proteasome activity results in accumulation of polyubiquitinated tau aggregates and cell death. Alternatively, a decline in p62 expression may impair the rate of polyubiquitinated tau degradation and might likewise result in an accumulation of polyubiquitinated tau.

(Duran *et al*. 2004), we are currently addressing these questions.

Recently, two groups have reported that tau is K63 polyubiquitinated by the E3-CHIP (Petrucelli *et al*. 2004; Shimura *et al*. 2004). It is possible that tau may be the substrate of more than one E3. However, our findings reveal that TRAF6 is a major E3 involved in the ubiquitination of tau *in vivo*, as tau polyubiquitination was completely abrogated in the absence of TRAF6. Inhibition of tau polyubiquitination did not lead to significant accumulation of tau in the TRAF6 knockout brain, thus suggesting that non-ubiquitinated tau might be degraded by the lysosome. Analysis of PHF-tau from AD brain detected the presence of K48-linked Ub chains (Morishima-Kawashima *et al*. 1993). Given the increase in sensitivity of mass spectrometry analysis over the past 10 years, it is possible that they may have failed to detect the K63-linkage. Studies are underway to re-examine the type of chain employed in the PHF-tau isolated from AD brain, as well as to map the site of K63 Ub within tau. Polyubiquitination also appears to control the partitioning of tau between soluble and insoluble fractions, as well as its ability to interact with microtubules. In this regard, it is worth noting that endogenous tau is polyubiquitinated at sites that correspond to the microtubule interaction domain (Morishima-Kawashima *et al*. 1993), which confirms our observation that inhibition of polyubiquitination resulted in microtubule interaction.

We observe a novel role for p62 in shuttling of tau for proteasomal degradation, via interaction of the K63 polyubiquitin chains of tau with the UBA domain of p62. This observation is consistent with p62's ability to selectively interact with K63-polyubiquitinated substrates and its N-terminus to interact with the proteasome (Seibenhener *et al*. 2004). It is generally accepted that K48 chains of ubiquitin provide a signal for sorting and degradation of target substrates by the proteasome (Madura 2002); however, K63 polyubiquitin chains have not yet been shown to directly mediate proteasomal degradation (Pickart 2001). In this regard, our study is the first to clearly reveal a degrading function for the K63 chains of a substrate. These findings are consistent with an earlier report that tau is degraded by the

proteasome (David *et al*. 2002). Interestingly, Hofmann and Pickart 2001) initially reported that K63 chains were as efficiently degraded by the proteasome compared with K48 chains. Thus, we confirm this observation with a specific substrate, K63-polyubiquitinated tau. It has been postulated that different types of polyubiquitin chains (K63 vs. K48) serve different intracellular targeting functions. However, an alternative possibility is that the chains are recognized by targeting the proteins that direct their intracellular localization. Therefore, our findings open the door for reinterpretation of the role that different polyubiquitin chains may play in directing the trafficking of substrates to the proteasome for degradation. Therefore, it is possible that all seven types of polyubiquitinated chains (K6, 11, 27, 29, 33, 48, 63) utilized in substrate ubiquitination (Peng *et al*. 2003) may be recognized by shuttling factors through their respective UBA domains

The major histopathological brain lesions in AD are NFTs consisting of aggregated hyperphosphorylated tau and, the decline in cognitive function correlates with the presence of NFTs (Johnson and Bailey 2003). The activity of the proteasome is decreased during the course of AD (Keller *et al*. 2000) either as a direct or indirect result of the neurotoxic amyloid beta peptide ($A\beta_{1-42}$) binding to the proteasome to block activity (Gregori *et al*. 1995; Lam *et al*. 2000). Recent studies on immunotherapeutic clearance of tau aggregates in a mouse model of AD reveal a requirement for the proteasome (Oddo *et al*. 2004). For this therapy to be effective in humans, our results suggest that one must also evaluate the expression of p62 as a critical determinant for tau clearance. If p62 levels are below a critical threshold, we predict that tau clearance may not occur and that tau may accumulate in the form of aggregated insoluble polyubiquitinated protein. These possibilities are currently being tested in brain of the p62 knockout mice.

In summary, tau is K63-polyubiquitinated and shuttled for degradation to the proteasome by p62 (Fig. 7). From these studies it is clear that tau degradation and aggregation are tightly coupled. Small changes in p62 expression could lead to disturbances in tau degradation, accumulation and aggregation. Further studies of tau degradation/aggregation in a genetic background lacking p62 will yield important insights into p62's role in regulating tau shuttling.

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