

Essential Role of Sequestosome 1/p62 in Regulating Accumulation of Lys⁶³-ubiquitinated Proteins*

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Sequestosome 1 (SQSTM1)/p62 is an interacting partner of the atypical protein kinase C ζ/ι and serves as a scaffold for cell signaling and ubiquitin binding, which is critical for several cell functions *in vivo* such as osteoclastogenesis, adipogenesis, and T cell activation. Here we report that in neurons of p62^{-/-} mouse brain there is a detectable increase in ubiquitin staining paralleled by accumulation of insoluble ubiquitinated proteins. The absolute amount of each ubiquitin chain linkage measured by quantitative mass spectrometry demonstrated hyperaccumulation of Lys⁶³ chains in the insoluble fraction recovered from the brain of p62^{-/-} mice, which correlated with increased levels of Lys⁶³-ubiquitinated TrkA receptor. The increase in Lys⁶³ chains was attributed in part to diminished activity of the TRAF6-interacting the Lys⁶³-deubiquitinating enzyme (DUB), cylindromatosis tumor suppressor (CYLD). The interaction of CYLD with TRAF6 was dependent upon p62, thus defining a mechanism that accounts for decreased activity of CYLD in the absence of p62. These findings reveal that p62 serves as an adapter for the formation of this complex, thereby regulating the DUB activity of CYLD by TRAF6 interaction. Thus, p62 has a bifunctional role in regulation of an E3 ubiquitin-protein ligase, TRAF6, and a DUB, CYLD, to balance the turnover of Lys⁶³-polyubiquitinated proteins such as TrkA.

Sequestosome 1/p62 was cloned as the interacting partner of the atypical protein kinase C (1) and has been shown to contain several motifs that enable the protein to serve as a signaling scaffold (2). The C-terminal ubiquitin-associating (UBA)² domain of p62 has been shown to interact with polyubiquitinated proteins and, upon overexpression, to promote the for-

mation of ubiquitin-containing inclusions (3), whereas a UBA-deleted mutant, p62 Δ UBA, acts as a dominant negative for that function. Interestingly, p62-containing, ubiquitin-rich inclusions have been observed in surviving cells and may therefore play a role in the protection of cells from the toxicity of misfolded proteins (3–5). An attractive hypothesis would be that the UBA domain of p62 may have a role in sequestering ubiquitinated proteins to cytoplasmic inclusion bodies. However, there is limited information about the role that p62 could have in the formation of inclusions in, for example, the brain, which would be relevant to understanding its role in the initiation or progression of neurodegenerative diseases. Therefore, studies in animal models or genetically inactivated p62 would be instrumental for the establishment of such a potential novel role for p62. Interestingly, p62 has been demonstrated to be critically involved in the control of receptor trafficking, specifically of the neurotrophin receptor family, tropomyosin-related kinase (Trk) A, B, and C (7, 8). We have shown that TrkA ubiquitination is essential for neurotrophin-dependent receptor internalization, cell differentiation, and signaling (8). In this regard, the fact that p62 interacts with all members of the Trk family in a ligand-dependent manner (7) suggests that p62 may serve a common and conserved function in the neurotrophin signaling cascades. Importantly, there is a growing realization that the receptor trafficking pathways and pathways whereby inclusion bodies form, a hallmark of neurodegenerative diseases, share overlapping constituents (8). Here, we have established, using mice with genetic inactivation of p62, that this protein plays a critical role in regulating the turnover of lysine 63-ubiquitinated proteins, providing a new perspective on the role of this protein in its function as an adapter.

EXPERIMENTAL PROCEDURES

Materials—Mouse TrkA (B-3), ubiquitin, HA, CYLD (cylindromatosis tumor suppressor), Myc, and rabbit Trk (C-14) were from Santa Cruz Biotechnology, San Diego, CA. FLAG and tubulin antibodies were obtained from Sigma. 2.5 S nerve growth factor (NGF) was from Bioproducts for Science (Indianapolis, IN).

Animal Model—Knock-out mice (p62^{-/-}) were generated as described previously (9). All animals employed in this study were handled according to the Auburn University Institutional Animal Care and Use Committee, which abides by National Institutes of Health guidelines.

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² The abbreviations used are: UBA, ubiquitin-associating; Ub, ubiquitin; WT, wild type; Trk, tropomyosin-related kinase; FA, formic acid; NGF, nerve growth factor; DUB, deubiquitinating enzyme; HEK, human embryonic kidney; PC12, pheochromocytoma; PGPH, peptidyl glytamy peptide-hydrolyzing; CYLD, cylindromatosis tumor suppressor; TRAF6, tumor necrosis receptor-associated factor 6; RIPA, radioimmunoprecipitation assay; GST, glutathione S-transferase; HA, hemagglutinin.

Regulation of E3 Ubiquitin-Protein Ligase/DUB Activity by p62

Biochemical Fractionation—Soluble and insoluble fractions (RAB, RIPA, and formic acid (FA)) were prepared as described (10). Protein was determined using the DC protein assay (Bio-Rad Laboratories). For co-interaction/-immunoprecipitation, the cells were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 2 mg/ml leupeptin and aprotinin (3, 8). For the immunoprecipitation of ubiquitin conjugates, 1% SDS was added to the lysis buffer. The samples were separated by SDS-PAGE and Western blotted with appropriate antibodies.

Ubiquitin (Ub)-AQUA (Absolute Quantification of Proteins) Analysis—For mass spectrometry analysis, the FA fraction from wild type (WT) and p62^{-/-} brain was neutralized with Tris-HCl, pH 8.0 (10), and solubilized in RIPA buffer. Total ubiquitinated proteins or Trk receptors (C-14 antibody) were immunopurified and resolved by SDS-PAGE. Duplicate samples were processed for silver staining and Western blot. The gel region containing ubiquitin or Ub-TrkA (B-3) was aligned with the blot, excised, and digested using 15 ng/ml trypsin. Heavy isotope-labeled internal peptides corresponding to all seven types of human poly-Ub linkages were synthesized and quantified by amino acid analysis (Cell Signaling Technology, Inc., Beverly, MA). After in-gel digestion, the sample and heavy isotope-labeled internal standard were analyzed by LCQ-DECA XP ion mass spectrometer. This included analysis of di-glycine-tagged signature peptides at Lys⁶, Lys¹¹, Lys²⁷, Lys²⁹, Lys³³, Lys⁴⁸, and Lys⁶³. All seven linkages were quantified simultaneously in the same run as described (11). The ubiquitin chains were quantified by the ratio between endogenous peptide *versus* the internal standard. Each sample was quantified three times to obtain the relative standard deviation in measurements.

Proteasome Assay—The peptidyl glutamyl peptide-hydrolyzing activity (PGPH) activity of the proteasome was measured with benzyloxycarbonyl-Leu-Leu-Glu-7-amido-4-methylcoumarin (Z-LLE-AMC), a specific fluorogenic peptide substrate (12). The subunit composition of the proteasome was analyzed by Western blotting with antibodies to the 20 S subunits β 1, β 2, and β 5 (Biomol International, Plymouth Meeting, PA).

Histochemistry—The brains were dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Each brain was processed and embedded in paraffin; 5- μ m sections were cut for immunohistochemical staining with antibody to ubiquitin or TrkA (13) and processed employing the Histostain DS kit from Zymed Laboratories, San Francisco, CA.

Cell Culture and Transfection—Human embryonic kidney 293 (HEK) cells and pheochromocytoma (PC12) cells were grown as described (7, 8). HEK 293 cells were transfected employing a calcium phosphate method using the mammalian cell transfection kit (Chemicon Int., Temecula, CA) and PC12 cells using Lipofectamine 2000 (Invitrogen).

NGF Internalization—Iodinated NGF was prepared, and NGF internalization was examined by the acid wash technique (7). Specific binding was calculated in the presence or absence of cold NGF.

Deubiquitinating Enzyme (DUB) Assay—Lys⁶³ chains were kindly provided by Cecile Pickart, Johns Hopkins University. The DUB assay was conducted as described (14). In brief, CYLD

was immunoprecipitated from 2 mg of 6-month-old WT and p62^{-/-} brain homogenate prepared in 25 mM Tris, pH 7.2, 100 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, 10 mM *N*-ethylmaleimide, and protease inhibitors and collected with agarose-coupled secondary antibody. The DUB activity of CYLD was measured in a 50- μ l assay (DUB buffer: 50 mM HEPES, 0.5 mM EDTA, 100 μ g/ml bovine serum albumin, and 1 mM dithiothreitol) along with 1 ng of Lys⁶³ ubiquitin chains, with continuous shaking at 37 °C for 4 h, and then washing three times with DUB buffer. The chains were released by boiling in SDS-PAGE sample buffer, electrophoresed on 12% SDS-PAGE, and Western blotted with anti-ubiquitin and anti-CYLD.

GST-UBA Pulldown Assay—JM109 *Escherichia coli* cells were induced with isopropyl 1-thio- β -D-galactopyranoside and lysed in NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The GST-tagged UBA domain of p62 was bound to glutathione-agarose overnight at 4 °C followed by washing five times with NETN buffer (3). The purity of the preparation was validated by 12% SDS-PAGE, Coomassie blue staining, and/or Western blot analysis with GST antibody. The GST-UBA domain captured on agarose beads was washed three times with binding buffer prior to use in an interaction assay. Five μ g of GST-UBA domain was added to 750 μ g of PC12 cell lysate and rotated for 2 h. The beads were washed three times with binding buffer, and SDS-PAGE sample buffer was added and analyzed by 7.5% SDS-PAGE followed by immunoblotting with HA antibody to detect bound ubiquitinated TrkA.

Experimental Design—Unless otherwise noted, all experiments employing brain lysates were conducted on WT and p62^{-/-} mice 6 months of age. All experiments were repeated at least three times, and statistical significance was tested employing Student's *t* test.

RESULTS

Ubiquitin Accumulates in p62^{-/-} Brain—To analyze the potential role of p62 in the accumulation of ubiquitinated proteins in brain, sections of brain from WT and p62^{-/-} mice were immunostained with anti-ubiquitin antibody (Fig. 1A). Light microscopic analysis failed to detect any plaques, aggregates, or inclusions in neurons localized to the thalamus, hippocampus, cortex or amygdala of p62^{-/-} mouse brain taken at 6 months of age or at later ages (up to 18 months; not shown). However, prominent staining of ubiquitin in the cell body and axons was noted. The solubility of polyubiquitin was examined by sequential extraction in high salt buffer (RAB) without and with detergent (RIPA). Finally, the RIPA-insoluble material was subjected to extraction with FA. This differential extraction method is often used as a quantitative measure of protein aggregation (10), where the solubility of the aggregated insoluble protein shifts to the FA fraction. When lysates from WT and p62^{-/-} brain were extracted in this manner, a shift in the solubility of the extracted ubiquitinated proteins from the soluble to the highly insoluble FA fraction was observed in p62^{-/-} samples compared with those from WT mice (Fig. 1B). To better understand the timed sequential nature of this observation,

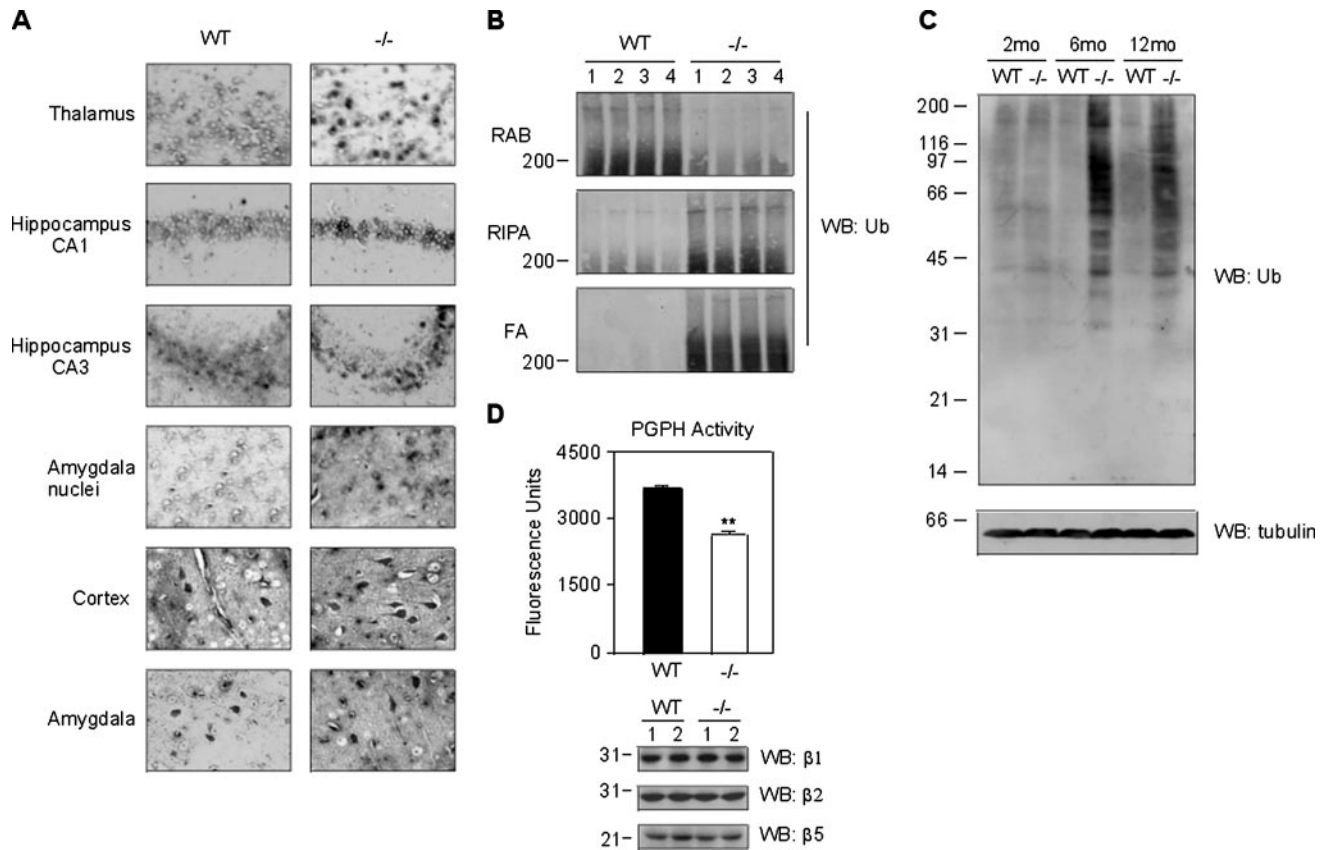


FIGURE 1. Deletion of p62 promotes accumulation of insoluble polyubiquitin. *A*, paraffin sections from WT or p62^{-/-} mouse brain were stained with antibody to ubiquitin and observed by light microscopy as shown ($\times 60$ magnification). *B*, mouse brain lysates (50 μ g) from four individual mice, WT or p62^{-/-}, were extracted sequentially with RAB, RIPA, and FA and immunoblotted with antibody to Ub. *C*, mouse brain lysate (50 μ g) from 2-, 6-, and 12-month-old WT or p62^{-/-} was sequentially extracted with RAB, RIPA, and FA. The resulting FA fraction (50 μ g) was immunoblotted with antibody to Ub or tubulin. *D*, proteasome activity was assayed in brain lysates prepared from WT or p62^{-/-} mice (p62^{-/-} < WT; mean \pm S.E., **, $p < 0.05$). 20 S proteasome subunits were examined by Western blotting (WB) with antibody to $\beta 1$, $\beta 2$, and $\beta 5$.

FA fractions isolated from 2-, 6-, and 12-month-old animals reveal that the p62^{-/-} mice did not accumulate significant amounts of polyubiquitin until 6 months of age (Fig. 1C). Because the proteasome may be overloaded by the accumulation of polyubiquitin-containing aggregates (15), the activity of the proteasome was tested by measuring PGPH activity (12). Compared with WT, lysates from p62^{-/-} brain exhibited a slight, but significant, decline in PGPH activity; however, no differences were observed in the activity of trypsin or chymotrypsin (not shown) or in the expression of proteasome subunits (Fig. 1D). No significant differences were observed in proteasome activity recovered from mice up to 12 months of age (not shown). Because such a distinct pattern of polyubiquitin accumulation was observed in the FA fraction from the p62^{-/-} mice, we wondered whether there was a selective increase in a particular type ubiquitin chain. To address this question, quantitative mass spectrometry of ubiquitin revealed selective hyperaccumulation of Lys⁶³-linked polyubiquitin chains in the samples recovered from p62^{-/-} brain compared with WT (Fig. 2). To account for the selective increase in these chains, we reasoned that these chains might accumulate due to inhibition of an ubiquitin C-terminal hydrolase-L1 (UCH-L1). This enzyme has been shown to colocalize with p62 in cultured cells (16). However,

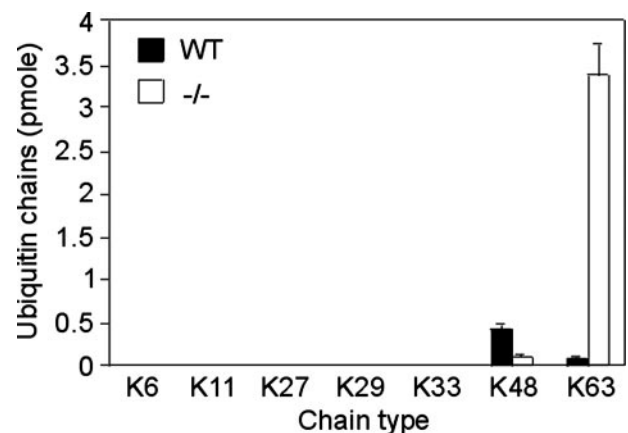


FIGURE 2. Hyperaccumulation of Lys⁶³-ubiquitin chains in p62^{-/-} brain lysates. Absolute amounts (pmol) of ubiquitin chain linkage were measured in FA samples from WT or p62^{-/-} brain by quantitative mass spectrometry and expressed as mean \pm relative standard deviation.

analysis of UCH-L1 activity (17) in brain from WT and p62^{-/-} mice revealed no significant differences.³

Removal of p62 Results in Impaired Trafficking of Neurotrophin Receptors—Trk family members (A, B, and C) are ubiquitinated in a ligand-dependent manner (7), and interaction with

³ L. Yin, M. W. Wooten, and K. Wilkinson, unpublished observation.

Regulation of E3 Ubiquitin-Protein Ligase/DUB Activity by p62

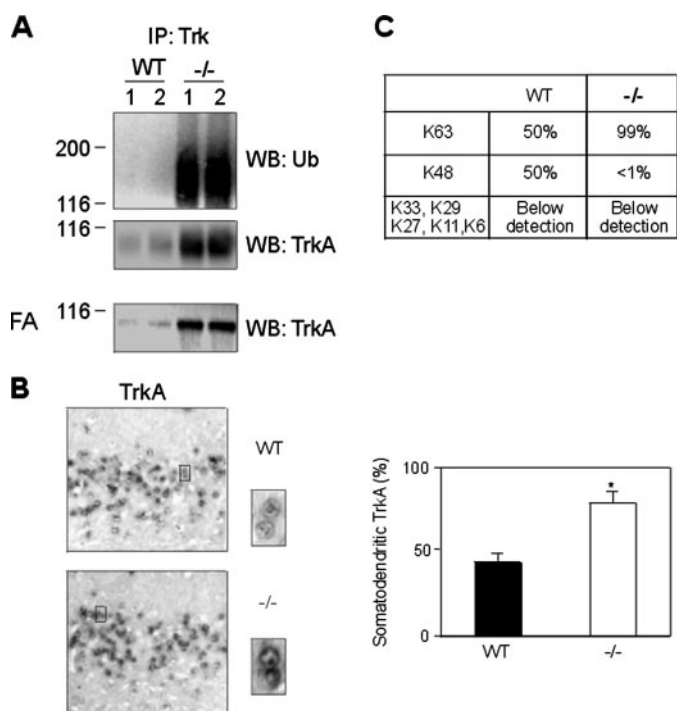


FIGURE 3. Accumulation of Lys⁶³-ubiquitinated TrkA in p62^{-/-} mouse brain. A, lysates (1 mg) from the brain of two mice each, WT and p62^{-/-}, were differentially extracted in RAB, RIPA, and FA. Members of the Trk family (A, B, and C) were immunoprecipitated (IP) with C-14 antibody and Western blotted (WB) for TrkA (B-3) and ubiquitin. A fraction of the lysate (50 μg) was blotted for TrkA with B-3 antibody. B, matched paraffin sections from WT and p62^{-/-} mouse brain was stained with TrkA antibody (B-3) and viewed at ×20. Inset: ×100. Three hundred pyramidal neurons were counted, and the presence or absence of somatodendritic TrkA was scored and covered to mean percentage ± S.D. (*, *p* < 0.05). C, Trks were immunopurified from the FA fraction of WT and p62^{-/-} mouse brain. The distribution of ubiquitin between WT and p62^{-/-} was expressed as the percentage of total ubiquitin (pmol).

p62 is necessary for TrkA signaling, internalization, and differentiation through a TRAF6-dependent mechanism (8). TRAF6 is a signaling adapter that plays an important role in the Lys⁶³ polyubiquitination of several signaling proteins (2). In light of these findings, together with the fact that the loss of p62 promotes the accumulation of Lys⁶³-polyubiquitinated proteins (Fig. 2), we reasoned that an absence of p62 may lead to misdirection of TrkA and its accumulation. To test this idea, we subjected brain lysates to sequential detergent/acid extraction (10). The resulting insoluble material was immunoprecipitated with a pan-Trk antibody that recognizes Trk A, B, and C receptors. Ubiquitinated TrkA receptors accumulated in the FA fraction of p62^{-/-} brain but not of WT (Fig. 3A). We next examined the immunostaining pattern of TrkA in WT and p62^{-/-} brain sections (Fig. 3B). The staining of TrkA in pyramidal neurons from p62^{-/-} mice was largely confined to the somatodendritic compartment (Fig. 3B, inset). Quantitative analysis of TrkA staining confirmed this observation was significant (Fig. 3B). This distinct localization pattern in p62^{-/-} neurons suggest that p62 may affect TrkA trafficking. Analysis of immunopurified Trk by mass spectrometry revealed equal distribution of Lys⁶³- and Lys⁴⁸-linked ubiquitin chains associated with the receptor in samples from WT mice. However, Trk receptor recovered from p62^{-/-} brain was predominantly of the Lys⁶³ form (Fig. 3C).

consistent with hyperaccumulation of Lys⁶³-ubiquitinated proteins in the p62^{-/-} brain (Fig. 2). These findings validated earlier studies employing cotransfection of tagged ubiquitin mutants (8), which show that TrkA is Lys⁶³-ubiquitinated and reveal a novel role for p62 in the control of the TrkA polyubiquitination state under basal conditions *in vivo*.

The NGF-stimulated Lys⁶³-ubiquitination site in TrkA has been mapped to Lys⁴⁸⁵ (8). Mutation of this site in TrkA impairs NGF-dependent ubiquitination, internalization, and signaling (8). Thus, we sought to determine whether the ubiquitinated receptor would interact with p62. Co-expression studies conducted in HEK cells revealed that the K485R mutation blocked interaction of p62 with TrkA (Fig. 4A). These findings suggest that ubiquitinated TrkA interacts with p62. To gain insight into the structural interactions between TrkA and p62, the domain of p62 necessary for interaction with TrkA was mapped by undertaking coexpression studies between full-length and either N-terminal or C-terminal UBA deletion mutants of p62 and TrkA (Fig. 4B). Expression of a p62 mutant lacking its UBA domain was defective in TrkA interaction. To further validate that the UBA domain of p62 binds Lys⁶³-polyubiquitinated TrkA, various Lys to Arg mutants of ubiquitin (Lys²⁹, Lys⁴⁸, Lys⁶³) were expressed in PC12 cells followed by immunoprecipitation of either the endogenous receptor or of the tagged ubiquitin, HA (Fig. 4C). Results from this experiment show that expression of K63R ubiquitin mutant exerts a dominant-negative effect on TrkA interaction with p62. A parallel experiment was conducted wherein the interaction of ubiquitinated TrkA with the UBA domain of p62 was examined in a pulldown assay. Mutation at either Lys²⁹ or Lys⁴⁸ did not inhibit interaction of polyubiquitin with the p62 UBA domain, whereas K63R completely blocked interaction with the UBA domain (Fig. 4D). Collectively, these findings reveal Lys⁶³-polyubiquitinated TrkA interacts with the UBA domain of p62. We reasoned that if TrkA interaction with the UBA domain of p62 were needed for NGF internalization, the overexpression of a construct lacking the UBA domain might exert a dominant negative effect. Evaluation of NGF internalization revealed that ligand uptake was severely reduced in cells expressing the UBA deletion of p62 (Fig. 4E). Therefore, interaction of ubiquitinated TrkA with the UBA domain of p62 is necessary for TrkA-NGF internalization. Thus, an absence of p62 could lead to accumulation of Lys⁶³-ubiquitinated TrkA.

Association of p62 with a Lys⁶³-DUB, CYLD, Regulates Its Activity—We have previously shown that p62 serves as a scaffold for the recruitment of the E3 ubiquitin-protein ligase, TRAF6, leading to TrkA Lys⁶³ ubiquitination (8). Those observations would be in apparent contradiction with the accumulation of Lys⁶³-ubiquitinated TrkA, a TRAF6 substrate (8), in p62^{-/-} brain. An attractive hypothesis that could explain these apparent paradoxical sets of data would be that p62, in addition to its well established role in the activation of TRAF6, might also regulate a DUB enzyme with specificity for Lys⁶³ chains. Intriguingly, CYLD encodes a DUB with specificity toward proteins ubiquitinated through Lys⁶³-linked ubiquitin chains (14) and has been shown to interact with TRAF6. Therefore, we first examined CYLD DUB activity in brain lysates from WT and p62^{-/-} mice and observed that its deubiquitinating activity was

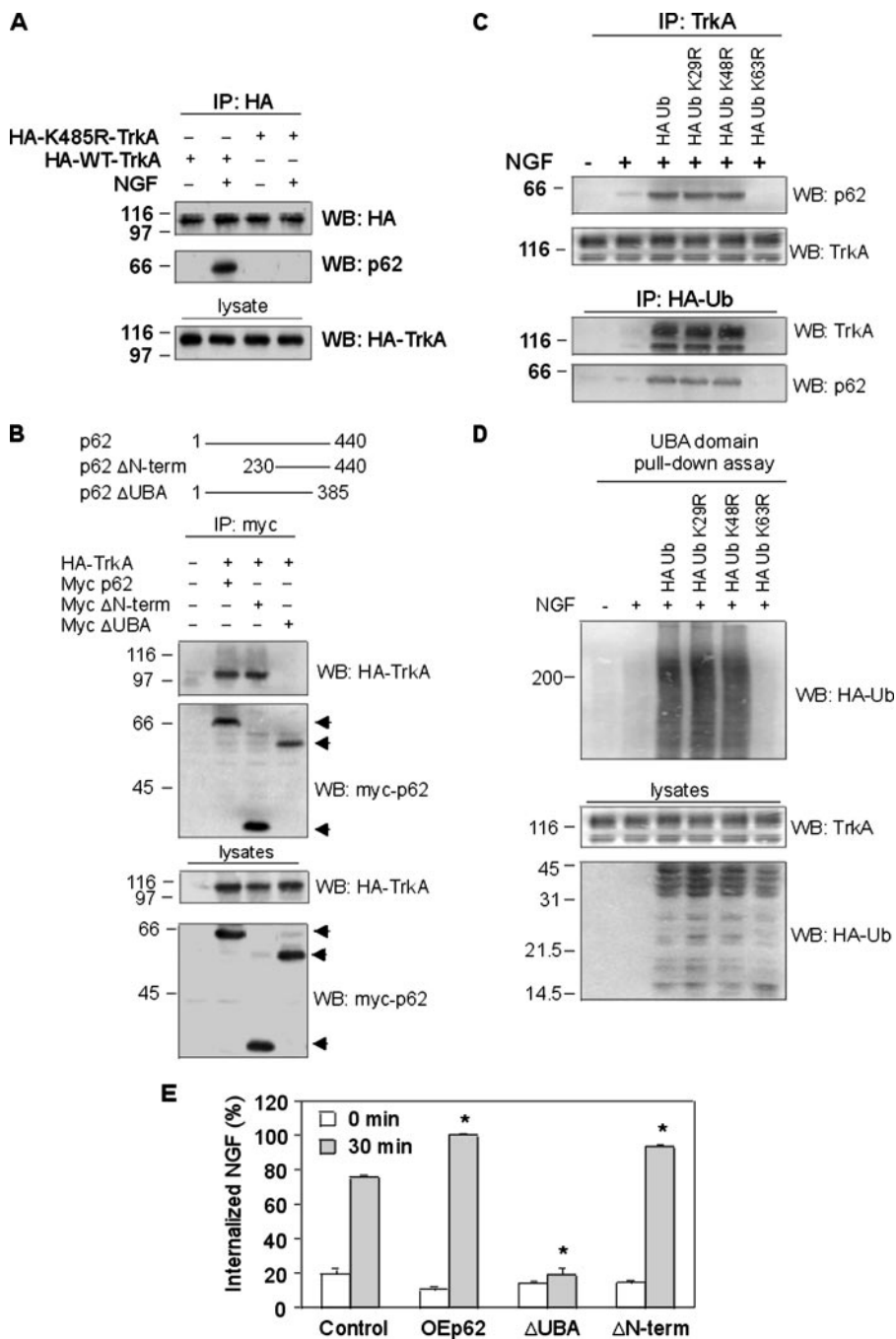


FIGURE 4. TrkA interacts with the UBA domain of p62. *A*, HA-WT TrkA or HA-K485R TrkA was transfected in HEK cells followed by treatment with NGF (50 ng/ml) for 15 min. The cells were lysed and immunoprecipitated (IP; 1 mg) with anti-HA and immunoblotted with anti-HA and p62. The lysates (50 μ g) were also Western blotted (WB) with HA to examine the expression of TrkA. *B*, HA-tagged TrkA along with Myc-tagged WT p62, Δ N-term, or Δ UBA (as shown) was expressed in HEK 293 cells followed by NGF stimulation (50 ng/ml) for 15 min. The cells were lysed with Triton lysis buffer, and the association of p62 with TrkA was determined by immunoprecipitation of the lysates with antibody to Myc and Western blotting with antibody to HA and Myc. As a control, a fraction of the lysate (50 μ g) was blotted with HA antibody to examine expression of TrkA. Expression of Myc-tagged p62 constructs is shown with an arrow. *C*, PC12 cells were transfected with HA-Ub and various ubiquitin mutants (K39R, K48R, K64R) followed by treatment with NGF for 15 min. The cells were lysed with Triton lysis buffer followed by immunoprecipitation with Trk (C-14) antibody and Western blotting for p62 and TrkA (B-3). Alternatively, the lysates were immunoprecipitated with HA and Western blotted for TrkA (B-3) and p62. The same lysates (500 μ g) were employed in a GST-p62 UBA domain pull-down assay, *D*, the pull-downs were blotted with HA to detect bound ubiquitinated TrkA. *E*, PC12 cells were transfected with either Myc-tagged p62 (OEp62), Δ UBA, or Δ N-term constructs. After 48 h the cells were incubated with 0.4 μ Ci/ml [125 I]NGF alone or with an excess of unlabeled NGF (200 ng/ml) at 4 $^{\circ}$ C for 1 h and chased at 37 $^{\circ}$ C for 0 and 30 min. The cells were lysed, and the specific internalized counts ([125 I]NGF internalized - [125 I]NGF internalized in the presence of excess NGF) were determined by γ -counting. The mean percentage \pm S.D. of NGF internalized was calculated from three different experiments. The data were analyzed using Student's *t* test (*, $p < 0.001$).

significantly diminished in brain lysates taken from p62 $^{-/-}$ mice as compared with WT controls (Fig. 5A). Furthermore, the autodeubiquitination of CYLD, a marker of activity (18), was likewise absent in p62 $^{-/-}$ lysates (Fig. 5B), which is consistent with reduced CYLD DUB activity (Fig. 5A). These findings suggest that p62 serves as an adapter to regulate the ubiquitination of CYLD, which in turn regulates its ability to hydrolyze Lys⁶³ chains. Because p62 has a TRAF6 binding site (2), we first examined whether CYLD was physically associated with p62 (Fig. 5C). The association between CYLD and TRAF6 or p62 and CYLD was readily detected in brain lysates of WT but not p62 $^{-/-}$ mice, suggesting that p62 serves as an adapter for both TRAF6 and CYLD. Because CYLD DUB activity/ubiquitination was reduced in p62 $^{-/-}$ lysates, we postulated that p62 should enhance basal level of CYLD activity if p62 were serving a regulatory role. To test this idea, we cotransfected CYLD and TRAF6 in the presence or absence of p62 in HEK cells (Fig. 5D). Therein, we observed enhanced ubiquitination of CYLD upon the addition of p62, thus confirming that p62 regulates CYLD activity. Recent findings have shown that DUB activity can be modulated by ubiquitination (18). CYLD is another example showing that ubiquitination of the DUB modulates its function. Altogether, our findings reveal that p62/TRAF6 serves as a platform for the recruitment and regulation of the Lys⁶³-DUB, CYLD. In keeping with this idea, we hypothesized that diminished Lys⁶³-DUB activity of CYLD in p62 $^{-/-}$ brain will influence the level of a TRAF6 substrate, TrkA, and its state of polyubiquitination.

Therefore, we next examined the interaction of TrkA with CYLD by coimmunoprecipitating cells treated with NGF. Interestingly, the results shown in Fig. 6A clearly demonstrate that both proteins interact in a ligand-dependent manner, suggesting that CYLD might be

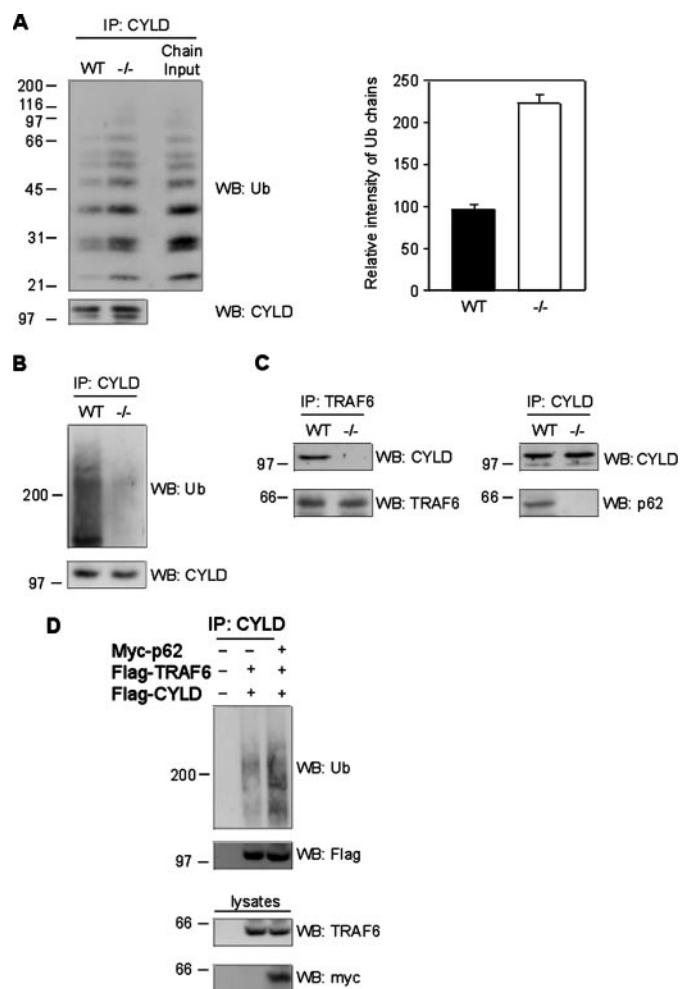


FIGURE 5. Absence of p62 impairs the DUB activity of CYLD. *A*, DUB activity of CYLD was measured in WT and $p62^{-/-}$ brain lysates with Lys⁶³-linked ubiquitin chains. Blots from three separate experiments were scanned and normalized. The data are shown as mean \pm S.E. ($p < 0.05$). *B*, brain lysates (1 mg) were immunoprecipitated (IP) with antibody to CYLD and Western blotted (WB) for Ub and CYLD. *C*, brain lysates (1 mg) WT and $p62^{-/-}$ were immunoprecipitated for TRAF6 and Western blotted for CYLD and TRAF6 or immunoprecipitated for CYLD and blotted for CYLD and p62. *D*, HEK cells were transfected with Myc-p62, Flag-TRAF6, or Flag-CYLD as shown followed by immunoprecipitation (1 mg) with CYLD and Western blotting with Ub and FLAG antibodies. The lysates (50 μ g) were blotted with antibody to TRAF6 and Myc. All experiments were conducted three separate times with similar results.

a physiologically relevant regulator of the TrkA ubiquitination state. We postulated that in conditions where the DUB activity of CYLD is low, TrkA would accumulate, whereas under normal circumstances, TrkA would be ubiquitinated transiently by TRAF6 and deubiquitinated by CYLD. To examine this possibility, we first tested the role of p62 in regulating this function. The interaction of TrkA and CYLD was absent in brain lysates from $p62^{-/-}$ mice (Fig. 6*B*). Next, we examined the potential effects of CYLD on receptor ubiquitination. PC12 cells were transfected with WT or a catalytically inactive form of CYLD (HN) (14) followed by treatment with NGF (Fig. 6*C*). The ubiquitination and degradation of TrkA was enhanced by expression of WT-CYLD; however, the expression of the catalytically inactive HN-CYLD mutant led to hyperaccumulation of polyubiquitinated TrkA forms, similar to what was observed in brain lysates obtained from $p62^{-/-}$ mice, which possess dimin-

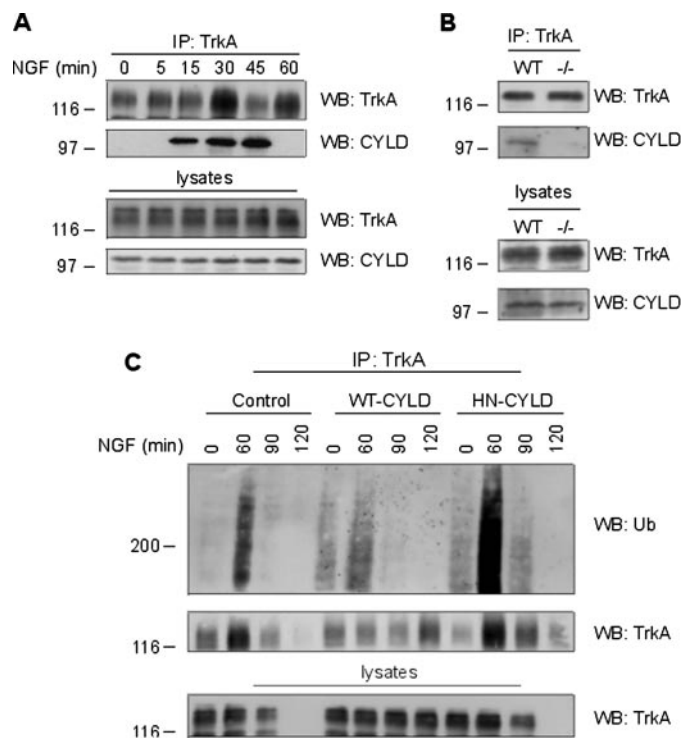


FIGURE 6. TrkA interacts with CYLD. *A*, PC12 cells were treated with NGF (50 ng/ml) for 0–60 min as shown followed by immunoprecipitation (IP) with TrkA and Western blotting (WB) for CYLD or TrkA. *B*, brain lysates (1 mg) were immunoprecipitated with antibody to TrkA (B-3). Both the immunoprecipitates and the lysates (50 μ g) were Western blotted for TrkA and CYLD. *C*, PC12 cells were transfected with WT-CYLD or the mutant HN-CYLD followed by NGF treatment (50 ng/ml) as shown. TrkA (C-14) was immunoprecipitated and blotted for Ub and TrkA (B-3). Lysates (50 μ g) were blotted with the same antibodies. All experiments were conducted three separate times with similar results.

ished CYLD activity. Because TrkA contains both Lys⁴⁸- and Lys⁶³-linked ubiquitin chains (Fig. 3*C*), the residual ubiquitin signal observed on the blot in WT-CYLD-expressing cells may likely be because of Lys⁴⁸-linked chains, which are not hydrolyzed by CYLD (14). Altogether, these findings support a model whereby CYLD interaction with TRAF6/p62 regulates its activity and, in the absence of p62, contributes to enhanced accumulation of Lys⁶³-polyubiquitinated proteins such as TrkA.

DISCUSSION

Growing evidence suggests that Lys⁶³-linked ubiquitin chains may represent a common signal for internalization and sorting of receptor tyrosine kinases (6, 8, 19). Our findings support a model whereby the UBA domain of p62 specifically interacts with the TrkA receptor to regulate its internalization and sorting. We have previously shown that p62 interaction with TRAF6 regulates synthesis of Lys⁶³ chains on target substrates such as TrkA (8, 20). We now extend these findings to demonstrate that the Lys⁶³-specific DUB, CYLD, is also regulated by the p62 adapter. This is the first time that the activity of a DUB and ubiquitination of its target substrate have been shown to be regulated by the same protein, implying that a finely tuned mechanism must exist to regulate these two antagonistic functions by p62. The p62 knock-out mice possess a complex phenotype, with development of mature onset obesity and insulin resistance (9). The hyperaccumulation of polyubiquitin was

coincident with the onset of obesity at 6 months of age. How the obese state impacts this system remains to be defined. Interestingly, the accumulation of Lys⁶³ chains appears to exert a negative effect on Lys⁴⁸ ubiquitination. This is consistent with similar observations from other laboratories (21). Ubiquitination/deubiquitination has emerged as an important mechanism for regulating various cellular functions. The data presented here are of great relevance because they demonstrate that diminished Lys⁶³ CYLD DUB activity, as a consequence of the loss of p62 in knock-out mice, accounts at least in part for the hyperaccumulation of Lys⁶³ chains and substrates, such as TrkA, in p62^{-/-} brain. However, it still remains possible that p62 also has a role in the turnover of polyubiquitinated substrates to proteasome or lysosome through a shuttling function (3). Therefore, the absence of p62 would impair turnover of TrkA and lead to further accumulation of the polyubiquitinated form, thereby further contributing to the pool of Lys⁶³-ubiquitinated proteins that accumulate. Thus, p62^{-/-} tissue represent a valuable resource to identify other p62-dependent Lys⁶³-ubiquitinated substrates by proteomic analysis.

Alterations in neurotrophins or Trk receptor levels have been documented in several neurodegenerative disorders (reviewed in Ref. 22). Recent studies have revealed that p62 may be involved in the pathogenesis of protein misfolding diseases and be localized to inclusions associated with Huntington disease (23), spinocerebellar ataxia type 3 (5), amyotrophic lateral sclerosis (24), Parkinson disease (4), frontotemporal dementia (25), and Alzheimer disease (26). In most of these diseases, proteasome function is also impaired leading to formation of ubiquitinated aggresomes.

Recent findings have revealed that Parkin aggresomes contain Lys⁶³-ubiquitinated proteins (27); this type of chain may be a common signature for accumulated proteins associated with these various diseases. It will be interesting to assess whether there are any differences in p62 expression in the progression of these pathologies or the effect that crossing mice harboring mutant genes for these diseases onto a p62^{-/-} background would have on disease progression. The extent of neuronal cell death is more likely related to the levels of specific ubiquitinated proteins, such as Trk receptors, and their mistrafficking. Altogether, our study has unveiled a new mechanism for the p62 adapter in regulation of the Lys⁶³-DUB, CYLD. We have also observed that p62 and TRAF6 colocalize with tau *in vitro* and in aggresomes isolated from Alzheimer disease brain (28). This finding raises the tantalizing possibility that a balance between Lys⁶³-TRAF6 ubiquitination and Lys⁶³-CYLD deubiquitination may play an important role in preventing neurofibrillary tangle formation or accelerating its pathology if the balance is tipped. Further experiments will be needed to define the mechanisms whereby p62 may modulate the pathogenicity of tau and other proteins associated with human neurodegenerative disease. That the SQSTM1/p62 gene is highly conserved from human to *Drosophila* suggests that p62 is likely to have a

conserved role as both an adapter (2) and a receptor for ubiquitinated proteins (3).

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