The Homeostasis of p62/SQSTM1 Short Isoform Regulates Selective Autophagy

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Abstract

Autophagy, a highly conserved lysosomal degradation pathway, has been shown to play a pivotal role in many physiological and pathological processes. Sequestosome 1 (SQSTM1/p62) which serves as autophagy receptor is a multifunctional protein involved in signal transduction, protein degradation and cell transformation. Human SQSTM1 has two isoforms, p62L and p62S, which are derived from alternative splicing at the 5'donor sites. However, few studies focus on p62S, and its function needs to be further explored. Here we found that p62S, but not p62L is mainly degrades by ubiquitin-proteasome signaling pathway. E3 ligase TRIM72 was identified as an interacting partner for p62S, and promotes the ubiquitination and degradation of p62S. Furthermore, we demonstrate that p62S competes with the autophagy receptor p62L which binds to ubiquitinated autophagy cargoes, thus playing a dominant negative role in autophagy regulation, while this inhibitory effect could be attenuated by TRIM72-dependent ubiquitination of p62S. Delineation of the mechanism and regulatory roles of p62S sheds a new light on the proposed pathological implications of p62 in cell physiology. TRIM72 and p62S are promising therapeutic targets for autophagy-related diseases.

Introduction

Proteins within cells are continually being degraded to amino acids and replaced by newly synthesized proteins, and this process was highly selective and precisely regulated. Eukaryotic proteins are mainly degraded through two degradation systems: ubiquitin-proteasome system (UPS) and autophagic-lysosomal system (ALS)[1, 2]. Autophagy is an evolutionarily conserved catabolic process that involves the sequestration and transport of organelles, macromolecules or invading microbes to the lysosomes for degradation[3, 4, 5]. As a dynamic and inducible catabolic process that responds to environmental or stimuli, the autophagy pathway drives rapid cellular changes that necessary for proper cellular process. In addition, autophagy is also crucial for intracellular quality control and this role for maintaining homeostasis is particularly important to the liver and other tissues which cell lines are capable of terminal differentiation, such as neurons and myocytes[6, 7, 8].

Some cytoplasmic proteins were found to be selectively taken up into nascent autophagic vesicles through a family of autophagy receptors, which commonly bear a functionally conserved LC3-interacting region (LIR) domain. Currently reported autophagy receptors include SQSTM1/p62, NBR1, NDP52, Nix, Cbl, Stbd1, OPTN, Tollip, etc. [9, 10, 11]. SQSTM1/p62 was the first protein shown to bind both target-associated ubiquitin (Ub) and LC3 conjugated to the phagophore membrane, thus acting as an important autophagic receptor for ubiquitinated targets[12, 13]. Therefore, p62 serves as both the autophagy substrate and the autophagy receptor for protein aggregates and damaged or excess organelles as well as for invading microbes.

At the N-terminus of p62 contains a Phox and Bem1p (PB1) domain, which is responsible for di- and multi-merization of the protein as well as interacting with other structurally related proteins, such as NBR1, PKCζ, MEKK3, ERK and MEK5[14, 15]. Followed the PB1 domain is a ZZ-type zinc finger domain,

which contains the binding site for receptor-interacting serine-threonine kinase 1 (RIP1). Near the ZZ-type zinc finger domain, there is a TNF receptor-associated factor 6 (TRAF6) binding domain (TBS), which contains the binding site for the E3 ligase TRAF6[14, 15]. p62 also harbors a LC3 interacting region (LIR) and a KEAP1 interacting region (KIR) motif, which are responsible for the interaction with human microtubule-associated protein 1 light chain 3 (LC3) and Kelch-like ECH-associated protein 1 (KEAP1), respectively. These two regions are important for its function as autophagy receptor and oxidative stress response regulator. The C-terminus of SQSTM1 contains an ubiquitin-associated (UBA) domain which is required for its binding to mono- and poly-ubiquitin[10, 16, 17].

Alternative splicing pathways generate different mRNAs encoding distinct protein products, thus increasing the coding capacity of genes and the diversity of proteins. Previous study has showed that ZIP, the homologous protein of p62 in rat, has two alternative splicing isoforms ZIP1 and ZIP2, which co-exist in the same cell type and are elevated differentially by neurotrophic factors, also differentially stimulate phosphorylation of Kvβ2 by PKC-ζ, thus regulating neuronal excitability[18]. In 2003, Cristina Croci's team have reported a third isoform of ZIP, ZIP3, as a new member of the PKC-ζ-interacting protein family that is highly expressed in the mammalian retina and demonstrated its *in vitro* interaction with PKC-ζ, $GABA_C$ receptor ρ subunits and Kvβ2[19].

In this study, we found that the two isoforms of human SQSTM1, p62L and p62S, which have different protein degradation pathways. Interestingly, p62S is degraded through ubiquitin-proteasome system, and exists as an endogenous antagonist protein of p62L due to its competitive bingding with ubiquitylated autophagy cargoes, thus interfering with autophagic process. In addition, an E3 ligase for p62S were identified and validated, and its functions were extensively studied. Our study sheds a new light on the proposed pathological implications of p62 in cell physiology.

Material And Methods

Cell culture and transfection

Human cell lines HEK293T, Hela, SHSY5Y, H1299, HCT116, SMMC-7721, HepG2, Hskmc and Hepatocyte were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum), 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco, USA) in a 37℃ humidified atmosphere of 5% CO_2. Plasmids that used in this study were transfected into Hela cells using a Lipofectamine 2000 (Life Technologies, USA) according to the manufacturer's instructions.

Plasmid construction

The shRNAs for p62S and TRIM72 were synthesized as oligos (Biosune, China), annealed and inserted into the pLKO.1 vector that was digested with EcoRI and AgeI (NEB, USA), the specific sequences for shRNAs seen in Table 1. The plasmids containing p62S, p62L and TRIM72 were amplified from the cDNA human HEK293T or Hela cells, and inserted into pCADNA3.0, pET22B, pACT2, pGBKT7 or pGEX4T-1 vector. The plasmids of TRIM72 (ΔRING) and TRIM72 (C14A) were generated using the QuikChange Site-

Directed Mutagenesis Kit (Stratagene, USA). Plasmids that expressing Ubiquitin (pRK5-HA-UB) were kindly provided by Professor Ronggui Hu (Chinese Academy of Sciences, Shanghai, China).

Table 1
Sequences of the shRNAs for p62S and TRIM72.

shRNA	Target site sequence (5'3')
Scramble	GCGCGATAGCGCTAATAATTT
shp62S	CAGTTTGGCGTGCAACATGGG
shTRIM72	CAGACTGAGTTCCTCATGAAA

Reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR)

Total RNA was extracted from Hela cells using the RNAsimple total RNA kit (Tiangen, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Japan). Reverse transcription PCR (RT-PCR) were performed with 2xTaq MasterMix (Tiangen, China) on gene amplification system (BIOER, China) to assess the abundances of *p62S* and *p62L* mRNAs in different cell lines using specific primers (Table 2), *GAPDH* acts as internal control. Quantitative PCR (qPCR) was performed on ABI 7500 fast real-time PCR system (ABI, USA) to assess the relative abundances of *p62S* and *p62L* mRNAs using specific primers (as primers used in RT-PCR) with staining by SYBR Green (Selleck, China). The relative abundances of *p62S* and *p62L* were normalized to that of the *GAPDH* gene, using the $^{\Delta\Delta}$Ct method, and data were obtained from three independent experiments.

Table 2
Sequences of the primers used in RT-PCR and qPCR.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	GAGTCAACGGATTTGGTCGTATTG	ATTTGCCATGGGTGGAATCATATTG
p62L	GCCTACCTTCTGGGCAAGGA	ACTGGAAAAGGCAACCAAGTC
p62S	CTGAACTAAGGAGAAAGTCCTACA	GTTCCTACCACAGGCCCATT

Yeast two-hybrid screening

The p62S ORF (open reading frame) was cloned into pGBKT7, generating the bait plasmid, pGBKT7-P62S, which contained the in-frame fusion of GAL4 DNA binding domain. Yeast two-hybrid (Y2H) screening was performed by transforming yeast strain (Mav203 strain) that harbored bait vector, with the pACT2 prey vectors for human E3 cDNA expression library. Yeast transformants were first grown on to the plate on SD-2 (deficient in Leu, Trp) for selection of yeast cells containing both bait and prey vectors, and then transferred to SD-4 (deficient in Leu, Trp, His and Ura) plates to screen for E3 ligase proteins that

potentially interact with human p62S. The interaction was confirmed by transforming yeast Mav203 cells with the indicated bait and prey vectors, and allowing the transformants to grow on the SD-2 or SD-4 agar plates for approximately 3 days at 30℃. Images of the colonies on both plates were recorded.

Co-immunoprecipitation, immunoprecipitation and immunoblotting

For co-immunoprecipitation (Co-IP), Hela cells transfected with plasmids were lysed in 800μl Co-IP buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA and 1% NP-40, PH 7.4) supplemented with a protease inhibitor cocktail (Roche, USA), cell lysates were centrifuged at 4℃ with 13,000g for 10min, incubated with Anti-myc immunomagnetic beads (L-1010, Biolinkedin Biotech, China) overnight at 4℃, washed three times with Co-IP buffer. For immunoprecipitation, Hela cells transfected with plasmids were lysed in 1ml immunoprecipitation (IP) buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA, 0.1% SDS and 1% NP-40, PH 7.5) supplemented with a protease inhibitor cocktail, cell lysates were centrifuged at 4˚C with 15,000g for 10min, incubated with anti-Flag affinity gels (L-1013, Biolinkedin Biotech) overnight at 4℃, washed three times with IP buffer. The immunoprecipitates from Co-IP and IP were denatured at 100℃ for 10 min in 2X SDS-PAGE loading buffer. The inputs, immunoprecipitates and other cell lysates were subjected to SDS-PAGE, transferred to a PVDF membrane (Bio-Rad, USA), the membrane was blocked with 10% non-fat milk at room temperature for 1 hour, then incubated with the appropriate antibodies against p62 (made in our laboratory), GAPDH (1:5000, 60004-1-Ig, ProteinTech, China), Myc tag (1:2000, 16286-1-AP, ProteinTech), Flag (1:5000, 20543-1-AP, ProteinTech), ubiquitin (1:500, sc-47721, Santa Cruz, USA), HA (1:2000, SAB3500908, Sigma, USA), TRIM72 (1:500, 22151-1-AP, ProteinTech) or LC3 (1:1000, L7543, Sigma) overnight at 4℃, washed three times with TBST (50mM Tris-HCl, 150mM NaCl and 0.1% Tween-20, PH 7.4), then incubated with HRP-conjugated secondary antibodies (goat anti-mouse IgG (H+L), SA00001-1, 1:5000 dilution; goat anti-rabbit IgG(H+L), SA00001-2, 1:5000 dilution, ProteinTech) at room temperature for 1 hour, washed three times with TBST, and the signals were detected by enhanced chemiluminescence (ECL, 180-5001, Tanon Science and Technology, China) with exposure to X-ray film. The density of bands was calculated with Image J 1.8.0 (Rawak Software, Germany) when needed.

Expression and purification of recombinant proteins

The pGEX4T-1-GST-p62S, pGEX4T-1-GST-p62L and pET22b-LC3-His6 plasmids were expressed in BL21 *E. coli*, monoclonal were picked, cultured in 37ºC in 2ml LB medium (10g/L Tryptone, 10g/L Yeast extract and 10g/L NaCl) with respective resistance overnight, then transfer the bacterial to 500ml LB medium and cultured for 6 hours at 37℃, following isopropyl-β-d-thiogalactopyranoside (IPTG, Sangon, China) induction at 16℃ overnight. Next day, bacterial cells were centrifuged and lysated in PBS buffer, incubated with glutathione or Ni^{2+}TA beads (GE Healthcare) to enrich the respective proteins, followed by elution with 50mM reduced L-glutathione or with 1M imidazole dissolved in PBS buffer. The eluted products were dialyzed in PBS buffer supplemented with 15% glycerol prior to being aliquoted and preserved at -80℃.

GST pull-down assay

Purified LC3-His6 (50μg), GST-p62S (50μg), GST-p62L (50μg) and Glutathione Sepharose 4B were incubated at 4℃ overnight in 1ml pull-down buffer (20mM Tris-Cl, 100mM NaCl, mM $MgCl_2$, 1mM EDTA, 1mM DTT, 0.5%NP-40 and 20μg/ml BSA, pH 7.6). The beads were centrifuged with 2,000g at 4℃, and washed five times with pull-down buffer. Subsequently, the recovered beads were denatured at 100℃ for 10 min in 2X SDS-PAGE loading buffer and subjected to immunoblotting analysis.

Fluorescence microscopy analysis

Hela cells were transfected with plasmids contain GFP-LC3, p62S, TRIM72 or its mutants, treated with Rapamycin (2μM) for 12 hours, fixed with 4% paraformaldehyde for 20 min, and the cell nucleus was counterstained with 4, 6-diamidino-2-phenylindole (DAPI). The fluorescence microscopy analyses were carried out on Olympus BX51 microscope. Puncta formation by GFP-LC3 was quantitated as follows: n=200 cells assessed from three fields in three independent experiments.

Detection of the cellular oxidatively damaged cellular proteins

Hela cells were tranfected with plasmids contain p62S, TRIM72 or its mutants, treated with or without Rapamycin (2μM) for 12 hours. Protein oxidation was determined using OxyBlot Protein Oxidation Detection Kit (Millipore, USA), according to the manufacturer's instruction as previously described[11, 12]. Briefly, cell lysates were incubated for 20min with 12% SDS, derivatized with DNP (2,4-dinitrophenylhydrazine), stopped by Oxyblot Neutralization solution and 3μg of total proteins for each sample were resolved in SDS-PAGE, followed transferred to nitrocellulose membranes. The membranes were blocked with the blocking buffer for 45min at room temperature before incubation with antibodies against DNP (1:2000, D9656, Sigma, USA) for two hours, and subsequently with goat anti-rabbit HRP (horseradish peroxidase)-coupled secondary antibodies (1:5000, ProteinTech) for 1 hour at room temperature. Finally, the signals were detected by enhanced chemiluminescence (ECL) with exposure to X-ray film.

Salmonella infections assay

An overnight culture of Salmonella strain SL1344 was diluted 1:25 and bacteria were grown at 37℃ until the OD600 was typically 1.0-1.2. Hela cells were transfected with plasmids contain p62S, p62L, TRIM72 or its mutants for 48 hours, washed twice with PBS and infection was performed in antibiotic-free medium at multiplicity of infection of 100. Salmonella were allowed to invade cells for 30 min, washed three times with PBS. Then cells were lysed and subjected to plate assay. The Salmonella colony numbers were counted and calculated. The experiment was repeated three times.

Statistics

Data were analyzed by two tailed unpaired t-test or one-way ANOVA with Bonferroni post-hoc test using Graph-Pad Prism 7 (GraphPad Software Inc., USA). *P < 0.05 was considered to be of significant difference; **P < 0.01 was considered to be of very significant difference.

Results

Human p62 has a shorter isoform p62S that lacking PB1 domain at N terminus

The human *p62/SQSTM1* has alternative 5'donor sites during splicing of immature messenger-RNA, which results in two isoforms of p62/SQSTM1: p62L and p62S (Fig. 1A). The different of 5'-UTR (untranslated region) and CDS (coding sequence) region between two transcript variants lead to a lack of PB1 domain at N-terminus in p62S compare to p62L (Fig. 1A). However, the function of p62S remains poorly understood. To explore the physiological function of p62S, we first proved its existence in cells. Two pairs of primers were designed to amplify DNA fragments of these isoforms in nine different human cell lines. The expression of p62L was detected in all cell lines, whereas the p62S were detected in most cell lines, except Human Skeletal Muscle Cell (Hskmc) and adult hepatocyte (Fig. 1B).

An interference shRNA that specifically targeting *p62S* but not *p62L* were designed, results of quantitative PCR showed that the designed shRNA did only knockdown p62S, but not p62L, which was further confirmed by immunoblotting analysis using a specific p62 antibody produced in our laboratory that has the ability to detect both longer and shorter form of p62 (Fig. 1C).

p62S is mainly degraded through proteasome

As a multi-function protein, p62 not only serve as an autophagy adaptor to regulate autophagic process but also as an autophagy substrate. However, through which pathway p62S might be degraded is still unclear. Hela cells were treated with proteasome inhibitor bortezomib (BTZ), and observed that compared to p62L, p62S protein increased more obviously (Fig. 2A). Further studies indicated that proteasome inhibitor bortezomib BTZ but not autophagy inhibitor bafilomycin (BAF) inhibited the degradation of p62S (Fig. 2B and 2C). These results suggested that p62S is degraded mainly through proteasome but not autophagy pathway.

TRIM72 interacts with, and promotes the degradation of p62S

Next, we tried to identify the E3 ligases for p62S. Using the yeast two-hybrid (Y2H) screening system, TRIM72, a member of tripartite motif-containing (TRIM) family protein, was identified as an interacting partner for p62S but not p62L (Fig. 3A). GST pull-down assay indicated that recombinant TRIM72 protein directly interacted with P62S but not P62L *in vitro* (Fig. 3B), suggesting the PB1 domain of p62L might fold back to block the region that interact with TRIM72. Further co-immunoprecipitation (Co-IP) assay showed that p62S indeed form a complex with TRIM72 in Hela cells (Fig. 3C).

Then the effect of TRIM72 on p62S stability was detected, and found that TRIM72 promoted the degradation of p62S in a dose-dependent manner (Fig. 3D). TRIM72 overexpression in Hela cells reduced the endogenous protein level of p62S, while TRIM72 knockdown increased p62S level (Fig. 3E).

Furthermore, a pulse-chase experiment was conducted and found that the protein levels of p62S decreased over time due to TRIM72 overexpression, while the protein levels of p62L was unchanged, which further indicated the specific effect of TRIM72 on p62S degradation (Fig. 3F). Whether TRIM72 could support the ubiquitination of p62S was investigated, and found that wild-type TRIM72, but not E3 ligase activity dead mutants ($TRIM72^{\Delta RING}$ and $TRIM72^{C14A}$), could supported the ubiquitination of p62S (Fig. 3G). Altogether, these results suggest that TRIM72 is an authentic E3 ligase for p62S.

TRIM72 reverse the effect that p62S antagonizes the autophagy receptor function of p62L

It is intriguing to ask the physiological function of p62S and functional consequences of TRIM72 mediated ubiquitination of p62S protein. p62 was known as an important autophagy receptor by targeting poly-ubiquitylated cargoes to autophagosome. As shown in Fig. 4A and 4B, p62S bound to and pulled down poly-ubiquitylated cargoes as efficiently as p62L, and was capable of binding with LC3, despite lacking PB1 domain at its N-terminus. Since p62S lacks PB1 domain at N-terminus, we presumed that p62S might play roles in regulating autophagy under various stimuli. Overexpression of p62S was found to decrease lipidation of LC3 (Fig. 4C, lane 3) and GFP-LC3 puncta formation (Fig. 4D) in Hela cells under rapamycin treatment, indicating that p62S have inhibitory function on rapamycin-induced autophagy, which could be restored by wild-type TRIM72 but not the E3 ligase activity dead mutants ($TRIM72^{\Delta RING}$ and $TRIM72^{C14A}$) (Fig. 4C and 4D).

Oxidative damaged (carbonylated) proteins of cells were mainly cleared by autophagy, and then the effect of TRIM72 and p62S on oncarbonylated protein degradation was detected. As shown in Fig. 4E, degradation of carbonylated proteins was accelerated in Hela cells upon rapamycin treatment, which was reversed by p62S. However, wild-type TRIM72 but not the E3 ligase activity dead mutants ($TRIM72^{\Delta RING}$ and $TRIM72^{C14A}$) almost totally abolished the effect of p62S.

As invading microbes were mainly degraded through autophagy, thus autophagic activities were next assessed in Hela cells that transfected with p62L, or p62S combined with wild-type TRIM72 or the E3 ligase activity dead mutants ($TRIM72^{\Delta RING}$ and $TRIM72^{C14A}$), followed by Salmonella infection assay. As shown in Fig. 4F, overexpression of p62L into Hela cells cleared approximately 40% of the infected Salmonella cells, in comparison with p62S only cleared approximately 15%. Interestingly, when p62S was down-regulated by wild-type TRIM72, more than 40% of the infected Salmonella cells were cleared.

These data strongly suggested that p62S antagonizes the autophagy receptor function of p62L, while TRIM72 promotes selective autophagy by ubiquitination and degradation of p62S.

Discussion

Alternative splicing is usually tightly regulated in a cell-type- or developmental stage-specific manner. Coordinated changes in alternative splicing patterns of multiple pre-mRNAs are an integral component of gene expression programmes like those involved in nervous system differentiation[20], apoptosis[21] and

cancer[22]. Different splice variants of a given protein can display different and even antagonistic biological functions[23, 24]. Growing evidence is supporting the notion that different splicing variants of a given protein display different and even antagonistic biological functions. In this study, we found that the shorter isoform of p62, named p62S, derived from alternative splicing at the 5'donor sites which lacks PB1 domain was extensively expressed in many cell types (Fig. 1).

Among many domains of p62, PB1, LIR and UBA, are vital for its function as autophagy regulator[25, 26, 27]. Autophagy receptors p62 and NBR1 are reported to oligomerize through their N-terminal PB1 domains and interact with other ubiquitylated autophagy cargoes via their C-terminal UBA domain, thus playing central role in recruiting and targeting cargoes to autophagosome. The oligomerization is vital for their ability to regulate autophagy, and the PB1 deletion mutant did not form oligomer and is deficient in autophagy regulation[28, 29]. Sine p62S lacks PB1 domain which was vital for oligomerization of p62 and important for autophagy regulation, p62S plays important roles in regulating autophagy under the stimulation of rapamycin and Salmonella infection (Fig. 4C-D and 4F).

Not like p62L, p62S mainly undergoes proteasome degradation but not autophagy (Fig. 2). An E3 liagse TRIM72 was identified as an interacting partner for p62S, but not p62L, by yeast two hybrid screening, and validated by other assays (Fig. 3A-3C). TRIM72 belongs to the tripartite motif (TRIM) proteins family which functions as important regulators for a variety of diseases including cancer, infectious diseases, neuropsychiatric disorders, etc[30]. TRIM72 promotes the ubiquitination and degradation of p62S, playing an important role in regulating the homeostasis of p62S (Fig. 3D-3G).

In mammals, autophagy is essential for cellular homeostasis and renovation through the elimination of large cytoplasmic components, such as abnormal protein aggregates and damaged organelles, via lysosomal degradation[5, 31]. Overexpression of p62S in Hela cells was found to decrease lipidation of LC3 under rapamycin treatment, indicating that p62S have inhibitory function on rapamycin-induced autophagy, while TRIM72 could reverse the effect (Fig. 4C-D and 4F).

Autophagy serves as an important defense mechanism to clear invading microbes, such as Salmonella which cause globally distributed diseases with massive morbidity and mortality in human[32, 33]. Interestingly, p62L, but not p62S, efficiently cleared the infected Salmonella cells, when p62S was down-regulated by TRIM72, more than 40% of the infected Salmonella cells were cleared (Fig. 4F). An ongoing effort is to test these functions in animal models and patient samples.

Conclusions

Our findings have demonstrated the mechanism and regulatory roles of p62S by E3 ligase TRIM72, which sheds a new light on the proposed pathological implications of p62 in cell physiology. TRIM72 and p62S are promising therapeutic targets for autophagy-related diseases.

Declarations

Ethics approval and consent to participate

No animals and patients' samples were involved in this study.

Consent for publication

Not applicable.

Availability of data and materials

All the data generated or analysed during this study are included in this published article and are available for other researchers after the manuscript is published.

Competing interests

The authors declare that they have no conflict of interest.

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Author Contributions

RH and WG conceived the project. RH and WG designed and supervised the whole project, and CW led the study. CW, HP and JY performed most of the experiments. RH, CL and ZW wrote the manuscript. All authors read and approved the manuscript.

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Figures

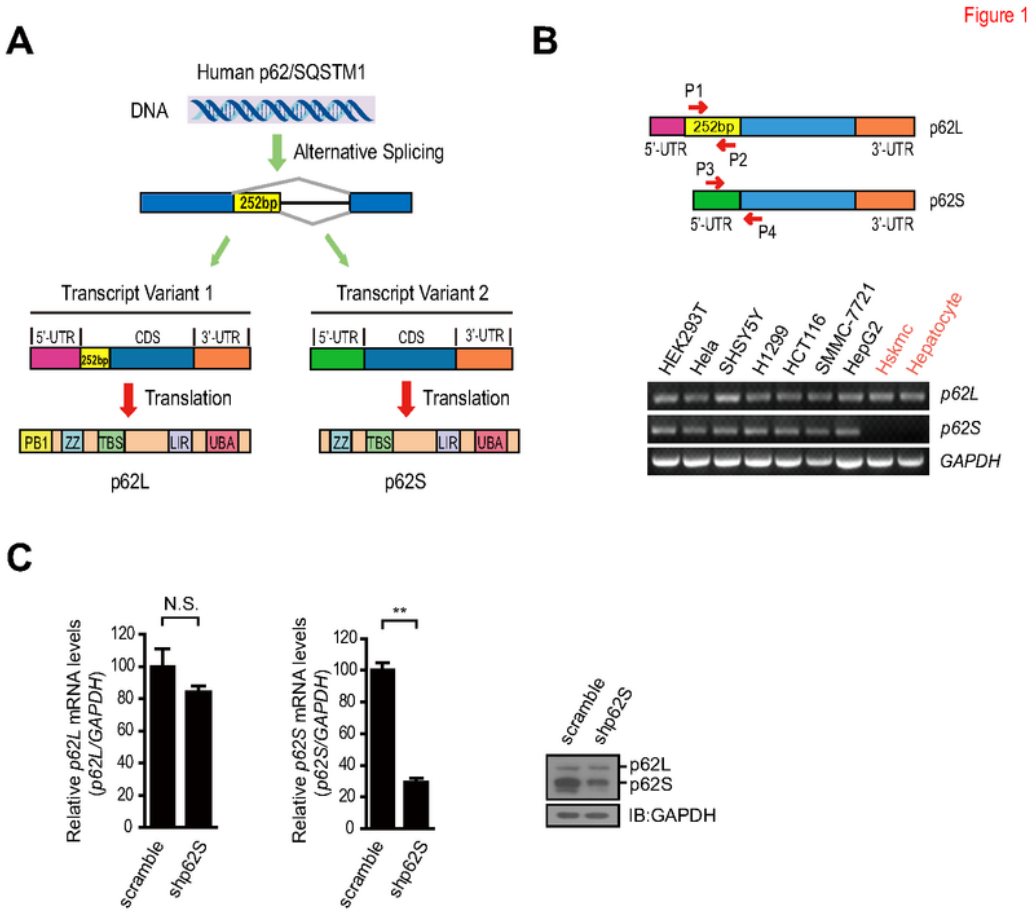

Figure 1

Human p62 has a shorter isoform p62S that lacking PB1 domain at N terminus. (A) Schematic domain structure of p62L and p62S, which are derived from alternative splicing of human p62/SQSTM1 gene. PB1, Phox and Bem1p domain; ZZ, zinc finger; TBS, TRAF6-binding site; LIR, LC3-interacting region; UBA, ubiquitin association region; UTR, untranslated region; CDS, coding sequence. (B) The mRNA expression levels of p62L and p62S in nine human cells were detected by reverse transcription PCR (RT-PCR), which

showed that p62L express in all nine different types of cells, and transcripts of p62S is not detectable in human skeletal muscle cell (Hskmc) and hepatocyte. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acts as internal control. (C) The shRNA were designed to only target p62S but not p62L to confirm the expression of p62S. Total mRNA extracted from Hela cells were used to measure the amount of p62L and p62S transcripts relative to GAPDH by RT-PCR, and cells lysates were also subjected to immunoblotting analysis with indicated antibodies. Data were presented as mean ± SD, two tailed unpaired t-test. N.S., no significant difference; **P < 0.01, very significant difference, three independent experiments.

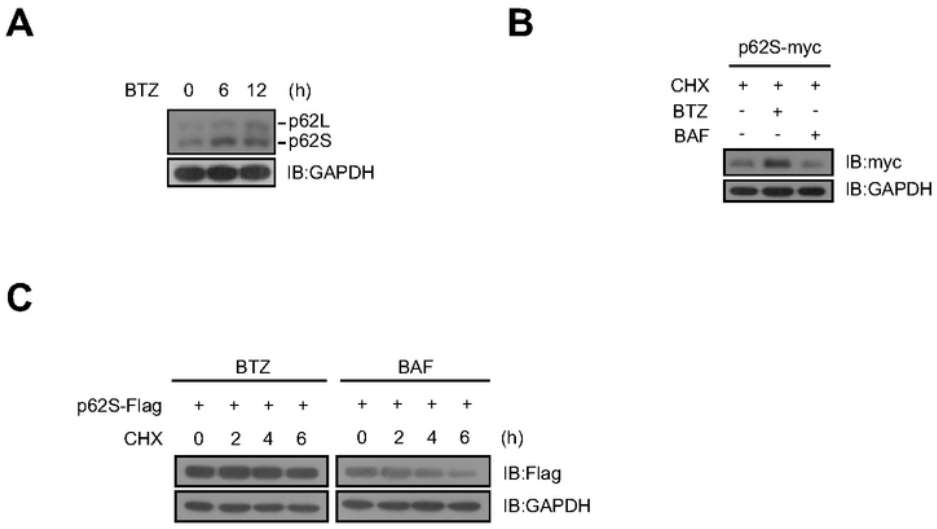

Figure 2

Human p62S is mainly degraded though the proteasome pathway. (A) Endogenous protein levels of p62L and p62S were both increased, especially p62S, when HeLa cells treated with proteasome inhibitor BTZ (1μM) for 6 or 12 hours, and subjected to immunoblotting analysis. BTZ, bortezomib, proteasome inhibitor. (B) p62S was mainly degraded through proteasome but not autophagy pathway. Hela cells were transfected with p62S-myc, and treated with BTZ (1μM) or BAF (200nM), as well as CHX (100μg/ml), for

8 hours before subjected to immunoblotting analysis with indicated antibodies. BAF, bafilomycin, autophagy inhibitor; CHX, cycloheximide, protein synthesis inhibitor. (C) BTZ but not BAF inhibited the degradation of p62S. Hela cells were transfected with p62S-Flag, and treated with BTZ (1μM) or BAF (200nM), as well as CHX (100μg/ml), during different time (0, 2, 4 or 6 hours) before subjected to immunoblotting analysis with indicated antibodies.

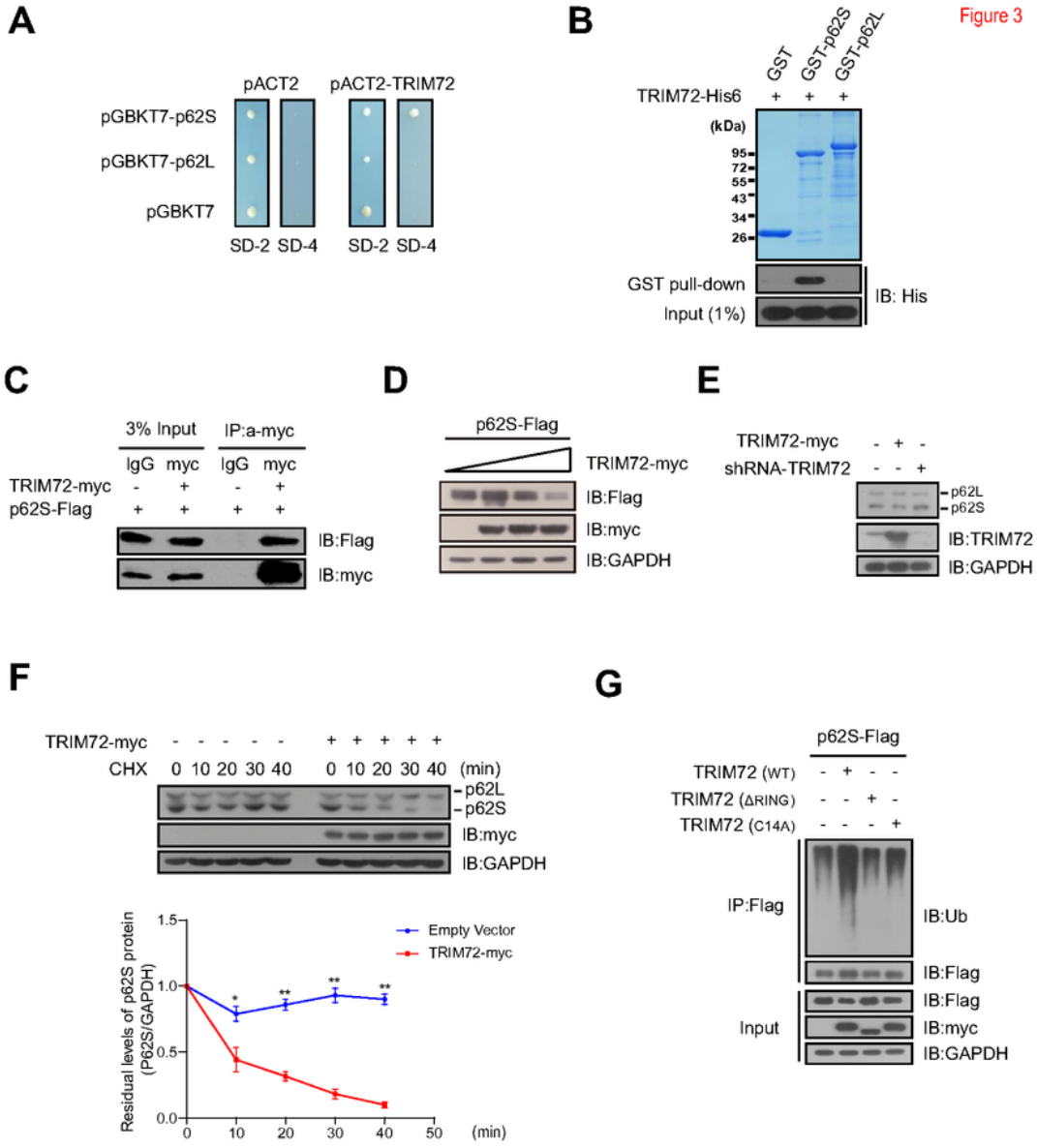

Figure 3

E3 ligase TRIM72 interacts with, and promotes the degradation of p62S. (A) Yeast two-hybrid (Y2H) screening identified TRIM72 as an interacting partner for p62S but not p62L. p62S was used as a bait, and the interaction of TRIM72 with p62L was also validated. SD-2, deficient in Leu and Trp; SD-4, deficient in Ura, His, Leu and Trp. (B) Recombinant p62S directly interacted with TRIM72 in vitro. GST pull-down assays were carried out with recombinant GST-tagged p62S or p62L and His-tagged TRIM72. GST served as a negative control. (C) TRIM72 and p62S formed a complex in Hela cells, as detected by a co-immunoprecipitation assay performed using anti-Myc antibody, followed by immunoblotting with anti-myc and anti-Flag antibodies. (D) TRIM72 promoted the degradation of p62S in a dose-dependent manner. Hela cells that transiently transfected with different amounts of TRIM72-myc or empty vectors, and p62S-Flag were subjected to immunoblotting analysis using indicated antibodies. (E) TRIM72 regulated the endogenous p62S protein level but not p62L. Hela cells that transiently transfected with TRIM72-myc or shRNA for TRIM72, and subjected to immunoblotting analysis using indicated antibodies. (F) TRIM72 promoted the degradation of endogenous p62S but not p62L. Hela cells were transiently transfected with TRIM72-myc or empty vectors, and treated with CHX during different times (0, 10, 20, 30 or 40 min) 48 hours later, followed by immunoblotting analysis using indicated antibodies. The intensity of p62S and GAPDH bands were analyzed and calculated. Data were presented as mean ± SD, two tailed unpaired t-test. *P < 0.05, significant difference; **P < 0.01, very significant difference, three independent experiments. (G) TRIM72 supported the poly-ubiquitylation of p62S in Hela cells. Hela cells were co-transfected with TRIM72(WT)-myc, TRIM72(ΔRING)-myc or TRIM72(C14A)-myc, and p62S-Flag. 48 hours later, cell lysates were immunoprecipitated with anti-Flag affinity gels and subjected to immunoblotting analysis using indicated antibodies.

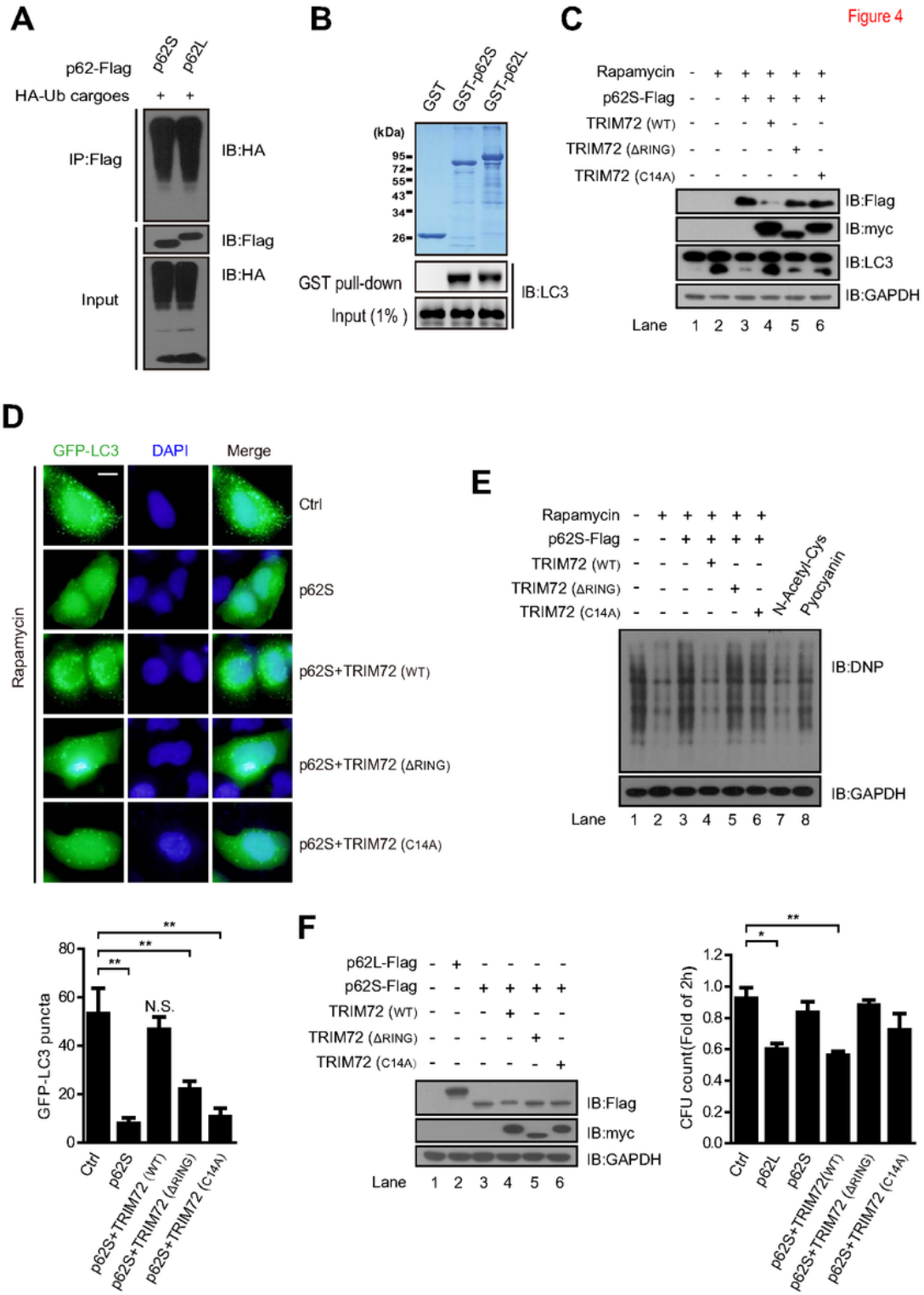

Figure 4

TRIM72 reverse the effect that p62S antagonizes the autophagy receptor function of p62L. (A) p62S bound to and pulled down poly-ubiquitylated cargoes as efficiently as p62L. Hela cells that expressing HA-tagged Ub conjugates were affinity-enriched with anti-HA chromatography. Flag-tagged p62S or p62L were incubated with the HA-tagged Ub conjugates, immunoprecipitated with anti-Flag affinity gels, followed by immunoblotting analysis using indicated antibodies. (B) p62S was capable of binding with

LC3, despite lacking PB1 domain at its N- terminus. GST pull-down assays were carried out with recombinant GST-tagged p62S or p62L and His-tagged LC3. GST served as a negative control. (C, D) p62S overexpression was found to decrease lipidation of LC3, while wild type TRIM72 but not E3 ligase activity dead mutants could reverse this effect. Hela cells were transfected p62-flag or not, combined with TRIM72(WT)-myc, TRIM72(ΔRING)-myc or TRIM72(C14A)-myc, and treated with 2 μM rapamycin for 12 hours before harvest, followed by immunoblotting analysis using indicated antibodies (C). Hela cells that co-transfected with GFP-LC3, p62S-Flag and TRIM72(WT)-myc, TRIM72(ΔRING)-myc or TRIM72(C14A)-myc were treated with 2μM rapamycin for 12 hr, and then subjected to fluorescent microscopy analysis. The intensity of fluorescent were analyzed and calculated, data were presented as mean ± SD, one-way ANOVA with Bonferroni post-hoc test. **P < 0.01, very significant difference; N.S., no significant difference, three independent experiments. Scale bars:10 μm. (E) p62S inhibited degradation of carbonylated proteins, while wild type TRIM72 but not E3 ligase activity dead mutants could reverse this effect. Hela cells were transfected p62-flag or not, combined with TRIM72(WT)-myc, TRIM72(ΔRING)-myc or TRIM72(C14A)-myc, and continued culture 24 hours before treated with DMSO or 10 μM Rapamycin for 6 hours. Pyocyanin (PCN) was used as a positive control, and N-acetyl-L-cysteine (NAC) was used as a negative control. Carbonylated proteins were visualized through derivatization with DNPH (2,4-Dinitrophenylhydrazine), followed by immunoblotting analysis with anti-DNP. (F) p62S did not clear infecting Salmonella as efficient as p62L. Hela cells were transfected p62-flag or not, combined with TRIM72(WT)-myc, TRIM72(ΔRING)-myc or TRIM72(C14A)-myc. 48 hours after transfection, cells were subjected to Salmonella infection for 30min, washed three times and followed by plate assay to count the number of surviving Salmonella cells. The colony numbers were counted and calculated. Data were presented as mean ± SD, one-way ANOVA with Bonferroni post-hoc test. *P < 0.05, significant difference; **P < 0.01, very significant difference, three independent experiments.