Supplemental Information

Keap1/Cullin3 Modulates p62/SQSTM1 Activity via UBA Domain Ubiquitination

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Figure S1. p62 WT and mutants schematics and comparison of p62 expression level (Related to Fig 1)
(A) Schematic of 7 putative ubiquitination sites on p62 and the three lysine to arginine constructs utilized; p62-7K/R, p62-6K/R and p62-K420R. Bottom schematic illustrates the site of p62 disease mutations including deletion of the UBA domain. (B) Anti-p62 immunoblot in p62-/- MEFs expressing HA-p62-WT, p62 +/- MEFs, and U20S cells. Anti-GAPDH was used as a loading control.
Figure S2. p62 expression level (Related to Fig 4)
(A) Anti-p62 immunoblot of p62-/-MEFs expressing empty vector, HA-p62-WT, K420R or ΔUBA. Anti-GAPDH was used as a loading control. (B) Anti-p62, anti-Myc and anti-V5-HRP immunoblots of p62-/-MEFs expressing HA-p62-WT or K420R along with Myc-Keap1/V5-Cul3. Anti-GAPDH was used as a loading control.
Figure S3. Endogenous p62 body size (Related to Fig 4)
Quantitation of endogenous p62 body size in U2OS cells expressing myc-Keap1/V5-cul3 or treated with 3 µM MLN4924 for 24hrs. All data are represented as mean ± SEM. *p<0.05. (n≥100)
Figure S4. FRAP assay in ATG5-/MEFs (Related to Fig 5)

(A-B) Cherry-p62 WT or K420R was transfected into ATG5-/MEFs for 24hrs and then subjected to FRAP assay. Also, p62 WT along with Cul3 and Keap1, or Keap1ΔBTB were transfected. R.F.I. was measured at each time point. The circle in (B) indicates the photobleached area. Data are represented as mean ± SEM. The scale bar indicates 1 µm (n ≥ 7 cells containing p62 bodies).
Figure S5. Cell Images of mCherry-GFP-p62 Quench Assay and Co-localization of Cherry-p62 with endogenous LC3B (Related to Fig6)

(A) Representative live cell images of p62-/MEFs expressing mCherry-GFP-p62-WT, K420R or T350A along with Myc-Keap1/V5-Cul3. Cells were treated with DMSO or (B) BafA for 4hrs. The scale bar indicates 1 µm. (C) Anti-LC3B immunostaining of p62-/MEFs expressing Cherry-p62-WT along with Myc-Keap1/V5-Cul3. The scale bar indicates 1 µm. The graph indicates the Pearson’s coefficient of Cherry-p62-WT and LC3B (n≥50). All data are represented as mean ± SEM. *p<0.05.
Figure S6. Live cell images of FRAP assay (Related to Fig7)
Representative live cell images of mCherry-p62-WT or p62-P392L, M404V and G425R following photobleaching and fluorescent recovery. The scale bars indicate 1 µm.
Supplemental Experimental Procedures

Reagents and antibodies

N-Ethylmalmide (NEM; #3876), BafilomycinA (#B1793) and cycloheximide (#C7698) is from Sigma. MLN4924 (#B1036) is from ApexBio. Anti-HA monoclonal antibody (Covance #MMS-101P, 1:1000 for WB), anti-HA polyclonal antibody (Abcam #9110, 1:1000 for WB), anti-Flag monoclonal antibody (Sigma #F1804, 1:1000 for WB, 1:100 for IF), anti-Flag polyclonal antibody (Sigma #F7425, 1:1000 for WB, 1:100 for IF), anti-p62 monoclonal antibody (Novus #H0008878-M01, 1:1000 for WB, 1:100 for IF), anti-p62 polyclonal antibody (Proteintech #18420-1-AP, 1:1000 for WB, 1:100 for IF), anti-Ub monoclonal antibody (FK2; Biomol #PW-8810, 1:500 for WB, 1:100 for IF), anti-Ub polyclonal antibody (Dako #Z0458, 1:5000 for WB, 1:100 for IF), anti-Gapdh polyclonal antibody (Cell Signaling #2118, 1:1000 for WB), anti-myc monoclonal antibody (Cell Signaling #2276, 1:1000 for WB), Anti-V5 HRP antibody (Invitrogen #B96125, 1:1000 for WB), anti-LC3B polyclonal antibody (Sigma #L7543, 1:500 for WB), anti-Keap1 polyclonal antibody (SantaCruz #sc15246, 1:500).

Cell culture and transient transfection

Immortalized wild type and p62/-MEFs were a kind gift by Dr. Komatsu, Niigata University (Komatsu et al., 2007). Keap1/-MEFs were from Dr. Wakabayashi, University of Pittsburgh (Kang et al., 2004). ATG5/-MEFs were from Dr. Virgin, Washington University in St. Louis. MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco #11965-084), 10% fetal bovine serum (FBS, Atlanta Biologicals #S10350H), and 50 μg/mL penicillin and streptomycin (P/S, Sigma #P4333), 1% sodium pyruvate (Gibco #11360070), and 1% non-essential amino acid (Gibco #11140050) at 37° C with 5% CO₂. U2OS cells were maintained in DMEM with 10% FBS, 50 μg/mL P/S at 37° C with 5% CO₂. Transfection was performed with lipofectamine2000 (Life Technologies #11668019) according to the manufacturer’s instruction. 24hr post-transfection, cells were washed three times with ice-chilled PBS and harvested in lysis buffer. HA-human p62-WT gift from Dr. Moscat, Sanford-Burnham Medical Research Institute (Rodriguez et al., 2006), HA-p62 K420R, HA-p62 ΔUBA, ΔPB1, D69A P392L, M404V, G411S, G425R generated using Quik Change Mutagenesis Kit (Agilent Technologies #200517). HA-p62 6 K/R commercially synthesized by Genewiz and HA-p62 7 K/R generated by mutagenesis. pDEST-mCherry-human p62 WT and pDEST-mCherry-eGFP-p62 WT gifted from Dr. Johansen, The Arctic University of Norway (Pankiv et al., 2007). pDEST-mCherry-human p62 P392L, M404V, G411S, G425R generated by mutagenesis. Flag-Ub-WT from Dr. Yarden, Weizmann Institute of Science (Mosesson et al., 2009). V5-Cul3, Cul3-Flag and Myc-Keap1 gifted from Dr. Diehl, University of South Carolina (Cullinan et al., 2004). DN-CUL3-FLAG from Addgene (#15820). HttQ72-CFP previously described (Fuentealba et al., 2010). His-Ub purchased from Addgene (#31815).
References