

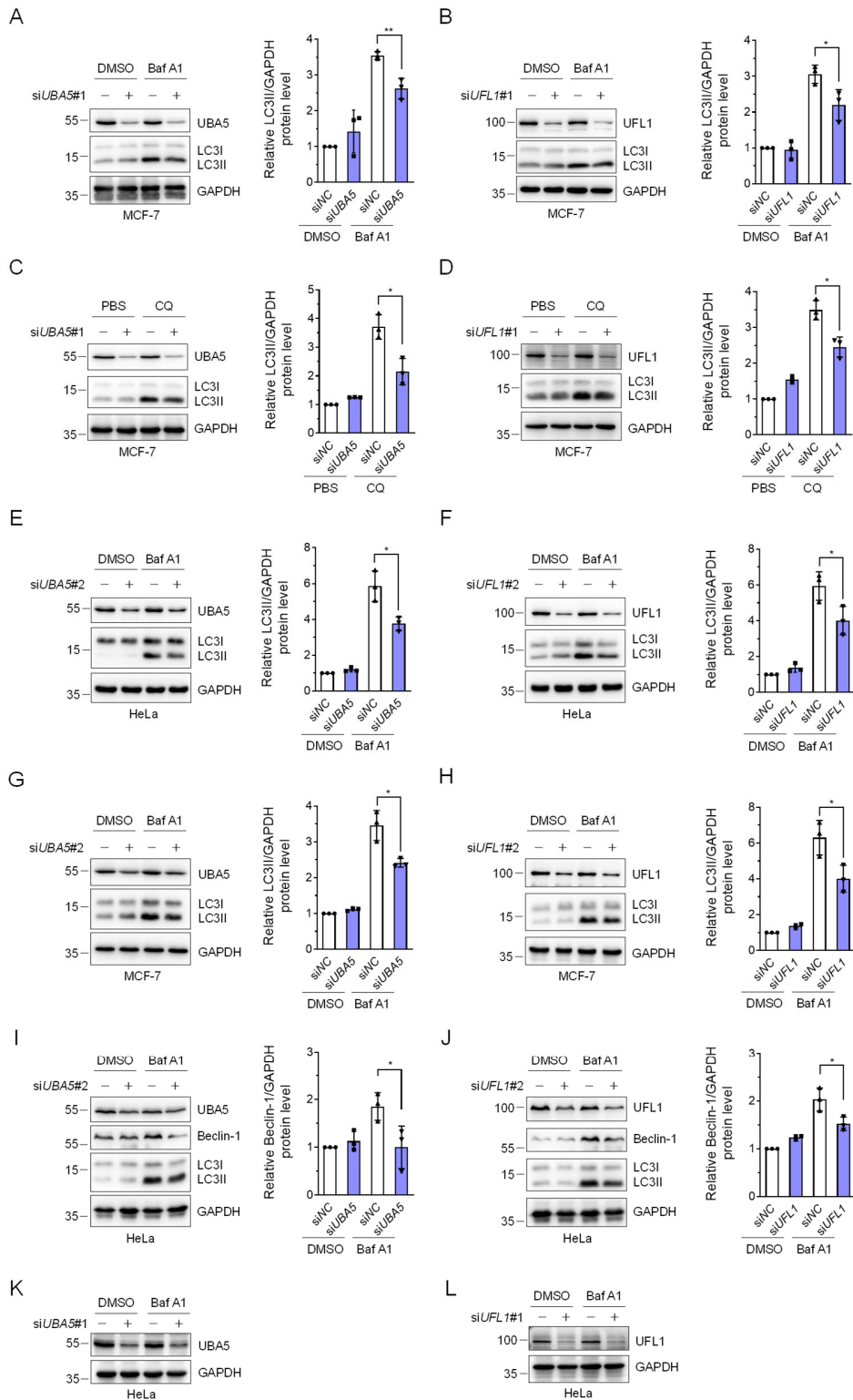
## **Supplementary Figures for**

### **SQSTM1/p62 UFMylation Enhances Autophagic Clearance of Pathogenic Mutant Huntingtin**

Xiaohui Wang, Xiaowei Lv, Honglv Jiang, Wenyun Zhu, Lindong Cao, Liang Zhou,  
Fang Lin, Rong Deng, Li-Fang Hu, Jingjing Ma\*, Jia-Bin Li\*, and Guoqiang Xu\*

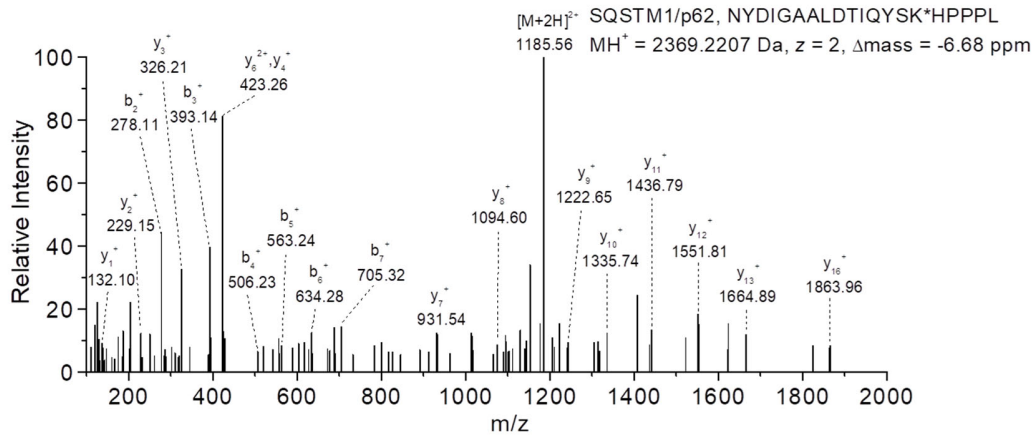
This file includes:

Figure S1 to S9.

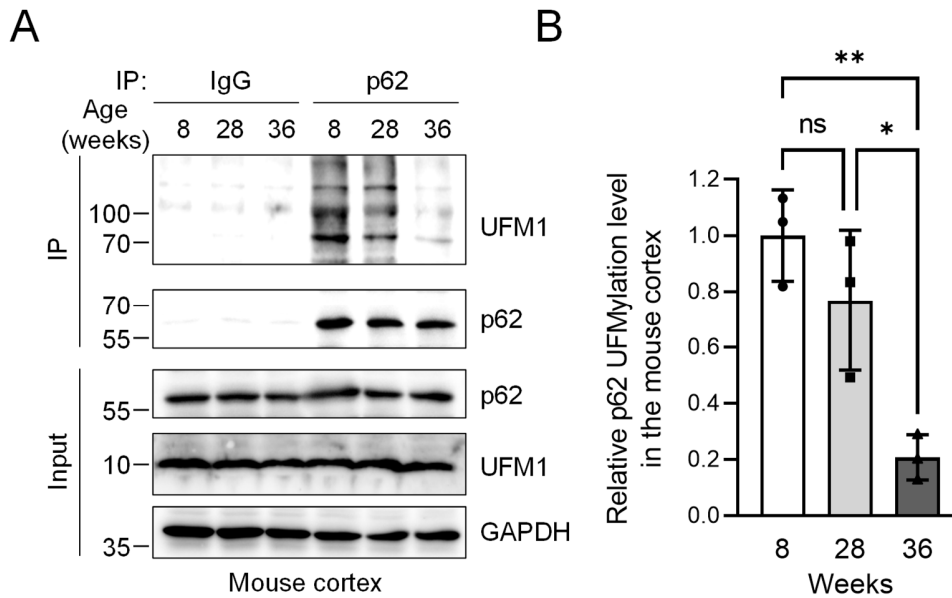


**Figure S1. Knockdown of *UFL1* or *UBA5* blocks autophagic activity. (A-D)** Control (siNC) and *UBA5*- or *UFL1*-knockdown (siUBA5#1 or siUFL1#1) MCF-7 cells were

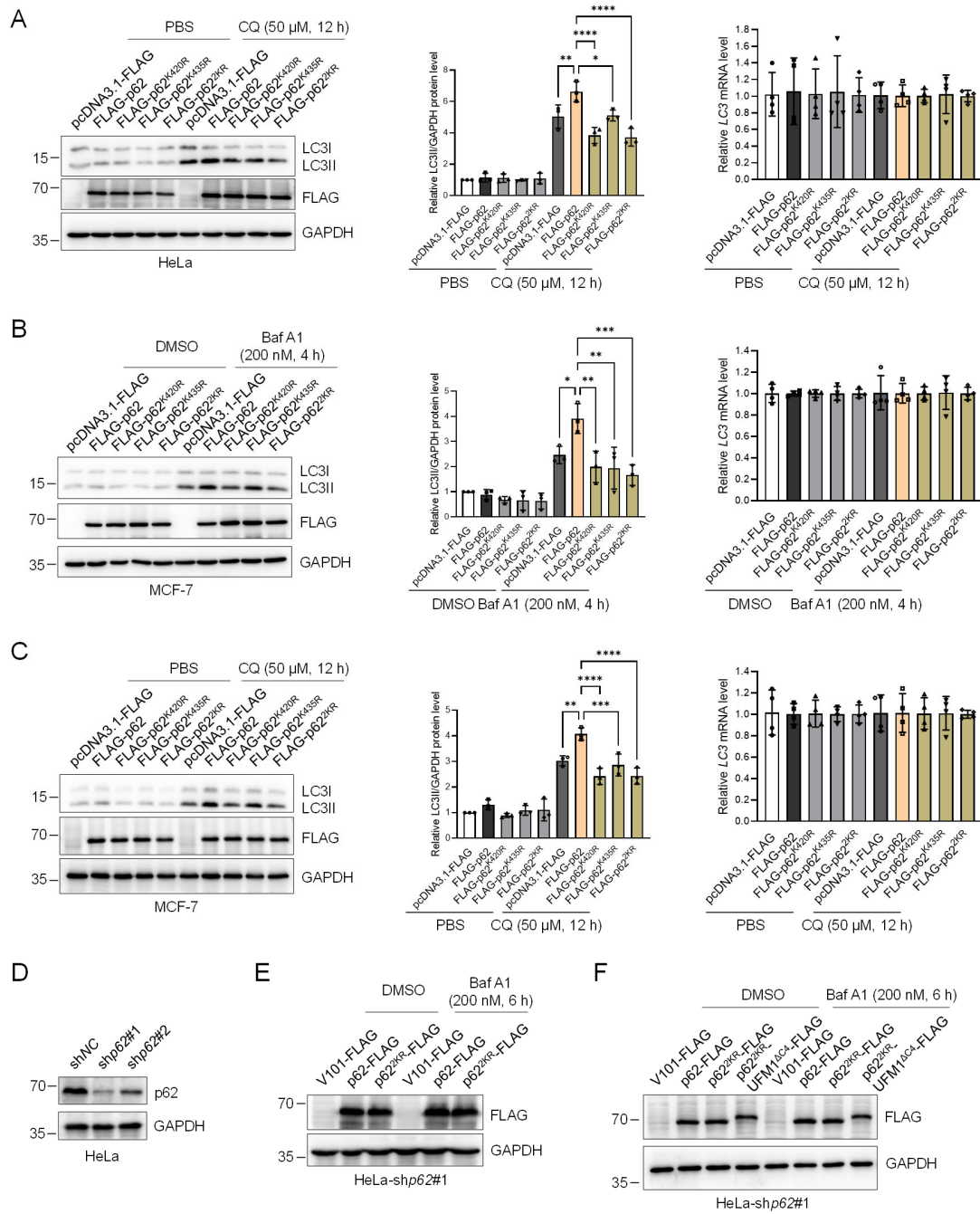
treated with Baf A1 (200 nM for 4 h) (A-B) or CQ (50  $\mu$ M for 12 h) (C-D). Cell lysates were immunoblotted for the indicated proteins. Mean  $\pm$  SD, n = 3 biological replicates, two-way ANOVA with Tukey's multiple comparisons post hoc test. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ . **(E-H)** Control (siNC) and *UBA5*- (E, G) or *UFL1*- (F, H) knockdown (si*UBA5*#2 or si*UFL1*#2) HeLa or MCF-7 cells were treated with Baf A1 (200 nM for 6 h) and cell lysates were immunoblotted for LC3, UBA5, or UFL1. The relative protein levels of LC3II were normalized to GAPDH and quantified. Mean  $\pm$  SD, n = 3 biological replicates, two-way ANOVA with Tukey's multiple comparisons post hoc test. \*:  $P < 0.05$ . **(I-J)** Control (siNC) and *UBA5*- (I) or *UFL1*- (J) knockdown (si*UBA5*#2 or si*UFL1*#2) HeLa cells were treated with Baf A1 (200 nM) for 6 h and cell lysates were immunoblotted for the indicated proteins. The relative protein levels of LC3II were normalized to GAPDH and quantified. Mean  $\pm$  SD, n = 3 biological replicates, two-way ANOVA with Tukey's multiple comparisons post hoc test, \*:  $P < 0.05$ . **(K-L)** Immunoblotting of cell lysates obtained from Figure 1E and 1F. GAPDH was used as the loading control.



**Figure S2. MS/MS spectrum of the p62 tryptic peptide containing the UFMylation site.** The peptide sequence, MH<sup>+</sup>, charge state (z), and Δmass (ppm) were provided. K\*: VG modified lysine.

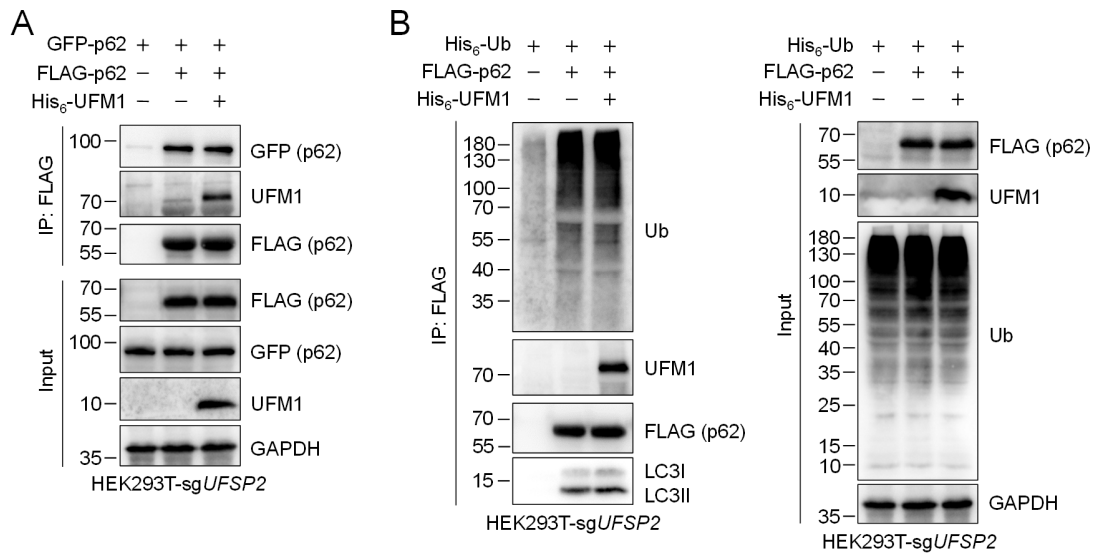


**Figure S3. Endogenous p62 in the mouse cortex is UFMylated.** (A-B) Cortices from 8, 28, or 36-week-old mice were dissected and lysed. The endogenous p62 was immunoprecipitated with IgG or anti-p62 antibodies. Lysates and immunoprecipitates were immunoblotted with the indicated antibodies (A) and the relative p62 UFMylation level was quantified (B). Mean ± SD, n = 3 biological replicates, one-way ANOVA with Tukey's multiple comparisons post hoc test, \*:  $P < 0.05$ , \*\*:  $P < 0.01$ , ns: not significant.

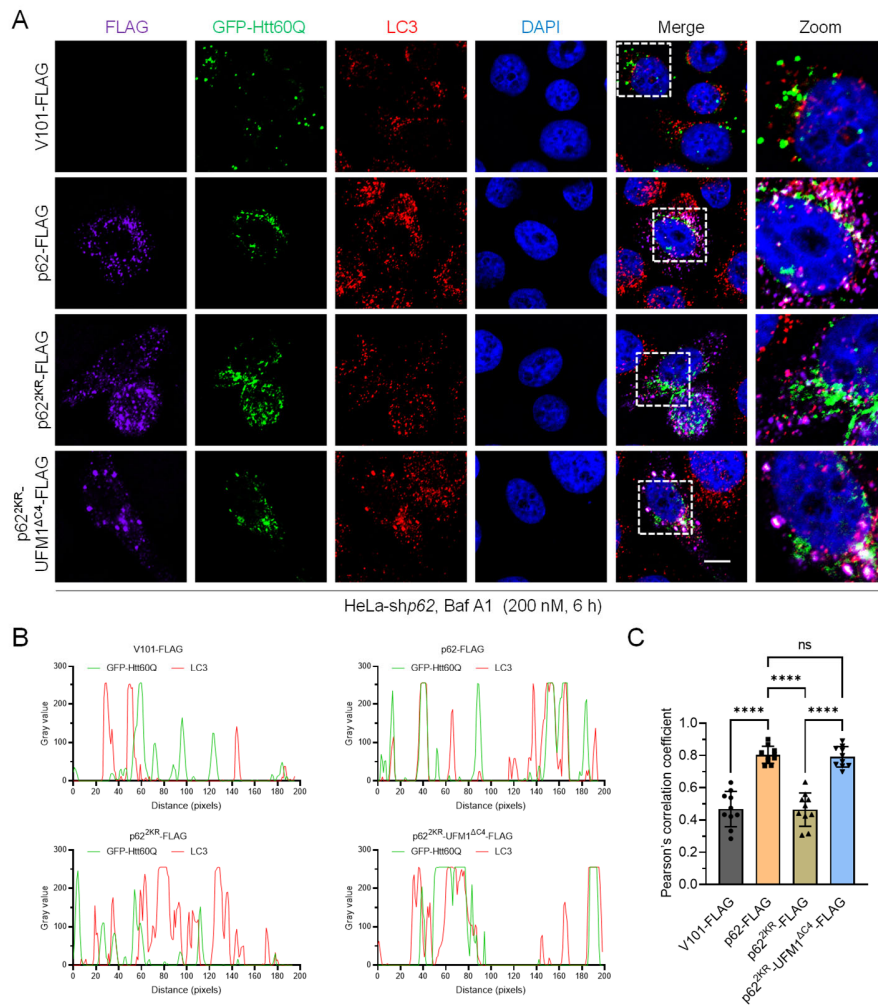


**Figure S4. UFMylation-deficient p62 mutant abolishes the effect of p62 on LC3. (A-C)** HeLa or MCF-7 cells were transfected with pcDNA3.1-FLAG, FLAG-p62, FLAG-p62<sup>K420R</sup>, FLAG-p62<sup>K435R</sup> or FLAG-p62<sup>2KR</sup> plasmid for 48 h and then treated with CQ (50  $\mu$ M for 12 h) (A), Baf A1 (200 nM for 4 h) (B), and CQ (50  $\mu$ M for 12 h) (C). Left

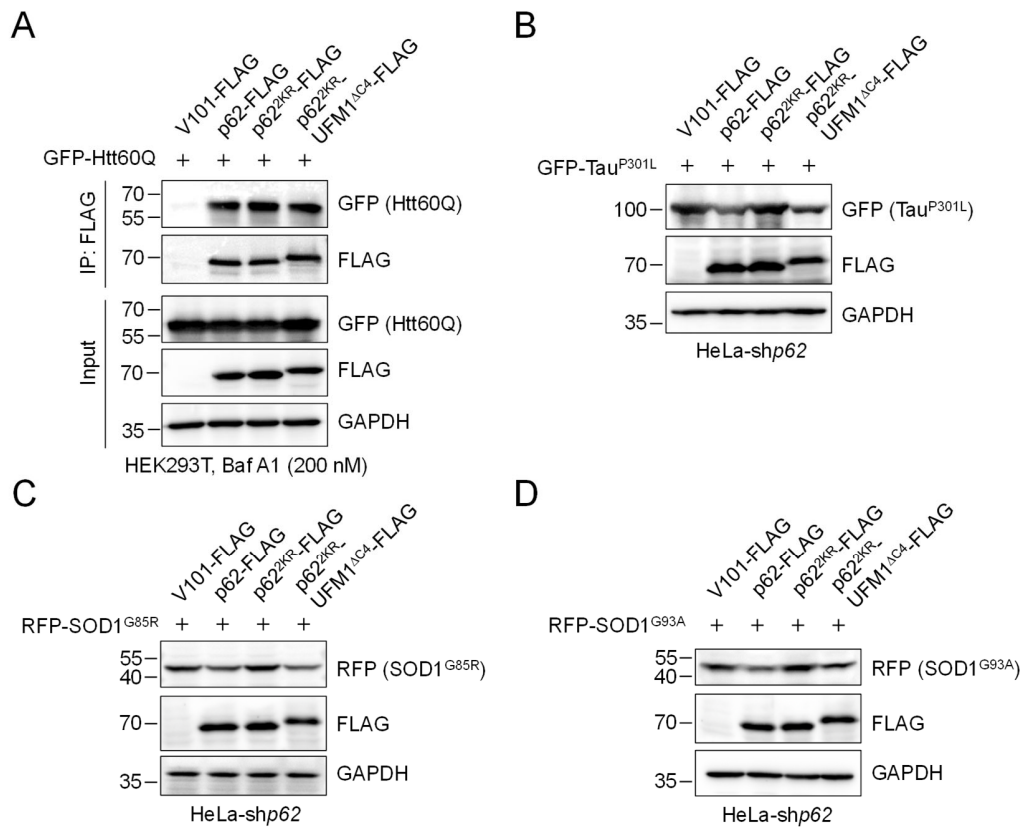
panels: Immunoblotting analysis of cell lysates. Middle panels: Quantification of immunoblotting results from three biological replicates. Right panels: qPCR analysis of *LC3* mRNA. The relative protein levels of LC3II were normalized to GAPDH and quantified. Immunoblotting data were presented as mean  $\pm$  SD (n = 3 biological replicates) and qPCR data were obtained from four technical replicates. Similar results were obtained from two biological replicates of qPCR experiments. Two-way ANOVA with Tukey's multiple comparisons post hoc test, \*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ . **(D)** Verification of *p62* knockdown efficiency in HeLa cells. **(E-F)** Cell lysates from Figure 4I and 5J were subjected to immunoblotting analysis.



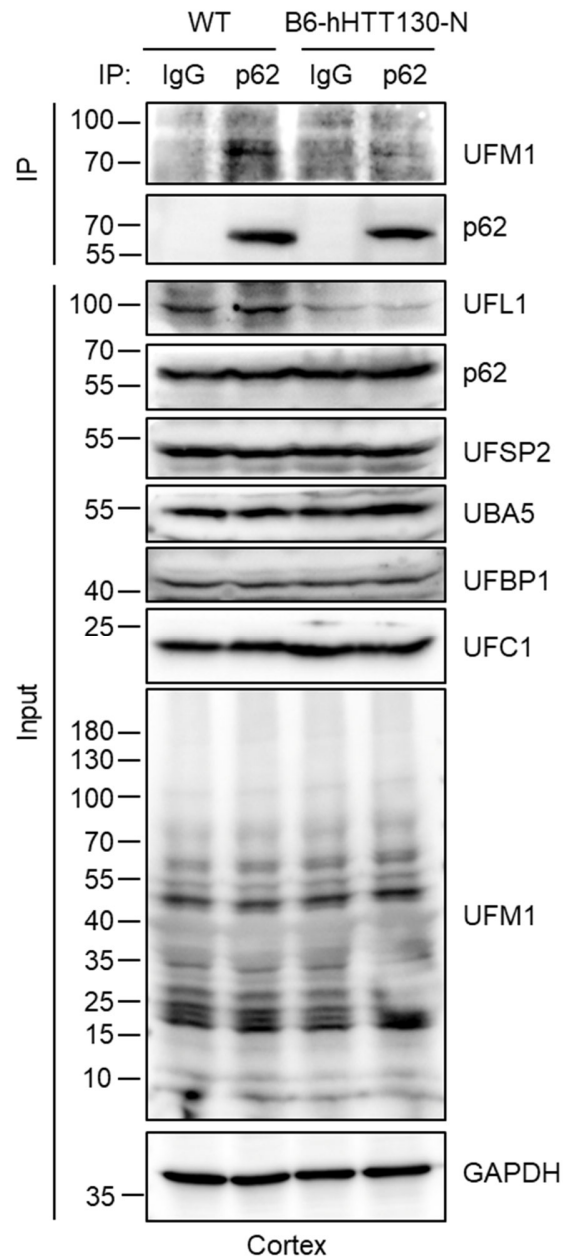
**Figure S5. UFM1 does not affect the p62 oligomerization and the interaction between p62 and ubiquitinated proteins.** (A) UFM1 does not affect p62 oligomerization. HEK293T-sg*UFSP2* cells were transfected with GFP-p62, FLAG-p62, and/or His<sub>6</sub>-UFM1 plasmids for 48 h. p62 was purified with anti-FLAG affinity gel and eluted with FLAG peptide (200 μg/mL). The cell lysates and affinity-purified samples were immunoblotted. (B) UFM1 does not influence the association of p62 with ubiquitinated proteins. HEK293T-sg*UFSP2* cells were transfected with His<sub>6</sub>-Ub, FLAG-p62, and His<sub>6</sub>-UFM1 plasmids for 36 h, and treated with MG132 (10 μM) for 12 h. Then, p62 was purified with anti-FLAG affinity gel and eluted with FLAG peptide (200 μg/mL). The cell lysates and affinity-purified samples were immunoblotted.



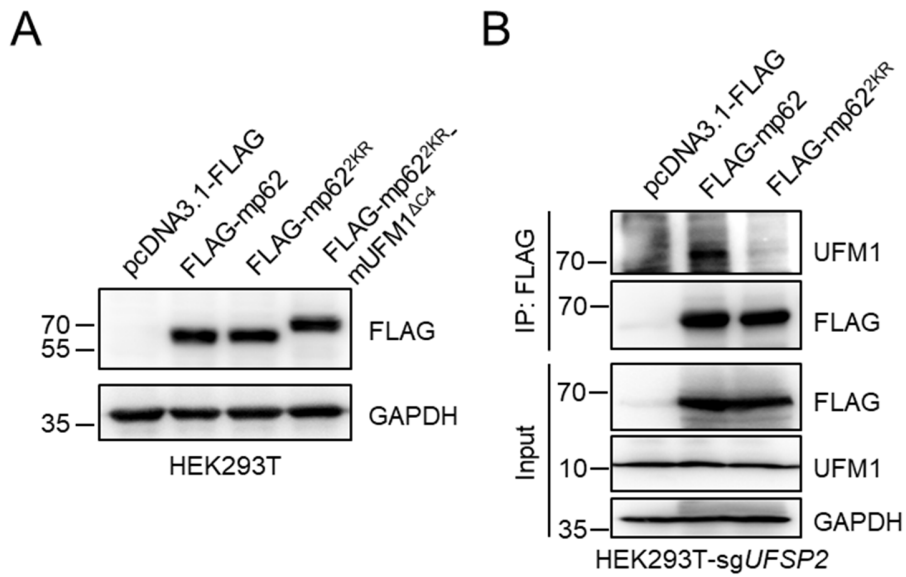
**Figure S6. The UFMylation of p62 promotes the colocalization of mHtt60Q with LC3 puncta.** (A-B) The *p62*-knockdown HeLa cells were transfected with V101-FLAG, p62-FLAG, p62<sup>2KR</sup>-FLAG, or p62<sup>2KR</sup>-UFM1<sup>ΔC4</sup>-FLAG, and GFP-Htt60Q plasmids for 42 h, treated with Baf A1 (200 nM) for 6 h, and then stained with FLAG, LC3, and DAPI. Representative confocal images (A) and colocalization analysis of GFP-Htt60Q with LC3 (B). Scale bar, 10  $\mu$ m. (C) The Pearson's correlation coefficient for the colocalization of GFP-Htt60Q and LC3. Mean  $\pm$  SD (n = 3 biological replicates with 10 cells), one-way ANOVA with Tukey's multiple comparisons post hoc test, \*\*\*\*:  $P < 0.0001$ , ns: not significant.



**Figure S7. The UFMylation of p62 is not required for substrate interaction but essential for the degradation of neurodegeneration-associated proteins.** (A) The UFMylation of p62 does not affect its binding to mHTT. HEK293T cells were transfected with V101-FLAG, p62-FLAG, p62<sup>2KR</sup>-FLAG, or p62<sup>2KR</sup>-UFM1<sup>ΔC4</sup>-FLAG, and GFP-Htt60Q plasmids for 42 h, treated with Baf A1 (200 nM) for 6 h, and then p62 was purified with anti-FLAG affinity gel and eluted with FLAG peptide (200 μg/mL). The cell lysates and affinity-purified samples were immunoblotted. (B-D) The UFMylation of p62 promotes the degradation of neurodegeneration-associated proteins. The p62-knockdown HeLa cells were transfected with V101-FLAG, p62-FLAG, p62<sup>2KR</sup>-FLAG, or p62<sup>2KR</sup>-UFM1<sup>ΔC4</sup>-FLAG, and GFP-Tau<sup>P301L</sup> (B), RFP-SOD1<sup>G85R</sup> (C), or RFP-SOD1<sup>G93A</sup> (D) plasmids for 48 h. Then the cell lysates were subjected to immunoblotting analysis.



**Figure S8. p62 UFMylation is decreased in the cortex of B6-hHTT130-N.** The cortex of WT or B6-hHTT130-N mice was lysed and endogenous p62 was immunoprecipitated with IgG or anti-p62 antibodies for immunoblotting analysis.



**Figure S9. Expression and UFMylation of murine p62 and its mutants.** (A) The expression of murine wild-type FLAG-mp62, UFMylation-deficient mutant FLAG-mp62<sup>2KR</sup> (K422R and K437R), and FLAG-mp62<sup>2KR</sup>-mUFM1<sup>ΔC4</sup> in HEK293T cells was determined by immunoblotting. (B) Biochemical validation of murine p62 UFMylation and its UFMylation sites. HEK293T-sgUFSP2 cells were transfected with His<sub>6</sub>-UFM1 and pcDNA3.1-FLAG, FLAG-mp62, or FLAG-mp62<sup>2KR</sup> (K422R and K437R) for 48 h. mp62 was purified with anti-FLAG affinity gel and eluted with FLAG peptide (200 μg/mL). The cell lysates and purified samples were immunoblotted.