NS1 binding protein regulates stress granule dynamics and clearance by inhibiting p62 ubiquitination

Pureum Jeon[1], Hyunji Ham[1], Haneul Choi[1], Semin Park[1], Jae-Woo Jang[1], Sang-Won Park[2], Dong-Hyung Cho[3], Hyun-Jeong Lee[4], Hyun Kyu Song[4], Masaaki Komatsu[5], Deok-Jin Jang[2] & Jin-A Lee[1,*]

[1]Department of Biological Sciences and Biotechnology, College of Life Sciences and Nanotechnology, Hannam University, Daejeon, Korea, [2]Department of Ecological Science, College of Ecology and Environment, Kyungpook National University, Sangju, Korea, [3]School of Life Sciences, BK21 FOUR KNU Creative BioRearch Group, Kyungpook National University, Daegu 41566, Korea, [4]Department of Life Sciences, Korea University, Seoul, Korea, [5]Department of Physiology, Juntendo University Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan

*Corresponding author

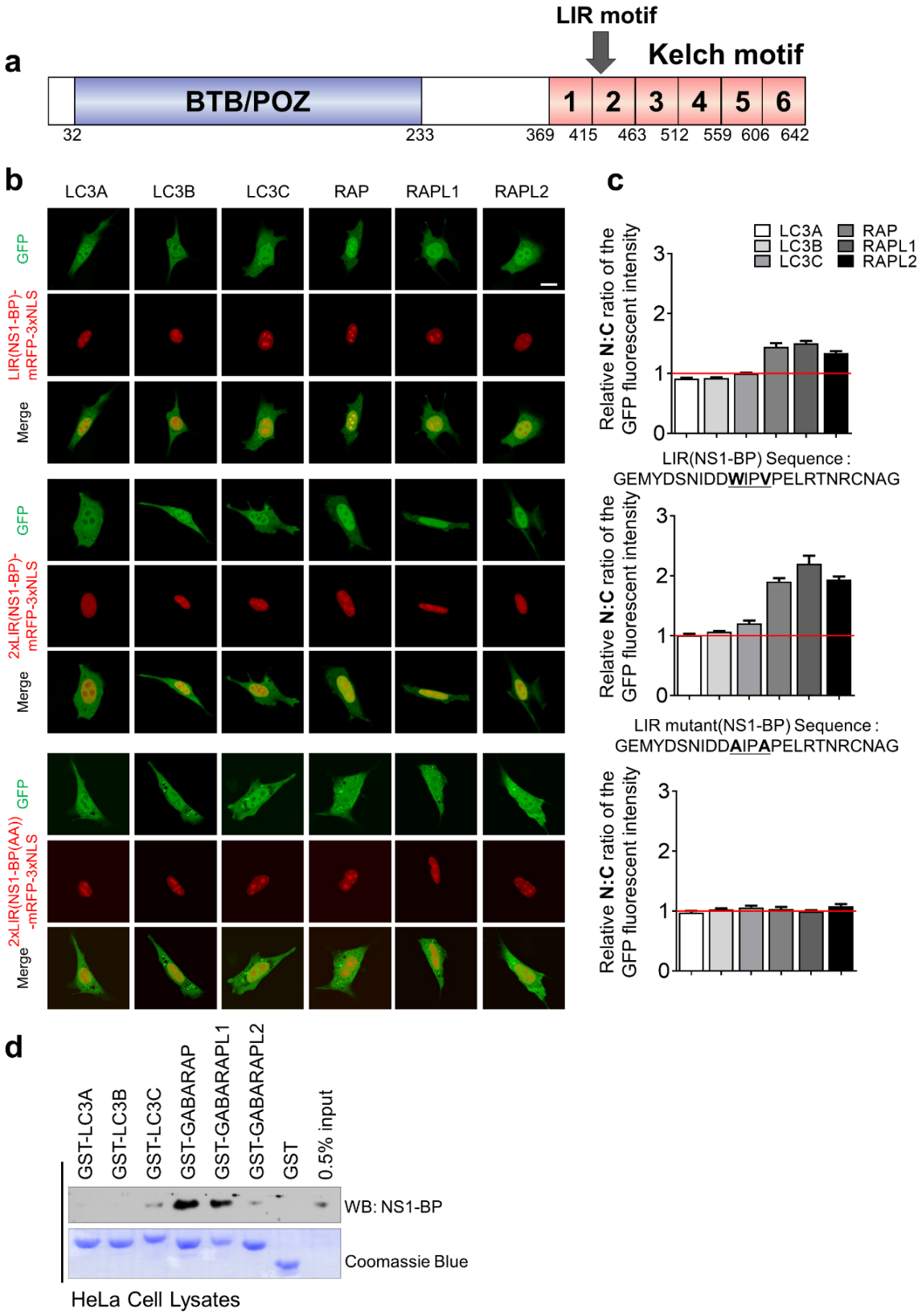
Jin-A Lee, PhD.;

ORCID : 0000-0002-6757-8371

1646, Yuseong-daero, Yuseong-gu, Daejeon 34054, Rep. of Korea

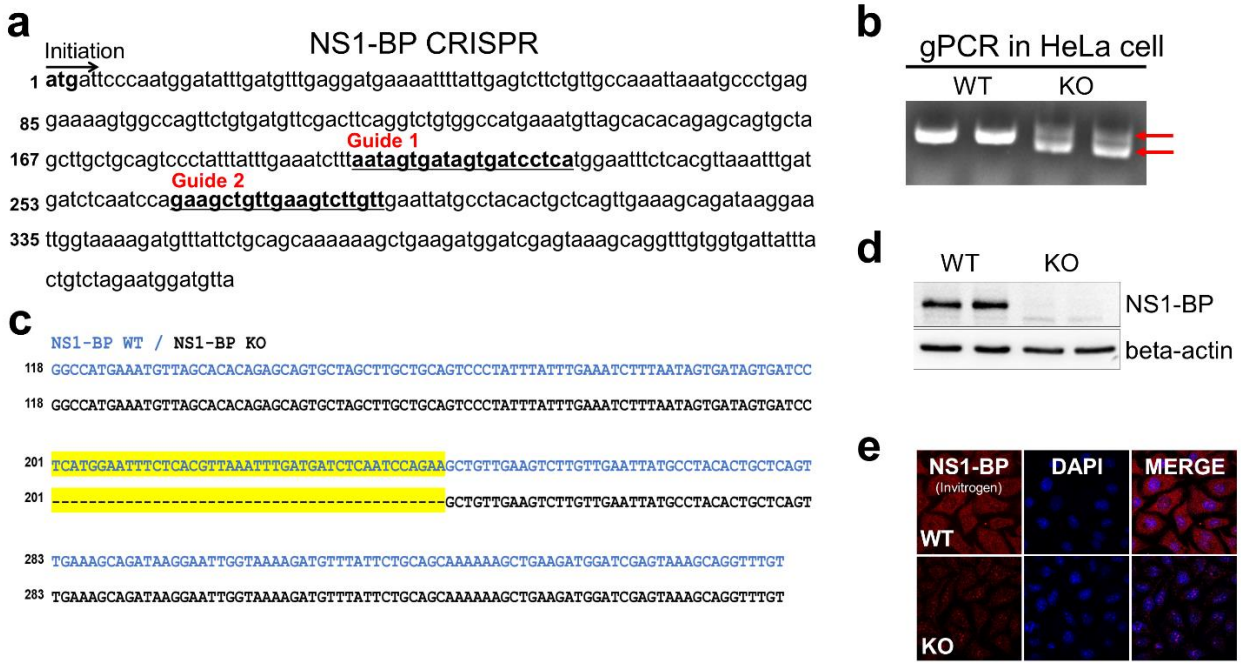
E-mail: leeja@hnu.kr

Tel: +82-42-629-8785; Fax: +82-42-629-8789

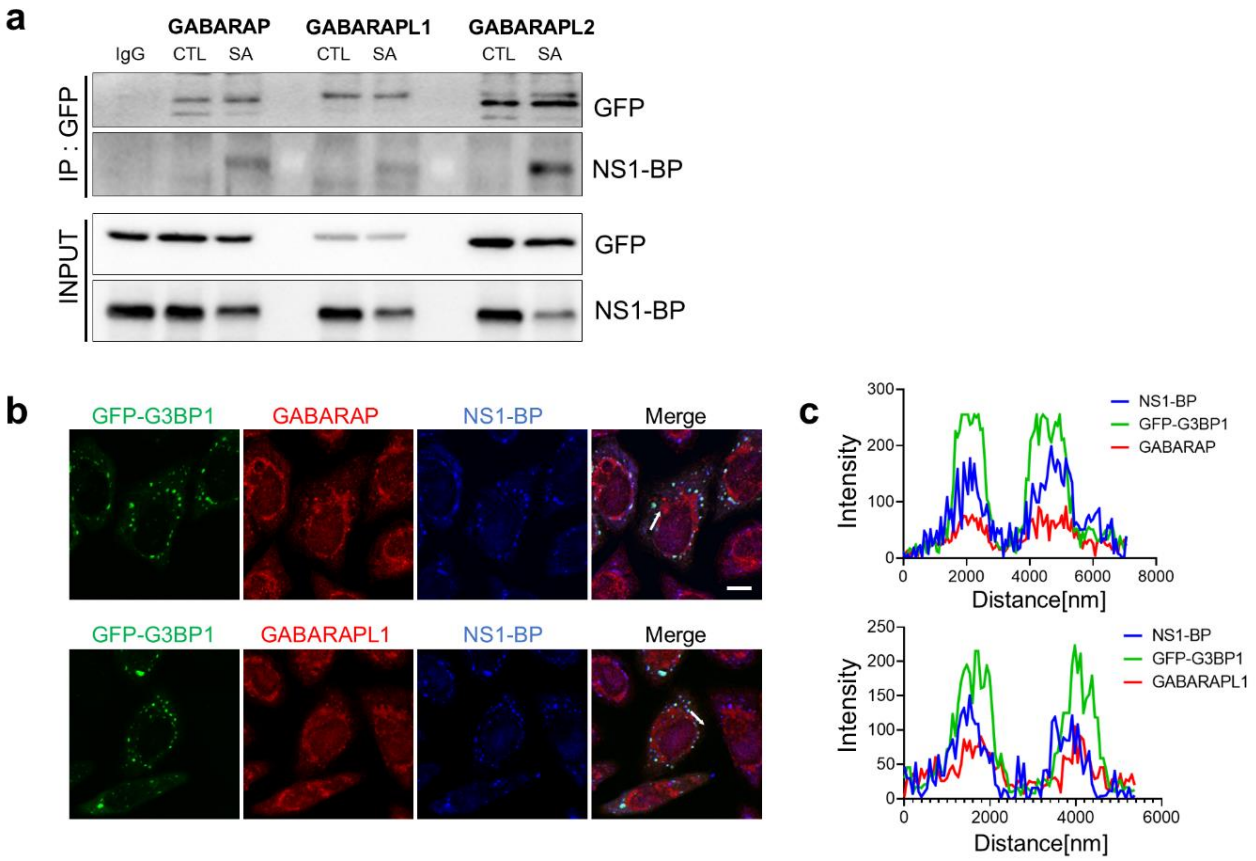


Supplementary Figure 1. Interaction of the LIR motif containing NS1-BP with GABARAP family proteins. **a** Schematic model of NS1-BP. **b** Representative images showing the cellular localization

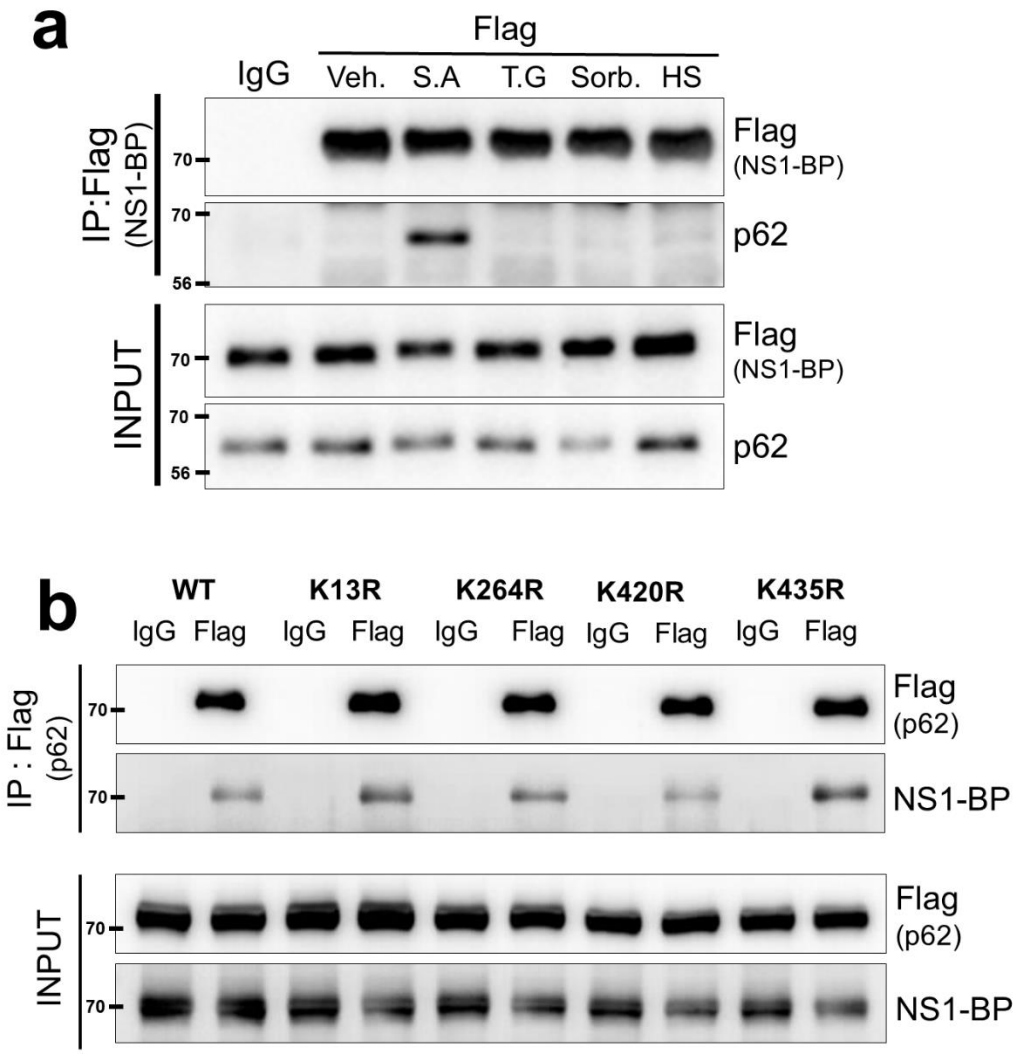
of EGFP-LC3/GABARAP with LIR(NS1-BP)-mRFP-3xNLS, 2xLIR(NS1-BP)-mRFP-3xNLS or 2xLIRmutant(NS1-BP(AA))-mRFP-3xNLS in MEFs. NLS; Nuclear localization signal. **c** Bar graphs showing the N/C ratio of the GFP fluorescent intensity. **d** GST-pulldown assay to analyze the binding of NS1-BP protein with LC3/GABARAP family proteins. The co-precipitated NS1-BP protein (upper panel) was analyzed through Western blotting (WB) using the anti-NS1-BP antibody. The immobilized GST fusion proteins are displayed on the Coomassie Brilliant Blue-stained gels (lower panel).



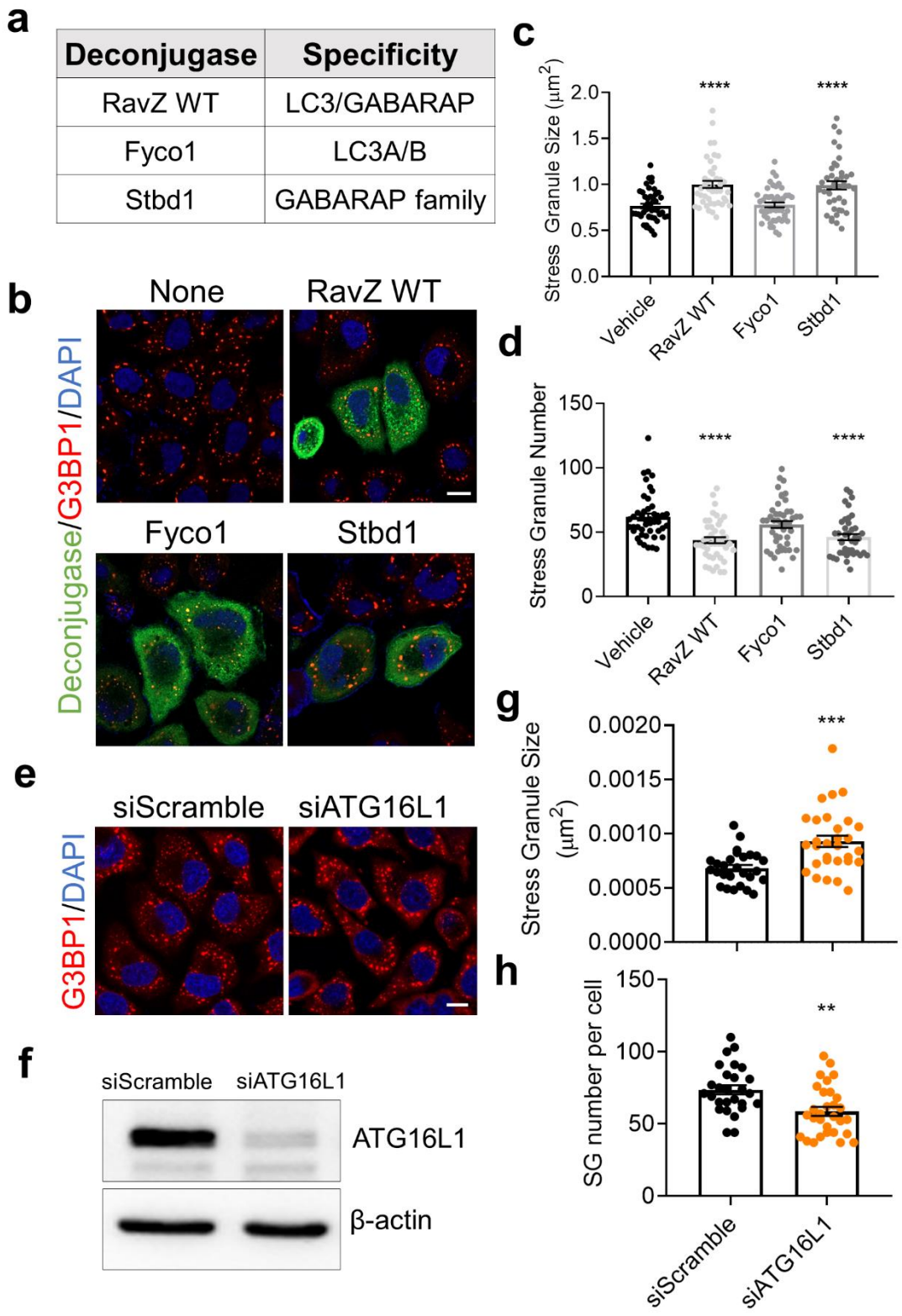
Supplementary Figure 2. Generation of NS1-BP KO HeLa cells using CRISPR/Cas9 technology. **a** Guide RNA sequence used for generating NS1-BP KO HeLa cells. **b** Genomic DNA PCR targeting of NS1-BP in NS1-BP WT and KO HeLa cells. The upper and lower red arrows indicate the WT and deletion regions within the NS1-BP gene. **c** Alignment of the region surrounding the CRISPR/Cas9 target sequence from genomic DNA extracted from NS1-BP WT and KO HeLa cells. **d** Immunoblotting showing NS1-BP protein levels in WT and KO cells. **e** Immunostaining depicting NS1-BP in WT and KO cells.



Supplementary Figure 3. NS1-BP interaction with GABARAP family proteins in a sodium arsenite-induced oxidative stress-dependent manner. **a** Co-immunoprecipitation (co-IP) and western blot using HEK293T cell lysates expressing GFP-GABARAP, GABARAPL1, GABARAPL2 with anti-GFP (or IgG) and anti-NS1-BP antibodies. Cells were treated with sodium arsenite(S.A) (0.5 mM, 1 h) or left untreated. **b** Representative images showing the cellular localization of GFP-G3BP1 with GABARAP or GABARAPL1 and NS1-BP under sodium arsenite treatment conditions. Scale bar, 10 μm. **c** Line scan graph showing the co-localization of GFP-G3BP1, GABARAP, or GABARAPL1, and NS1-BP.

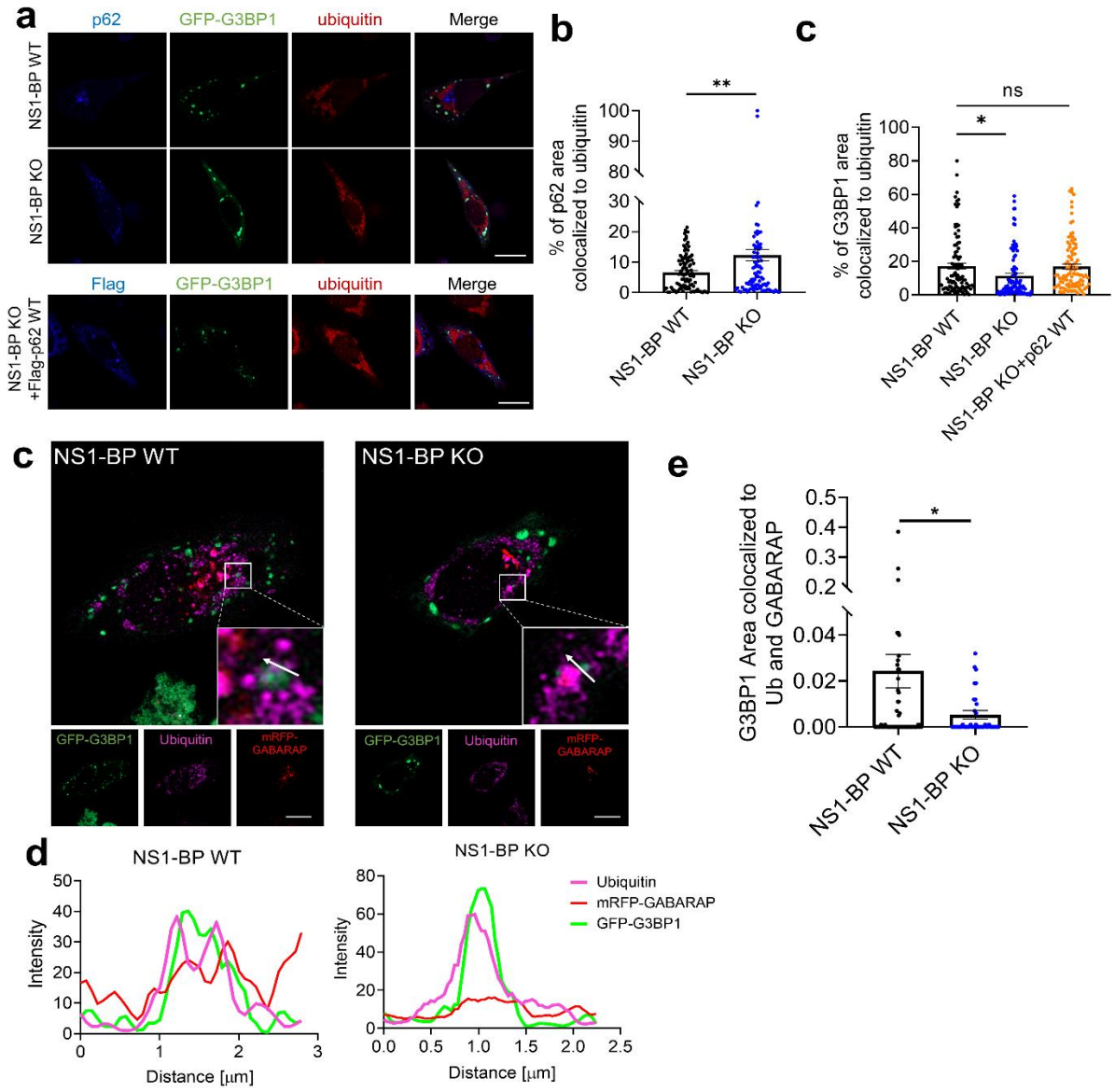


Supplementary Figure 4. NS1-BP interaction with p62 under oxidative stress conditions. **a** Immunoprecipitation and western blot using HEK293T cell lysates expressing Flag-NS1-BP with anti-Flag (or IgG) and anti-p62 antibodies. Cells were treated with various stressors (S.A 0.5 mM, 1 h; T.G 50 μM, 1 h; Sorbitol 0.4 M, 2 h; heat shock 42℃, 2 h). **b** Immunoprecipitation and western blot using HEK293T cell lysates expressing Flag-p62 WT or lysine mutant with anti-Flag (or IgG), anti-NS1-BP antibodies. Cells were treated with sodium arsenite (0.5 mM, 1 h).



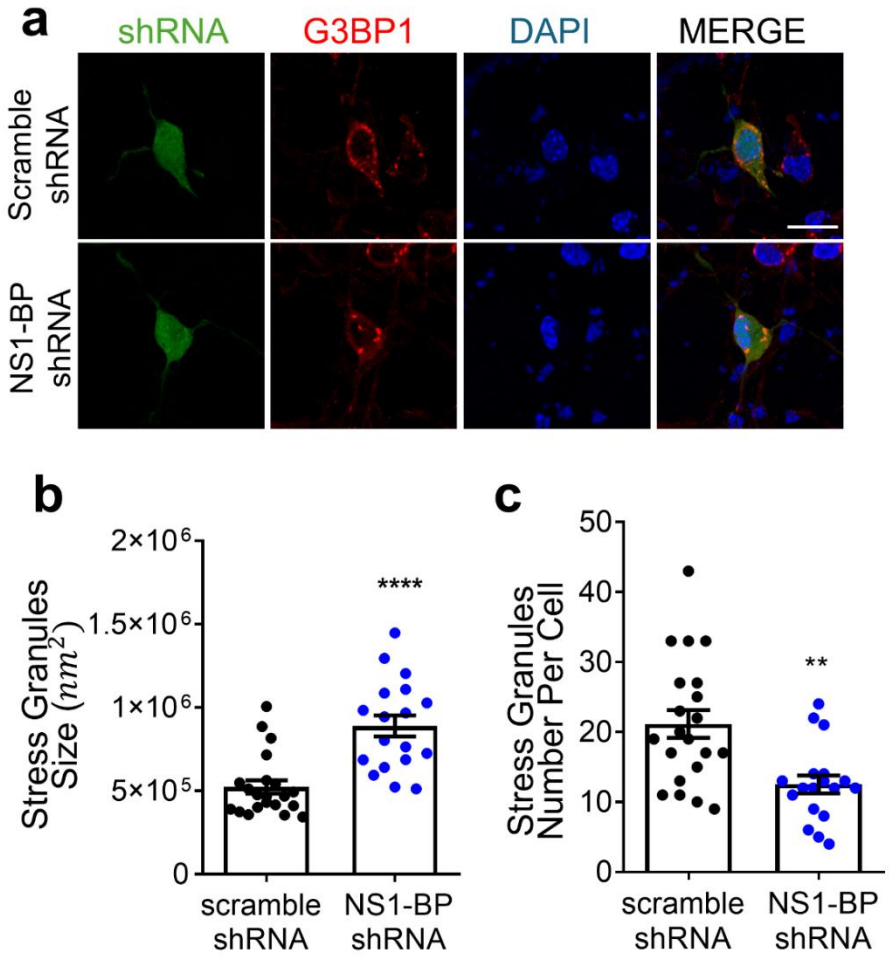
Supplementary Figure 5. Regulation of stress granules (SGs) by Autophagosome lipidated GABARAP family proteins and autophagy-related protein ATG16L1. **a** The table indicates the specificity of each deconjugase for LC3/GABARAP family proteins. **b** Representative images

showing G3BP1-positive SGs in HeLa cells expressing deconjugases (RavZ WT, Fyco1, Stbd1) upon sodium arsenite treatment condition. Scale bar, 10 μm. **c, d** Bar graphs showing the size(c) and number(d) of SGs. Data were quantified using a one-way ANOVA and presented as mean ± SEM (**** $p<0.0001$); Vehicle, n = 45; RavZ WT, n = 44; Fyco1, n = 46; Stbd1, n = 41. **e** Representative images showing G3BP1-positives SGs in ATG16L1 knockdown HeLa cells upon sodium arsenite treatment. Scale bar, 10 μm. **f** Western blot using anti-ATG16L1 and anti-β-actin in ATG16L1 siRNA expressing HeLa cell lysates. **g, h** Bar graphs showing the size(f) and number(g) of SGs. Data were quantified using a one-way ANOVA and presented as mean ± SEM (*** $p<0.001$); siScramble, n = 27; siATG16L1, n=30.



Supplementary Figure 6. Cellular localization of p62, ubiquitin, stress granules, and GABARAP. **a** Representative images showing the cellular localization of GFP-G3BP1 with endogenous p62,flag-p62 WT or ubiquitin and GABARAP under sodium arsenite treatment condition (S.A 0.5 mM, 1 h). Scale bar, 5 μm. **b, c** Bar graphs showing the percentage (%) of p62 area colocalized with ubiquitin (b) and percentage (%) of G3BP1 area colocalized with ubiquitin(c). Data were quantified using a two-tailed t-test and presented as mean ± SEM (** $p<0.005$); WT, n = 99; KO, n=100, KO + p62 WT, n=102. **c** Representative images showing the cellular localization of GFP-G3BP1 with ubiquitin and mRFP-GABARAP under sodium arsenite treatment condition (S.A 0.5 mM, 1 h). Scale bar, 5

μm. **d** Line scan graphs showing the localization of GFP-G3BP1, GABARAP, and Ubiquitin. **e** Bar graphs showing the co-localization and contact of GFP-G3BP1 and mRFP-GABARAP with Ubiquitin puncta in WT and NS1-BP KO cells. Data were quantified using a two-tailed t-test and presented as mean ± SEM (* $p<0.05$); WT, n = 74; KO, n = 47.



Supplementary Figure 7. Regulation of G3BP1-positive stress granules (SGs) by NS1-BP in mouse cortical neurons. **a** Representative images showing G3BP1 positive SGs in mouse cortical neurons expressing scramble or NS1-BP shRNA. Scale bar, 10 μm. **b** Graphs showing the number and size of SGs. Data were quantified using a two-tailed t-test and presented as mean ± SEM (**** $p<0.0001$); Scramble shRNA, n = 21; NS1-BP shRNA, n = 18.

Supplementary Table 1. Summary of the binding property of LIR motifs to each unlipidated mATG8(GA). Underline; core region in LIR motif.

The normalized N/C ratio: -1.4: - ; 1.4-2.0: +; 2.0-4.0: ++; 4.0: +++

LIR protein	LIR motif	LC3/GABARAP family interaction					
		LC3A	LC3B	LC3C	GABARAP	GABARAPL1	GABARAPL2
JMY	FALEETLESDWVAVRPHVFDEREKH	- (1.21±0.04; N=23)	- (1.18±0.03; N=24)	+ (1.44±0.05; N=26)	++ (3.13±0.29; N=27)	++ (3.12±0.22; N=25)	++ (2.30±0.15; N=27)
PLEKHM	QKVRPQQEDEWVNVQYPDQPEEPPE	++ (2.00± 0.13; N=19)	+ (1.76± 0.08; N=19)	+++ (4.46± 0.69; N=21)	++ (3.01± 0.27; N=19)	+++ (4.16± 0.43; N=21)	++ (2.68± 0.19; N=21)
TBC1D2B	RRTVFYTNEEWELLDPTPKDLEESI	+ (1.758±0.135, N=10)	+ (1.58±0.134, N=10)	+ (1.715±1.166, N=10)	+ (1.924±0.089, N=10)	++ (2.799±0.39, N=10)	++ (2.696±0.295, N=10)
UBR4	QEQSEVDHGDFEMVSESMVLETAEN	- (1.12± 0.02; N=20)	- (1.11± 0.02; N=20)	+ (1.46± 0.04; N=21)	++ (3.03± 0.20; N=20)	++ (2.14± 0.12; N=20)	+ (1.54± 0.07; N=21)
ZNF862	DITVYLLQEEWVLLSQQQKELCGSN	- (1.36±0.052, N=10)	+ (1.419±0.082, N=10)	++ (2.675±0.232, N=11)	+ (1.848±0.122, N=10)	++ (2.513±0.147, N=10)	++ (2.376±0.236, N=10)
IPO5/RANBP5	DMENMSDDDGWEFVNLGDQQSFGIK	- (1.385±0.052, N=5)	+ (1.835±0.087, N=5)	++ (2.851±0.476, N=5)	+++ (6.454±0.704, N=5)	+++ (7.401±0.913, N=5)	++ (3.583±0.387, N=5)
RANBP6	DVENMSDDDGWQFVNLGDQQSFGIK	- (1.14±0.052, N=6)	- (1.138±0.058, N=6)	+ (1.579±0.04, N=6)	+ (1.814±0.175, N=6)	++ (2.545±0.247, N=6)	+ (1.777±0.152, N=6)
PEX1	VEVEPLSADDWEILELHAVSLEQHL	+++ (4.47±0.683, N=5)	+++ (6.492±0.822, N=5)	+++ (5.309±0.403, N=5)	+++ (4.367±0.871, N=5)	+++ (9.565±2.16, N=6)	++ (3.927±0.447, N=5)
ADAMTS19	ELQFAPDREEWEVVFPALWRREPVD	+ (1.804±0.118, N=6)	+ (1.538±0.062, N=7)	+ (1.49±0.074, N=6)	++ (2.461±0.26, N=6)	++ (2.676±0.264, N=6)	++ (2.189±0.173, N=6)
ATXN2	MESILFKCSDFVVVQFKDMDSSYAK	- (1.148±0.061, N=5)	- (1.153±0.043, N=5)	++ (2.117±0.104, N=5)	+ (1.697±0.128, N=5)	+ (1.676±0.082, N=5)	+ (1.533±0.121, N=5)
NS1-BP	GEMYDSNIDDWIPVPELRTNRCNAG	- (0.912±0.016, N=21)	- (0.921±0.013, N=21)	- (0.992±0.013, N=23)	+ (1.445±0.061, N=20)	+ (1.502±0.042, N=20)	- (1.342±0.029, N=22)

Supplementary Table 2. Oligonucleotides used in this study

Name	Sequence (5' to 3')
p62 (ΔPB1) Forward	GTACAGAATTCGATGGCGCCCCGCAACATG
p62 (ΔPB1) Reverse	GTATGGGTACCTCACAACGGCGGGGG
p62 (ΔUBA) Forward	GACTAGAATTCGATGGCGTCGCTCACC
p62 (ΔUBA) Reverse	GTCATGGTACCTCACTGGGAGGGGTCCAG
p62 (KIR mutant) Forward 1	GACTAGAATTCGATGGCGTCGCTCACC
p62 (KIR mutant) Forward 2	TCAAAAGAAGTGGACAAGTCTACAGCGGAA
p62 (KIR mutant) Reverse 1	AGGGACTGGAGTTCCGCTGTAGACTTGTCC
p62 (KIR mutant) Reverse 2	GTATGGGTACCTCACAACGGCGGGGG
p62 (LIR mutant) Forward 1	GACTAGAATTCGATGGCGTCGCTCACC
p62 (LIR mutant) Forward 2	TCAGAGAAGCCCATCTCCAGCATCTGGGAG
p62 (LIR mutant) Reverse 1	TCTCCCAGATGCTGGAGATGGGCTTCTCTG
p62 (LIR mutant) Reverse 2	GTATGGGTACCTCACAACGGCGGGGG
p62 K7R Forward 1	CCGTCAGAATTAACCATGGACTACAAA
p62 K7R Forward 2	GCCCAGAAGGTAGGCCCTCACGGTG
p62 K7R Reverse 1	ATGGCGTCGCTCACCGTGAGGGCCT
p62 K7R Reverse 2	GTATGGGTACCTCACAACGGCGGGGG
p62 K13R Forward 1	TCTATATAAGCAGAGCTCGTTTAGTGA
p62 K13R Forward 2	GTCCTCGCGGCCCAGAA
p62 K13R Reverse 1	CTGGGCCGCGAGGACGC
p62 K13R Reverse 2	GCAGCTAGCGGTACCTCACAAC
p62 K157R Forward 1	GACTAGAATTCGATGGCGTCGCTCACC
p62 K157R Forward 2	GTCTGCGAGGGACGCGGCTTG
p62 K157R Reverse 1	CCGGTGCAAGCCGCGTCCCTC
p62 K157R Reverse 2	GTATGGGTACCTCACAACGGCGGGGG
p62 K165R Forward 1	TCTATATAAGCAGAGCTCGTTTAGTGA
p62 K165R Forward 2	GCGAGGCGGGTGTGCCC
p62 K165R Reverse 1	GCACACCCGCCTCGCATTC
p62 K165R Reverse 2	GCAGCTAGCGGTACCTCACAAC
p62 K189R Forward 1	GACTAGAATTCGATGGCGTCGCTCACC
p62 K189R Forward 2	CTCCGGAAGGTGCGCCACGGA
p62 K189R Reverse 1	CGAAGTGTCCGTGGCGCACCTT
p62 K189R Reverse 2	GTATGGGTACCTCACAACGGCGGGGG
p62 K264R Forward 1	GACTAGAATTCGATGGCGTCGCTCACC
p62 K264R Forward 2	CGGAGGGCGCAGAAGCCG
p62 K264R Reverse 1	CTTCTGCGCCCTCCGTGCTC
p62 K264R Reverse 2	GTATGGGTACCTCACAACGGCGGGGG
p62 K420R Forward 1	GACTAGAATTCGATGGCGTCGCTCACC
p62 K420R Forward 2	CCAGGCTCCTGCAGACCCGCAACTA

p62 K420R Reverse 1	GCTCCGATGTCATAGTTGCGGGTCT
p62 K420R Reverse 2	GTATGGGTACCTCACAACGGCGGGGG
p62 K435R Forward 1	TCTATATAAGCAGAGCTCGTTTAGTGA
p62 K435R Forward 2	GGGATGGCGTGAATACTGG
p62 K435R Reverse 1	TATTCACGCCATCCCCCGC
p62 K435R Reverse 2	CTCAAATGTCCCACCGGTT
p62 (751-1257) Forward	CTGGGCATTGAAGTTGATATCGATGTGGAG
p62 (751-1257) Reverse	GGTCTGCAGGAGCCTGGTGAG
p62 K420Q Forward	ACCAGGCTCCTGCAGACCCAGAACTATGACATCGGAGCGGCTCTGGA
p62 K420Q Reverse	TGCAGCTAGCGGTACCTTACAACGGCGGGGGATGCTTTGAATACTGGAT
p62 K435Q Forward	ACCAGGCTCCTGCAGACCAAGAACTATGACATCGGAGCGG
p62 K435Q Reverse	TGCAGCTAGCGGTACCTTACAACGGCGGGGGATGCTGTGAATACTGGAT
p62 K420/435Q Forward	ACCAGGCTCCTGCAGACCCAGAACTATGACATCGGAGCGGCTCTGGA
p62 K420/435Q Reverse	TGCAGCTAGCGGTACCTTACAACGGCGGGGGATGCTGTGAATACTGGAT
NS1-BP (ΔKelch) Forward	ATCCCAGATCTGCCACCATGATTCCCAATGGA
NS1-BP (ΔKelch) Reverse	AATGGGTACCTCACATGGGCTTTTC
NS1-BP (ΔBTB/POZ) Forward	TTACCGGAATTCTATGCAACAAGATGAG
NS1-BP (ΔBTB/POZ) Reverse	CGCGGATCC TTAAAACTGGAAAATCTT
CRISPR gRNA 1	AATAGTGATAGTGATCCTCA
CRISPR gRNA 2	GAAGCTGTTGAAGTCTTGTT
NS1-BP sgRNA Target 1	TATAGCGTGAGACACATCACATTTG
NS1-BP sgRNA Target 2	TATAGCCAGAAAATATTCCCCGGTG
pLVX-EF1a-3xFlag-p62 Forward (InFusion)	CGTGAATTCCTCGAGAGAACCGTCAGAATTAAC
pLVX-EF1a-3xFlag-p62 Reverse (InFusion)	CCGCTCTAGAACTAGGGTACCTCACAACGG