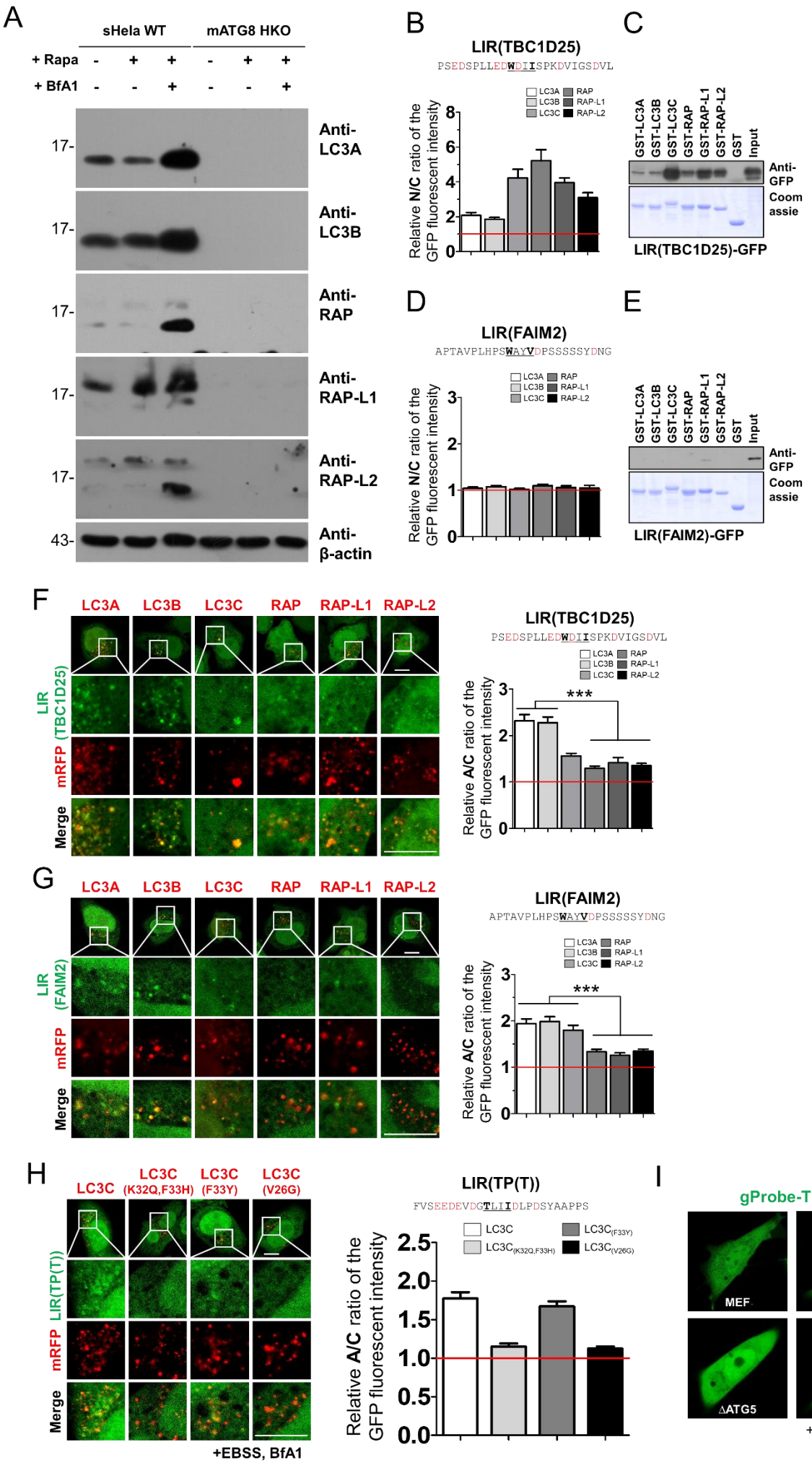
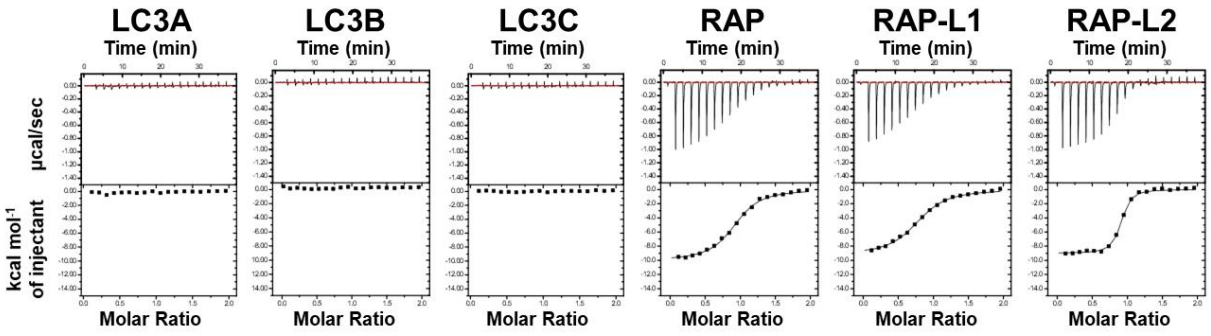

A
sHela WT
mATG8 HKO
+ Rapa
+ BfA1
17-
17-
17-
17-
17-
43-
Anti-LC3A
Anti-LC3B
Anti-RAP
Anti-RAP-L1
Anti-RAP-L2
Anti-β-actin
B
LIR(TBC1D25)
PSEDSPLLEDWDIISPKDVIGSDVL
LC3A
LC3B
LC3C
RAP
RAP-L1
RAP-L2
Relative N/C ratio of the GFP fluorescent intensity
C
GST-LC3A
GST-LC3B
GST-LC3C
GST-RAP
GST-RAP-L1
GST-RAP-L2
GST
Input
Anti-GFP
Coomassie
LIR(TBC1D25)-GFP
D
LIR(FAIM2)
APTAVPLHPSWAYVDPSSSSSYDNG
E
LIR(FAIM2)-GFP
F
LC3A
LC3B
LC3C
RAP
RAP-L1
RAP-L2
LIR (TBC1D25)
mRFP
Merge
LIR(TBC1D25)
PSEDSPLLEDWDIISPKDVIGSDVL
***
Relative A/C ratio of the GFP fluorescent intensity
G
LIR (FAIM2)
LIR(FAIM2)
APTAVPLHPSWAYVDPSSSSSYDNG
H
LC3C
LC3C (K32Q,F33H)
LC3C (F33Y)
LC3C (V26G)
LIR(TP(T))
FVSEEDEVDGTLIIDLPDSYAAPPS
LC3C(F33Y)
LC3C(K32Q,F33H)
LC3C(V26G)
+EBSS, BfA1
I
gProbe-TP(T)
MEF
HeLa
ΔATG5
ΔATG7
+Rapa,NH4Cl

Figure S1. Characterization of various LIR-containing constructs in HKO cells lacking all mATG8 proteins. a, Autophagic flux assay to verify the presence or absence of mATG8 in HKO cells. HeLa wild-type cells and mATG8 HKO cells were prepared in an untreated group, +Rapa (100 nM, 2 h)-treated group, and +Rapa (100 nM, 2 h) +BafA1 (100 nM, 2 h)-treated group. Each mATG8 protein except LC3C was analyzed by western blot (WB) using the indicated anti-LC3A, -LC3B, -GABARAP, -GABARAP-L1, and -GABARAP-L2 antibodies. **b-g,** LIR motifs with different binding properties for lipidated or non-lipidated mATG8. **b**, Quantification of the N/C ratios of GFP fluorescence in HKO cells expressing LIR(TBC1D25)-mRFP-3xNLS and each GFP-mATG8(GA) ($n \geq 20$ for each group). **c**, Glutathione S transferase (GST) pull-down assays to analyze the binding of LIR(TBC1D25)-GFP. The coprecipitated LIR(TBC1D25)-GFP protein was analyzed by western blot (WB) using the indicated anti-GFP. **d**, Quantification of the N/C ratio of GFP fluorescence in HKO cells expressing LIR(FAIM2)-mRFP-3xNLS and each GFP-mATG8(GA) ($n \geq 20$ for each group). **e**, Glutathione S transferase (GST) pull-down assays to analyze the binding of LIR(FAIM2)-GFP. The coprecipitated LIR(FAIM2)-GFP protein was analyzed by western blot (WB) using the indicated anti-GFP antibodies. **f**, Cellular localization of the LIR(TBC1D25)-GFP and each mRFP-mATG8 in HKO cells (left) and quantification of the autophagosome and cytosol (A/C) ratio of GFP fluorescence (right). Values are presented as means + SEM ($n \geq 20$ for each group). ***P < 0.001. **g**, Cellular localization of the LIR(FAIM2)-GFP and each mRFP-mATG8 in HKO cells (left) and quantification of the autophagosome and cytosol (A/C) ratio of GFP fluorescence (right). Values are presented as means + SEM ($n \geq 20$ for each group). ***P < 0.001. Scale bar: 10 μm. **h**, Effects of the A/C distribution of mRFP-LC3C mutants coexpressed with LIR(TP(T))-GFP. (H) Cellular localization of mRFP-LC3C, mRFP-LC3C(K32Q,F33H), mRFP-LC3C(F33Y,GA), and mRFP-LC3C(V26G) coexpressed with LIR(TP(T))-GFP in HKO cells (Left). Scale bar: 10 μm. Quantification of the relative autophagosome and cytosol (A/C) ratio of GFP fluorescence in cells (Right). Values are presented as means + SEM ($n \geq 20$ for each group). ***P < 0.001 compared with LC3C-expressing cells. LIR(TP(T)), LIR(TP(T))-GFP. **i**, Detection of LC3C-positive autophagic membranes in wild-type HeLa cells but not in ATG5 or ATG7 KO HeLa cells and MEF cells. Cellular localization of RavZ$(\Delta CA)_{TP(T)}$-GFP in wild-type HeLa, ATG5(−/−) or ATG7(−/−) HeLa, or MEF cells. Scale bar: 10 μm.

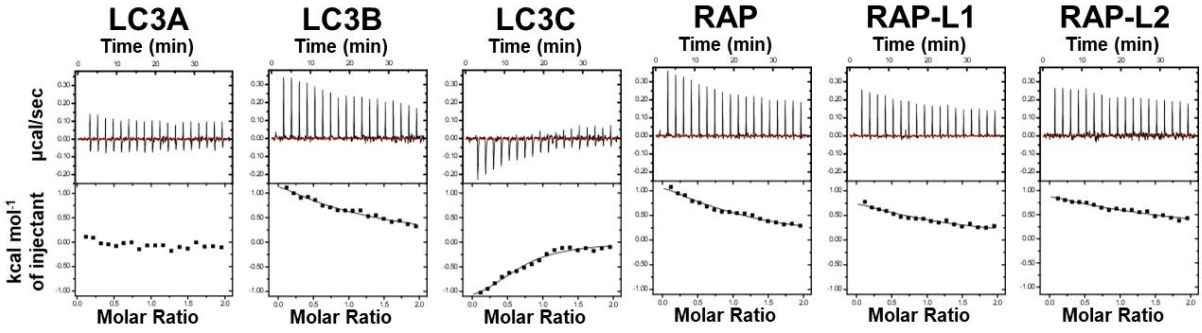
A



Summary of Kd values (µM) obtained by ITC

mATG8	LC3A	LC3B	LC3C	RAP	RAP-L1	RAP-L2
SpHfl1	N.D.	N.D.	N.D.	1.8±0.2	2.9±0.2	0.24±0.2

B

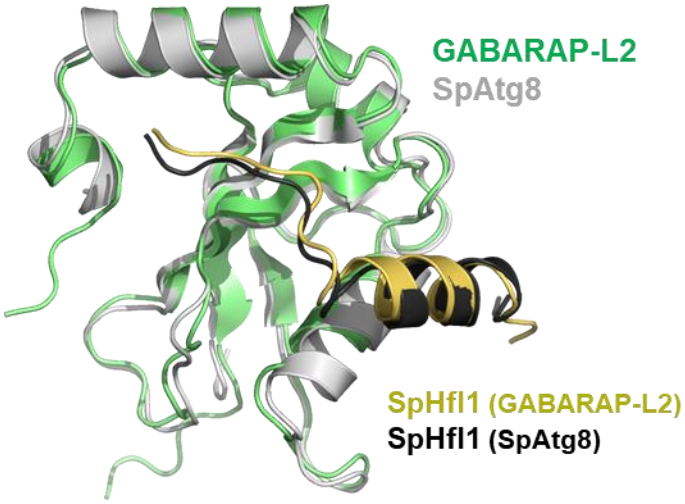


Summary of Kd values (µM) obtained by ITC

mATG8	LC3A	LC3B	LC3C	RAP	RAP-L1	RAP-L2
TP(T)	N.D.	228±127	26.9±6.9	132±17	196±33	671±97

Figure S2. ITC analysis of LIR(Sp) selective binding to GABARAP-L2 and LIR(TP (T)) selective binding to LC3C. a, ITC data of mATG8 protein binding to with LIR(Sp). **b**, ITC data of mATG8 protein binding to LIR(TP(T). RAP, GABARAP; RAP-L1, GABARAP-L1; RAP-L2, GABARAP-L2.

A



B

```
LC3A          ------MPSDRPFKQRRSFADRCKEVQQIRDQHPSKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELVKIIRRRLQLN
LC3B          ------MPSEKTFKQRRTFEQRVEDVRLIREQHPTKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELIKIIRRRLQLN
LC3C          MPPPQKIPSVRPFKQRKSLAIRQEEVAGIRAKFPNKIPVVVERYPRETFLPPLDKTKFLVPQELTMTQFLSIIRSRMVLR
GABARAP       --------MKFVYKEEHPFEKRRSEGEKIRKKYPDRVPVIVEKAPKAR-IGDLDKKKYLVPSDLTVGQFYFLIRKRIHLR
GABARAPL1     --------MKFQYKEDHPFEYRKKEGEKIRKKYPDRVPVIVEKAPKAR-VPDLDKRKYLVPSDLTVGQFYFLIRKRIHLR
GABARAPL2     --------MKWMFKEDHSLEHRCVESAKIRAKYPDRVPVIVEKVSGSQ-IVDIDKRKYLVPSDITVAQFMWIIRKRIQLP
SpAtg8        --------MRSQFKDDFSFEKRKTESQRIREKYPDRIPVICEKVDKSD-IAAIDKKKYLVPSDLTVGQFVYVIRKRIKLS
ScAtg8        --------MKSTFKSEYPFEKRKAESERIADRFKNRIPVICEKAEKSD-IPEIDKRKYLVPADLTVGQFVYVIRKRIMLP
Clustal Consensus               :*.  .:  *  :   *  :.  ::**: *:      :  :** *:*** .:.: ::  :** *: *

LC3A          PTQAFFLLVNQHSMVSVSTPIADIYEQEKDEDGFLYMVYASQETFGF--------------------
LC3B          ANQAFFLLVNGHSMVSVSTPISEVYESEKDEDGFLYMVYASQETFGMKLSV-----------------
LC3C          ATEAFYLLVNNKSLVSMSATMAEIYRDYKDEDGFVYMTYASQETFGCLESAAPRDGSSLEDRPCNPL
GABARAP       AEDALFFFVN-NVIPPTSATMGQLYQEHHEEDFFLYIAYSDESVYGL--------------------
GABARAPL1     PEDALFFFVN-NTIPPTSATMGQLYEDNHEEDYFLYVAYSDESVYGK--------------------
GABARAPL2     SEKAIFLFVD-KTVPQSSLTMGQLYEKEKDEDGFLYVAYSGENTFGF--------------------
SpAtg8        PEKAIFIFID-EILPPTAALMSTIYEEHKSEDGFLYITYSGENTFGTVFPF-----------------
ScAtg8        PEKAIFIFVN-DTLPPTAALMSAIYQEHKDKDGFLYVTYSGENTFGR--------------------
Clustal Consensus  . .*:::::: . :   :  :. :*.. :.:* *:*:.*:.:..:*
```

C

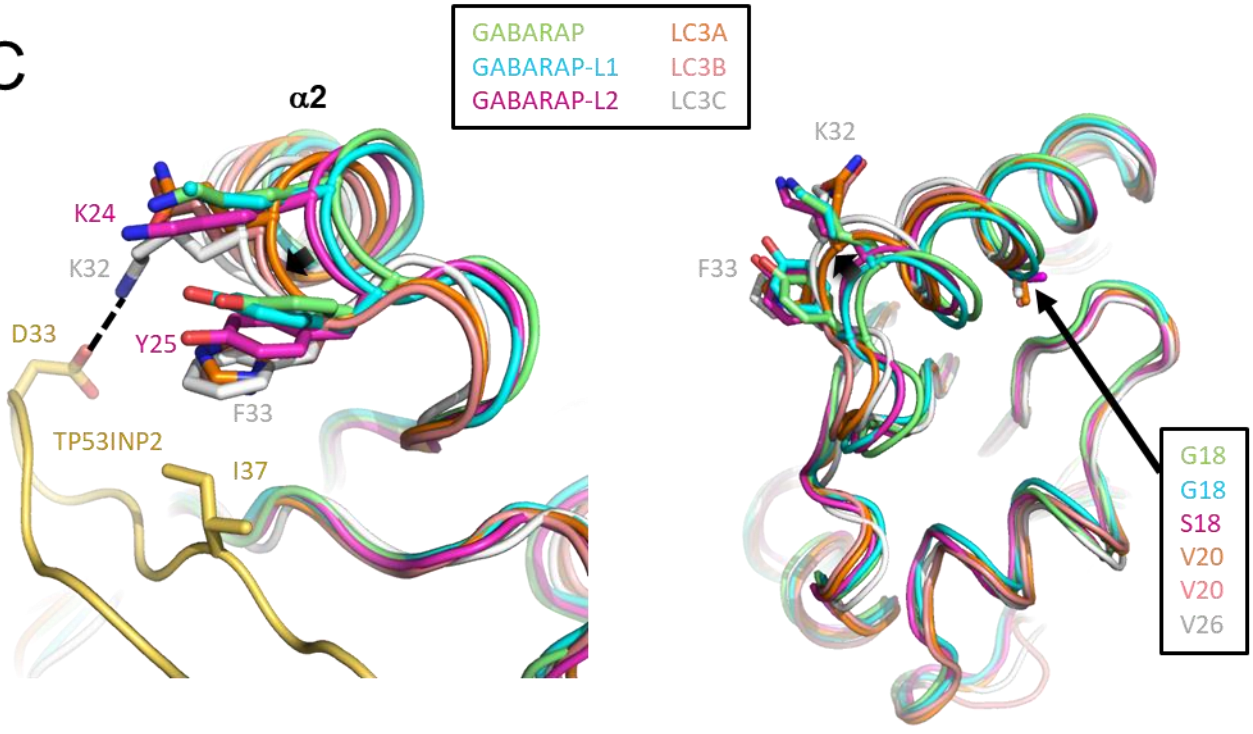


Figure S3. Residues responsible for specific interactions. a, Structural comparison of SpHFl1 LIR bound to GABARAP-L2 with that bound to SpAtg8 (PDBID 6AAF). The Cα atoms were used for superimposition. **b**, Sequence alignment of mATG8 proteins and fungal Atg8 proteins. Arrowheads indicate the residues that were involved in LIR binding but not strictly conserved among mATG8s. **c**, Structural comparison of the α2 helix of mATG8s. The Cα atoms were used for superimposition. The PDBIDs used were as follows: LC3A, 5CX3; LC3B, 5D94; LC3C, 3VVW; GABARAP, LIR(TP) complex (this study); GABARAP-L1, 6HOI; and GABARAP-L2, SpHfl1 LIR complex (this study).

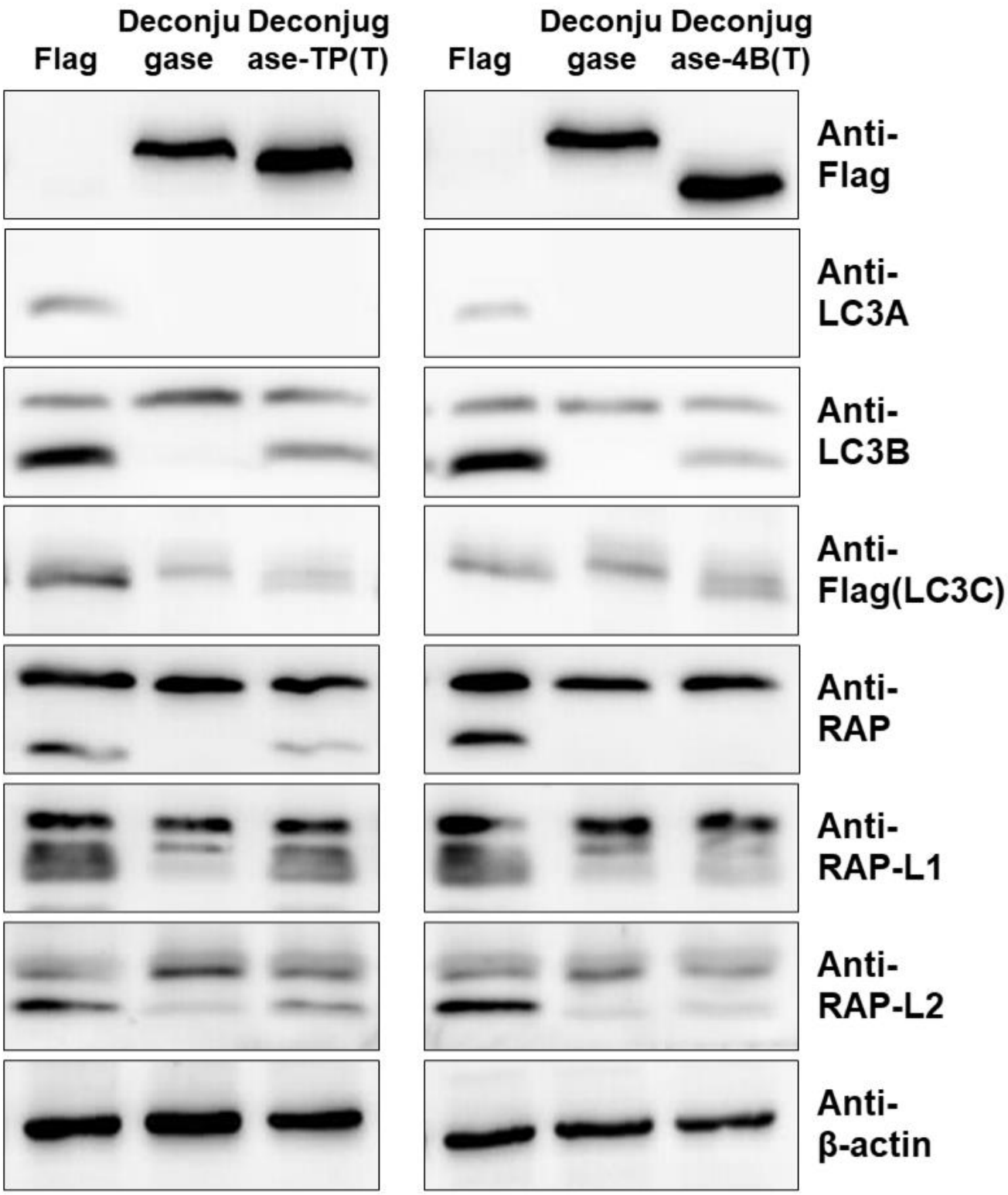


Figure S4. Non-selective delipidation of endogenous mATG8 by Deconjugase-TP(T) or Deconjugase-4B(T). Representative western blots of four independent experiments of endogenous mATG8 proteins after expression of Deconjugase-TP(T) and Deconjugase-4B(T) in HEK293T cells upon autophagy induction (100 nM rapamycin [Rapa] for 4 h).

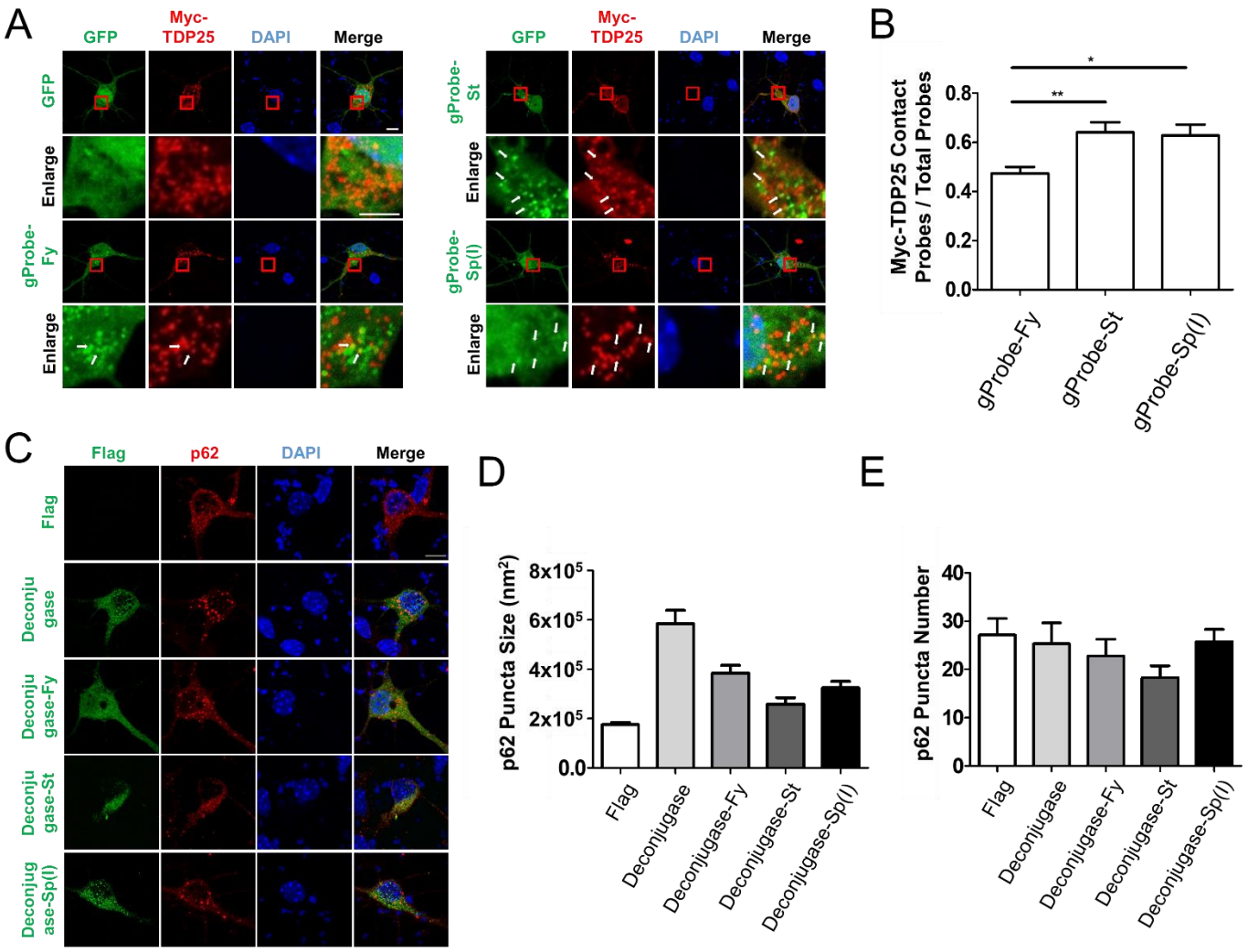


Figure S5. GABARAP subfamily-positive gProbe-X preferentially associated with TDP25-mediated aggregates, and the p62 puncta size was increased by Deconjugases. a, Cellular localization of Myc-TDP25 aggregates with gProbe-X. GFP empty vector was used as the control. X: Fy, St, and Sp(I). **b**, Quantification of relative Myc-TDP25 aggregates contacting the sensor. Values are presented as means + SEM ($n \geq 11$ for each group). *P < 0.05. **c**, Confocal images showing p62 puncta upon expression of Deconjugase-Y in mouse cortical neurons. Y: Fy, 4B(T), St, and Sp(I). **d**, Size of p62 puncta. **e**, Number of p62 puncta (E). Values are presented as means + SEM ($n \geq 11$ for each group).

Table S1: Related to Figure 3. Data collection and refinement statistics.

	LIR (SpHfl1) - GABARAL-L2	LIR (TP53INP2) - GABARAP
Data collection		
Space group	$P4_32_12$	$P4_1$
Cell dimensions		
a, *b*, *c* (Å)	45.85 45.85 152.59	46.39 46.39 75.40
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	43.91-1.86 (1.92-1.86)	46.39-1.75 (1.81-1.75)
R_{sym} or R_{merge}	0.154 (1.071)	0.099 (1.061)
$I / \sigma I$	10.7 (1.7)	10.8 (1.3)
Completeness (%)	99.9 (98.7)	99.7 (97.7)
Redundancy	12.6 (12.3)	7.0 (6.9)
CC1/2	0.996 (0.708)	0.994 (0.766)
Refinement		
Resolution (Å)	43.91-1.86	46.39-1.75
No. reflections	14553	16066
R_{work} / R_{free}	0.178/0.198	0.185/0.212
No. atoms		
Protein	1136	1079
Ligand	0	9
Water	129	75
B-factors		
Protein	37.2	49.2
Ligand		105.7
Water	40.7	53.5
R.m.s. deviations		
Bond lengths (Å)	0.007	0.007
Bond angles (°)	1.17	1.12

Values in parentheses are for highest-resolution shell.

Table S2. Summary of the binding property of RavZ(ΔCA)LIR(X)-EGFP probes to each lipidated mATG8. The A/C ratio: -1.4: - ; 1.4-2.0: +; 2.0-4.0: ++; 4.0: +++

Probe	LC3/GABARAP family interaction					
	LC3A	LC3B	LC3C	GABARAP	L1	L2
gProbe-Sp	+ (1.467±0.042, N=31)	+ (1.530±0.036, N=30)	+ (1.639±0.067, N=30)	+ (1.851±0.045, N=30)	+ (1.875±0.087, N=31)	+++ (5.270±0.205, N=22)
gProbe-Sp(I)	- (1.130±0.050, N=21)	- (1.183±0.045, N=20)	- (1.289±0.049, N=21)	- (1.306±0.079, N=20)	- (1.234±0.059, N=20)	+++ (4.952±0.393, N=20)
gProbe-TP(T)	- (1.202±0.056, N=20)	- (1.158±0.070, N=22)	+++ (4.085±0.322, N=23)	- (1.169±0.064, N=20)	- (1.235±0.055, N=20)	- (1.120±0.041, N=21)
gProbe-4B(T)	- (1.374±0.058, N=20)	- (1.375±0.055, N=20)	- (1.303±0.072, N=20)	+++ (4.666±0.182, N=20)	+++ (4.886±0.131, N=20)	- (1.367±0.050, N=20)
gProbe-Fy	+++ (4.898±0.132, N=29)	+++ (4.657±0.182, N=24)	+ (1.658±0.066, N=23)	- (1.067±0.033, N=20)	- (0.976±0.033, N=20)	- (1.071±0.041, N=20)
gProbe-St	- (1.249±0.044, N=22)	- (1.249±0.050, N=20)	- (1.279±0.058, N=20)	+++ (6.098±0.395, N=22)	+++ (5.971±0.463, N=22)	+++ (5.682±0.545, N=20)
gProbe-ULK2	- (1.125±0.038, N=21)	- (1.311±0.056, N=21)	+ (1.676±0.105, N=22)	+++ (5.262±0.261, N=22)	+++ (5.137±0.160, N=22)	+++ (4.227±0.262, N=22)
gProbe-Nix-p	++ (2.396±0.125, N=15)	++ (2.230±0.126, N=15)	+ (1.898±0.127, N=18)	++ (2.522±0.161, N=15)	+++ (4.539±0.175, N=15)	++ (2.375±0.129, N=15)