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Molecular determinants regulating selective binding of autophagy adapters and receptors to ATG8 proteins

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Autophagy is an essential recycling and quality control pathway. Mammalian ATG8 proteins drive autophagosome formation and selective removal of protein aggregates and organelles by recruiting autophagy receptors and adaptors that contain a LC3-interacting region (LIR) motif. LIR motifs can be highly selective for ATG8 subfamily proteins (LC3s/GABARAPs), however the molecular determinants regulating these selective interactions remain elusive. Here we show that residues within the core LIR motif and adjacent C-terminal region as well as ATG8 subfamily-specific residues in the LIR docking site are critical for binding of receptors and adaptors to GABARAPs. Moreover, rendering GABARAP more LC3B-like impairs autophagy receptor degradation. Modulating LIR binding specificity of the centriolar satellite protein PCM1, implicated in autophagy and centrosomal function, alters its dynamics in cells. Our data provides new mechanistic insight into how selective binding of LIR motifs to GABARAPs is achieved, and elucidate the overlapping and distinct functions of ATG8 subfamily proteins.

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Autophagy is an essential stress survival pathway transferring cytoplasmic material in autophagosomes to lysosomes for degradation, thereby restoring nutrients and molecular building blocks¹. Autophagy also ensures cellular health by selectively removing toxic macromolecules, damaged organelles, or intracellular pathogens^{2,3}. Accordingly, deregulation of autophagy has been implicated in a broad range of diseases, including cancer, neurodegenerative disorders, and infection.

The signaling and machinery proteins mediating autophagosome formation are highly conserved from yeast to human. In mammals, the ULK protein kinase complex initiates autophagosome formation by phosphorylating and activating the ATG14-Beclin1-phosphatidylinositol 3-phosphate (PI3P) kinase complex^{4,5}. Production of PI3P at autophagosome formation sites on the endoplasmic reticulum (ER) (omegasomes) recruits PI3P-binding effectors, DFCP1 and WIPI proteins⁶. WIPI2b is essential for the recruitment of the ATG12-5-16L1 complex⁷, which mediates in a ubiquitin-like conjugation reaction covalent attachment of cytosolic ATG8 proteins to phosphatidylethanolamine on the autophagic membrane^{8,9}.

Mammalian ATG8 proteins comprise two subfamilies, namely LC3s (LC3A, LC3B, and LC3C) and GABARAPs (GABARAP, GABARAP-L1, and GABARAP-L2)¹⁰. ATG8 proteins promote autophagosome formation, elongation, and closure, as well as fusion with lysosomes^{11–13}. In selective autophagy, ATG8 proteins (on the inner membrane) facilitate engulfment of cargo in autophagosomes by directly binding to autophagy receptors, such as p62¹⁴, NDP52¹⁵, or NBR1¹⁶. However, a growing number of ATG8 interactors are autophagy adaptors, which are not degraded by autophagy and fulfilling diverse functions ranging from regulation of autophagosome formation (e.g., ULK complex^{17,18}) and fusion with the lysosome (e.g., PLEKHM1¹⁹) to autophagosome transport (e.g., FYCO1²⁰).

Both autophagy receptors and adaptors contain an ATG8-interacting motif, more commonly known as LC3-interacting region (LIR) motif, which is recognized by ATG8 proteins^{21–23}. The canonical LIR motif is a small Θ -X₁-X₂- Γ ₃ motif, where Θ represents an aromatic residue (W/F/Y) and Γ an aliphatic residue (L/V/I) (whose side chains bind to hydrophobic pocket 1 (HP1) and HP2 of the LIR docking site (LDS), respectively) and X represents any amino acid (aa). The N-terminal region directly preceding the core LIR motif often harbors acidic or phosphorylated residues that stabilize ATG8 binding through electrostatic interactions^{21–23}.

Despite overlapping functions there is growing evidence for functional differences between LC3s and GABARAPs^{11–13,24}. Many LIR-containing proteins exhibit high selectivity for specific ATG8 proteins; however, the underlying mechanisms regulating binding selectivity are still poorly understood. A recent study focusing on residues in the core LIR motif, defined a GABARAP interaction motif (GIM), Θ -[V/I]-X₂-V, where valine or isoleucine in position X₁ and valine in position Γ ₃ promotes interaction with GABARAPs²⁵. However, no LC3 subfamily-specific interaction motif has been identified and not all GABARAP-specific LIRs possess a GIM, suggesting that there may be additional mechanisms regulating selective binding to LC3 and GABARAP subfamily proteins.

In this study, we examined the molecular determinants that regulate selective binding of the centriolar satellite (CS) marker protein PCM1 (pericentriolar material 1) and the ULK1 complex to GABARAPs. We identified key residues within the LIR motif and immediately C-terminal to the core LIR motif, as well as non-conserved, subfamily-specific LDS residues in ATG8 proteins, which confer binding specificity towards GABARAPs. Altering the binding specificity of the PCM1 LIR motif changes PCM1

dynamics in cells. Furthermore, selective degradation of the autophagy receptor NDP52 is impaired by rendering GABARAP more LC3B-like. Our data provide new mechanistic detail on how LIR binding specificity is achieved and will help elucidating the diverse biological functions of ATG8 proteins.

Results

PCM1 binds to GABARAP via a C terminally extended LIR motif

We recently showed that the CS protein PCM1 binds directly to GABARAP through a canonical LIR motif regulating GABARAP-specific autophagosome formation and GABARAP stability²⁶. PCM1 is a large scaffolding protein, coordinating assembly of CS, which are electron-dense granules surrounding the centrosome and implicated in centrosome assembly and ciliogenesis²⁷. We identified one main LIR motif (PCM1^{1955–1958}:FVKV) in the C-terminus of PCM1²⁶. Mutation of this LIR reduces GABARAP binding by more than 80%.

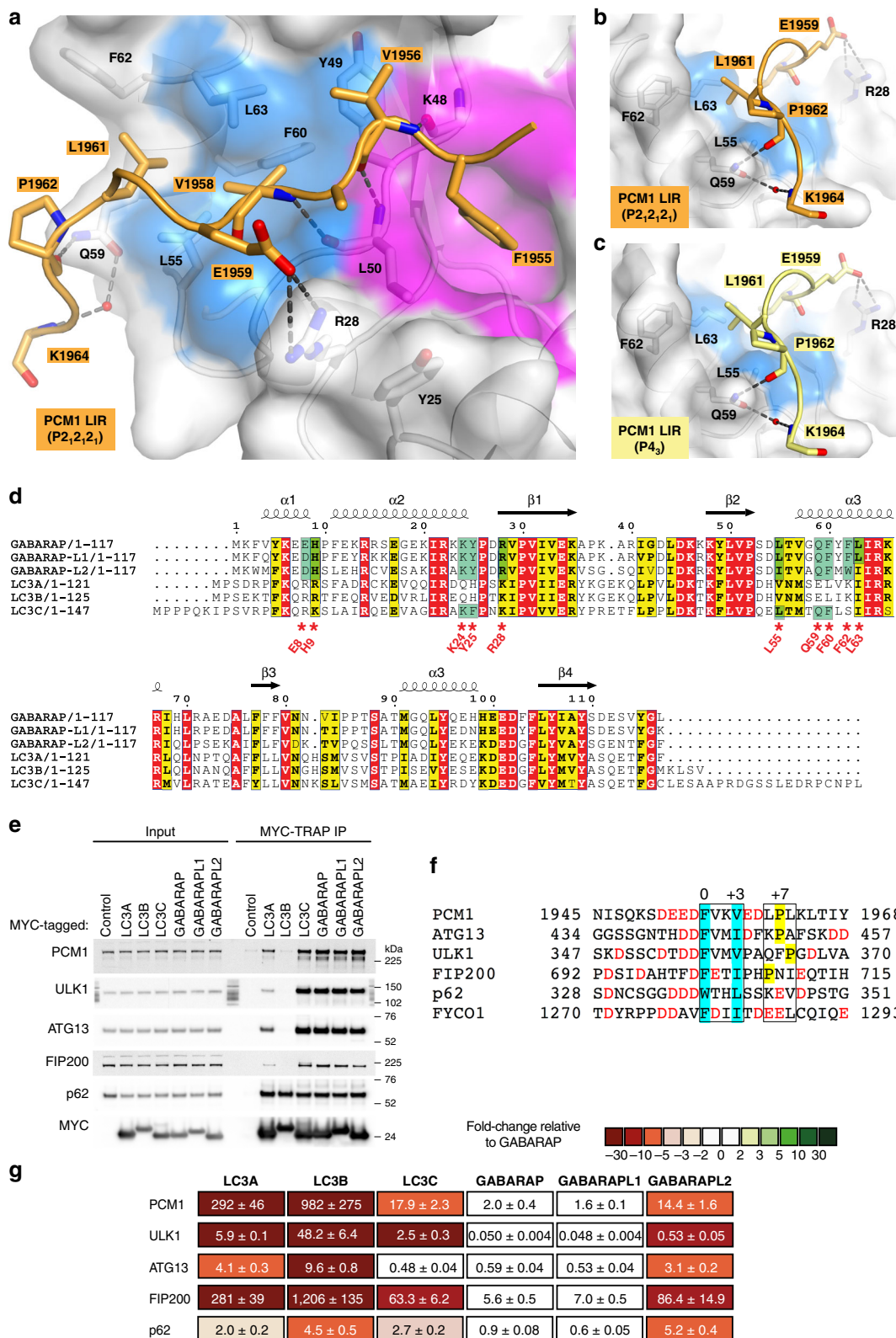
To elucidate the molecular basis of the PCM1-GABARAP interaction, we determined the crystal structure of the PCM1^{1951–1964} LIR motif bound to GABARAP. The structure was solved in two different space groups $P2_12_12_1$ (Fig. 1a, b and Supplementary Fig. 1b) and $P4_3$ (Fig. 1c and Supplementary Fig. 1a, c) at a resolution of 1.55 and 1.86 Å (Table 1), respectively. Both structures displayed identical canonical LIR interactions comprising PCM1 hydrophobic residues F1955 (Θ) and V1958 (Γ ₃) deeply bound to HP1 and HP2, as well as three hydrogen bonds formed between the main chains of PCM1 LIR residues V1956 (X₁) and V1958 (Γ ₃) and the main chains of GABARAP residues K48^{GAB} and L50^{GAB}. On top of these canonical LIR interactions additional specific contacts were observed in both PCM1:GABARAP structures. Within the core LIR motif, V1956^{PCM1} in position X₁ formed a hydrophobic interaction with Y49^{GAB} of HP2. Notably, the adjacent C-terminal region engaged in multiple interactions: (1) a salt bridge between E1959^{PCM1} (X₄) and R28^{GAB}, (2) a large hydrophobic contact between L1961^{PCM1} (X₆) and L55^{GAB}, F62^{GAB}, L63^{GAB}, and (3) two hydrogen bonds between the side chain of Q59^{GAB} and the main chain of P1962^{PCM1} (X₇), as well as K1964^{PCM1} (X₉) through a water molecule (Fig. 1a–c and Supplementary Fig. 1a). This proline residue at position X₇ was also critical for GABARAP binding to a mutational peptide array of the PCM1 LIR motif²⁶.

Most of the residues interacting with the C-terminal region of the PCM1 LIR are only conserved among GABARAP subfamily proteins (R28^{GAB}, L55^{GAB}, Q59^{GAB}, and F62^{GAB}) and LC3C (L64^{LC3C} and Q68^{LC3C}) (Fig. 1d). Thus, these residues may be critical for stabilizing association of the C-terminal region and mediating selective binding to the PCM1 LIR motif.

PCM1 binds strongly to GABARAP, GABARAP-L1, GABARAP-L2, and LC3C, and weakly to LC3A and LC3B²⁶ (Fig. 1e), which is the same binding behavior as reported for the ULK1 complex members ULK1, ATG13, and FIP200^{17,18,25} (Fig. 1e). The PCM1 LIR motif is similar to the human ULK1 LIR motif^{17,18,28} (Fig. 1f). PCM1, ULK1, and ATG13 LIR sequences contain GIMs²⁵ and interestingly the C-terminal regions of the PCM1, ATG13, ULK1, and FIP200 LIR motifs also exhibit proline residues at position X_{6–8}.

To better understand the selective ATG8 binding of these proteins, we used bio-layer interferometry (BLI) to determine the binding affinities (K_d values) of the individual LIR motifs to all recombinant ATG8 proteins (Fig. 1g).

The ULK1 LIR bound the strongest to GABARAP and GABARAP-L1 with K_d s of 50 and 48 nM, respectively, followed by the ATG13 LIR (K_d s of 0.59 and 0.53 μ M), which also showed a relatively strong affinity for LC3A (K_d of 4.1 μ M). Both PCM1



and FIP200 LIRs bound weakly to GABARAP/GABARAP-L1 with K_d s of 2.0/1.6 and 5.6/7.0 μ M, respectively. The LIR motif of the autophagy receptor p62 (SQSTM1) bound strongly to all six ATG8 proteins. The p62 LIR affinity to LC3B (4.5 μ M) is similar to the affinity (3.2 μ M) determined by isothermal calorimetry using a C terminally extended p62 LIR peptide²⁹. p62 binding to

LC3B (4.5 μ M) and GABARAP-L2 (5.2 μ M) was five-fold weaker than to GABARAP (0.9 μ M); however, these differences in binding are not detected in MYC-Trap immunoprecipitation (IP) experiments (Fig. 1e), where p62, due to all mammalian ATG8 proteins¹⁴. The binding specificities of ULK1, ATG13, FIP200,

Fig. 1 Pericentriolar material 1 (PCM1) binds to GABARAP via a C terminally extended LC3-interacting region (LIR) motif. **a** Structure of PCM1¹⁹⁵¹⁻¹⁹⁶⁴ LIR bound to GABARAP (P2₂,2₂). The PCM1 LIR sequence is shown in orange cartoon with interacting residues shown as sticks. GABARAP is displayed in white cartoon and transparent surface with hydrophobic pocket 1 and 2 colored in pink and blue surfaces, respectively. **b, c** Close-up view of Q59^{GAB} interaction with main chain carbonyl residues of P1962^{PCM1} and K1964^{PCM1} in two different crystal forms (**b**: P2₂,2₂; **c**: P4₃). **d** Sequence alignment of human ATG8-family orthologs using ESPript⁶¹. Identical (red) and similar residues (yellow) are boxed. Red asterisks and green boxes indicate non-conserved residues between LC3A/B and LC3C/GABARAPs further analyzed in this study. **e** MYC-TRAP immunoprecipitation (IP) of HEK293A cells expressing MYC-ATG8 constructs and immunoblot with indicated proteins. **f** Sequence alignment of LIR peptides. The core LIR motif is boxed and aromatic and hydrophobic residues in position Θ_0 and Γ_3 depicted in blue. Residues in position X₆₋₈ are boxed to highlight proline residues (yellow). Acidic residues are shown in red. **g** Affinities (K_d values) of LIR peptides to human ATG8 proteins determined by bio-layer interferometry (BLI). Color code indicates fold changes relative to wild-type (WT) GABARAP (data are mean \pm s.d., $n = 2-4$)

Table 1 Data collection and refinement statistics

	PCM1:GABARAP	PCM1:GABARAP	ATG13:GABARAP	ULK1:GABARAP
PDB ID	6HYL	6HYM	6HYN	6HYO
Resolution range	54.32-1.55 (1.61-1.55)	45.59-1.86 (1.92-1.86)	41.29-1.14 (1.18-1.14)	41.12-1.07 (1.10-1.07)
Space group	P2 ₂ ,2 ₁	P4 ₃	I23	I23
Unit cell	53.6 65.9 95.7 90 90 90	80.8 80.8 55.1 90 90 90	101.1 101.1 101.1 90 90 90	100.7 100.7 100.7 90 90 90
Total reflections	257,013 (24,635)	140,914 (13,154)	1,120,671 (76,985)	1,385,490 (108,563)
Unique reflections	49,150 (4840)	30,131 (3045)	62,488 (6252)	74,520 (7428)
Multiplicity	5.2 (5.1)	4.7 (4.3)	17.9 (12.3)	18.6 (14.6)
Completeness (%)	99.0 (95.7)	99.5 (97.7)	99.9 (99.9)	99.9 (100.0)
Mean $I/\sigma(I)$	11.1 (1.2)	19.7 (1.3)	23.7 (1.0)	23.9 (1.6)
Wilson B -factor	24.3	41.5	15.7	14.0
R -merge	0.05 (0.97)	0.02 (0.78)	0.04 (1.82)	0.04 (1.65)
R -meas	0.06 (1.08)	0.02 (0.89)	0.04 (1.90)	0.05 (1.71)
R -pim	0.02 (0.47)	0.01 (0.42)	0.01 (0.54)	0.01 (0.44)
CC1/2	0.998 (0.69)	1 (0.65)	1 (0.55)	1 (0.62)
CC*	1 (0.90)	1 (0.88)	1 (0.84)	1 (0.87)
Reflections used in refinement	48,794 (4680)	30,008 (2978)	62,465 (6252)	74,519 (7428)
Reflections used for R -free	2397 (261)	1524 (192)	3077 (326)	3593 (400)
R -work	0.24 (0.50)	0.18 (0.37)	0.22 (0.39)	0.14 (0.23)
R -free	0.28 (0.57)	0.21 (0.40)	0.23 (0.41)	0.16 (0.24)
Number of non-hydrogen atoms	2443	2366	1390	1414
Macromolecules	2209	2146	1219	1191
Ligands		28		52
Solvent	234	192	171	171
Protein residues	260	264	132	131
RMS (bonds)	0.00	0.02	0.01	0.01
RMS (angles)	0.52	1.24	1.30	1.39
Ramachandran favored (%)	99.6	99.6	99.2	98.5
Ramachandran allowed (%)	0.4	0.4	0.8	1.5
Ramachandran outliers (%)	0	0	0	0
Average B -factor	41.4	53.0	27.5	20.8
Macromolecules	41.1	52.6	26.3	17.9
Ligands		63.3		39.7
Solvent	44.1	56.4	36.2	35.6

and PCM1 LIR peptides were consistent with IP experiments (Fig. 1e). The strongest binding was measured for GABARAP and GABARAP-L1, followed by GABARAP-L2 and LC3C. LC3B was the weakest interactor with all four LIR peptides.

ATG13 and ULK1 bind GABARAP via extended LIR motifs.

Structural analyses of many LIR motifs demonstrated that N-terminal acidic residues directly preceding the core LIR motif participate in ATG8 binding²¹⁻²³. However, a role of the adjacent C-terminal region in stabilizing ATG8 binding, as reported, for example, for FYCO1³⁰⁻³², ALFY³³, and AnkG/AnkB³⁴, seems rather the exception.

Our affinity measurements showed that the LIR motifs of ATG13 and ULK1 exhibited very strong binding affinities for GABARAP. In order to find out whether their C-terminal regions are contributing to GABARAP binding in a similar way as observed for PCM1, we determined the crystal structure of ATG13⁴⁴¹⁻⁴⁵⁴ and ULK1³⁵⁴⁻³⁶⁶ LIR motifs bound to GABARAP

at a resolution of 1.14 and 1.07 Å, respectively (Table 1 and Supplementary Fig. 1d, e). The structure of ATG13 bound to LC3A, LC3B, and LC3C was determined in a previous study³⁵. However, residues C-terminal of the ATG13 core LIR motif were not included and ATG13 LIR structures in complex with GABARAP subfamily members have not been reported.

Both ATG13 and ULK1 structures (Fig. 2 and Supplementary Fig. 2) were solved near atomic resolution, which allowed the modeling of two alternate conformations for the ATG13 LIR in its C terminal moiety (Fig. 2a-c and Supplementary Fig. 2a, b). The ATG13 and ULK1 LIR motifs interacted with GABARAP in a very similar way (Supplementary Fig. 2c, d). In the N terminus, the main chain of the aspartate in position X₋₂ (D442^{ATG13} and D355^{ULK1}) formed a hydrogen bond with K48^{GAB} from HP1. Within the core LIR sequence (Θ_0 -X₁-X₂- Γ_3) the interactions were completely identical: (1) the canonical LIR interactions previously described for PCM1 structure, (2) in position X₁, a valine (V445^{ATG13}, V358^{ULK1}) was packed against the aromatic

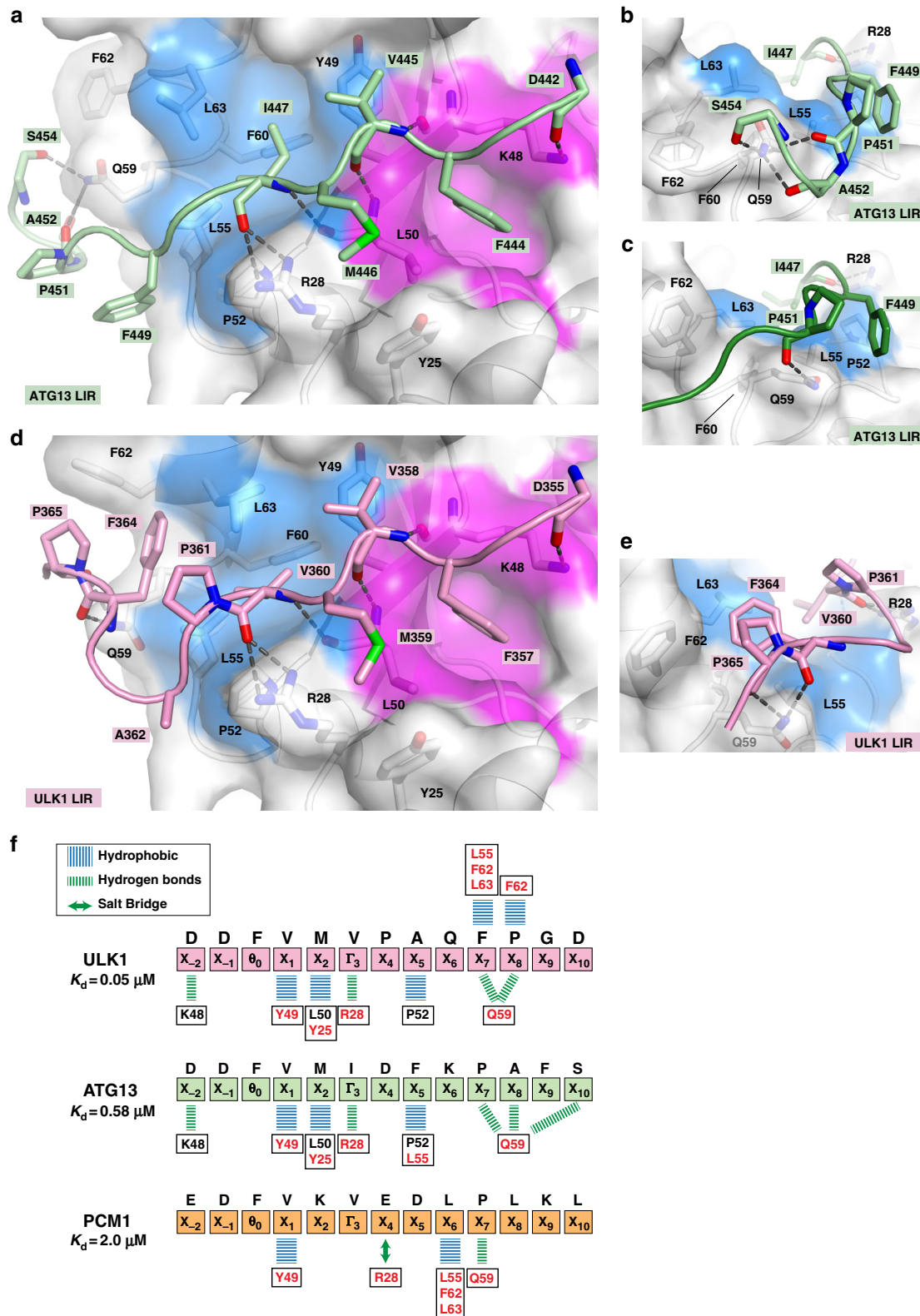


Fig. 2 ATG13 and ULK1 bind GABARAP via C terminally extended LC3-interacting region (LIR) motifs. **a** Structure of the ATG13⁴⁴¹⁻⁴⁵⁴ LIR bound to GABARAP in alternate conformation A. The ATG13 LIR sequence is shown in light green cartoon with interacting residues depicted as sticks. GABARAP is displayed in white cartoon and transparent surface with hydrophobic pocket 1 (pink) and 2 (blue). **b, c** Close-up view of Q59^{GAB} forming hydrogen bonds with main chain carbonyls of ATG13 LIR residues in alternate conformation A (light green) and B (dark green), respectively. **d** Structure of ULK1³⁵⁴⁻³⁶⁶ LIR-GABARAP complex. The ULK1 LIR sequence is shown in pink cartoon with interacting residues depicted as sticks. **e** Close-up view of interactions between GABARAP residues, Q59^{GAB}, F62^{GAB}, and L63^{GAB}, with ULK1 LIR residues in position X_{7,8} (F364^{ULK1}, P365^{ULK1}). **f** Schematic overview of non-canonical LIR interactions observed in the structures of GABARAP bound to ULK1 (pink), ATG13 (green), and PCM1 (orange) LIR motifs. Blue lines indicate hydrophobic interactions, green lines hydrogen bonds, and green double arrow salt bridges. Strictly conserved residues are boxed and displayed in black; non-conserved residues are boxed and displayed in red

ring of Y49^{GAB}, (3) in position X₂, a methionine was engaged in a large surface of hydrophobic interactions with L50^{GAB}/Y25^{GAB}, (4) in position Γ₃, the carbonyl of the hydrophobic residue formed two hydrogen bonds with the guanidinium group of R28^{GAB}. The C-terminal region of ATG13 and ULK1 LIR motifs showed similar interactions. F364^{ULK1} in position X₇ formed an extended hydrophobic interaction with L55^{GAB}, F62^{GAB}, and L63^{GAB} (similarly to L1961^{PCMI}, Supplementary Fig. 2d). This interaction was further stabilized by P365^{ULK1} in position X₈, which was packed against F62^{GAB} side chain and formed hydrophobic contacts with F364^{ULK1}. Thus F364^{ULK1} was sandwiched between P365^{ULK1} and L55^{GAB}/F62^{GAB}/L63^{GAB}. In position X₅, ATG13 has a phenylalanine residue, F449^{ATG13}, which engaged in more limited hydrophobic interactions with L55^{GAB} and P52^{GAB} from HP2 (Fig. 2a–c and Supplementary Fig. 2a, b). A361^{ULK1}, the corresponding X₅ residue in the ULK1 LIR, made hydrophobic contact only with P52^{GAB} (Fig. 2d). Finally, in both LIRs main chain carbonyls in position X₇, X₈, and X₁₀ (P451^{ATG13}, A452^{ATG13}, S454^{ATG13} and F364^{ULK1}, P353^{ULK1}) formed hydrogen bonds with Q59^{GAB} side chain (Fig. 2b–e and Supplementary Fig. 2c).

Overall, the structures of GABARAP bound to ATG13 and ULK1 LIRs exhibited similar interactions like the PCMI LIR-GABARAP complex (Fig. 2f and Supplementary Fig. 2c, d). Within the core LIR motif, a Valine in position X₁ formed hydrophobic contact with Y49^{GAB}. However, in contrast to M446^{ATG13} and M359^{ULK1}, the side chain of K1957^{PCMI} in position X₂ was partially disordered and the aliphatic part formed only limited hydrophobic contact with GABARAP.

In the C-terminal region, hydrophobic residues in position X_{5–7} interacted with the edge of the HP2, which is extended in GABARAP subfamily proteins by the presence of hydrophobic and aromatic residues (L55^{GAB}/GABL1/I55^{GABL2} and F62^{GAB}/GABL1/W62^{GABL2}), but not in LC3 subfamily proteins, which have fewer hydrophobic, and even charged, residues (V58^{LC3A/B}/L64^{LC3C} and K65^{LC3A/B}/S71^{LC3C}) in the corresponding positions (Fig. 1d). Most strikingly, hydrogen bonds and hydrophobic contacts of residues C-terminal to the core LIR motifs (positions X_{4–10}) seem to be critical in mediating strong association with GABARAP and involve residues, which are non-conserved between LC3 and GABARAP subfamily proteins. Hence, not only residues within the core LIR motif but also those C-terminal may confer binding selectivity to different ATG8 orthologs.

C-terminal region critical for ULK1 LIR binding specificity.

The C-terminal regions of the PCMI LIR and ULK complex LIR motifs are highly conserved (Supplementary Fig. 3a). To examine whether the C-terminal region may regulate selective binding to ATG8 proteins, we performed a peptide array analysis as well as BLI affinity measurements using truncated peptides of the ULK1 LIR motif (Supplementary Fig. 3b, c). Either six, eight, or all ten amino acids were deleted from the N or C terminus adjacent to the core LIR motif. Deletion of the whole sequence N-terminal to the core motif led to complete loss of binding (Supplementary Fig. 3b) and a more than 200-fold decrease in affinity to all six ATG8 proteins (Supplementary Fig. 3c) underscoring the importance of acidic residues in positions X₋₁ and X₋₄ for the binding affinity²¹. Strikingly, removal of the whole C terminus of the ULK1 LIR motif switched on binding to LC3A and LC3B (Supplementary Fig. 3b, c), suggesting that the proline residue and/or the alanine residue in position X_{4,5} (P361^{ULK1} and A362^{ULK1}) may inhibit LC3A and LC3B binding to the ULK1 LIR motif. Using this peptide array approach, removal of the C-terminal region of the PCMI LIR motif did not reveal any changes in binding specificity.

To further elucidate the role of P361^{ULK1} and A362^{ULK1} we analyzed the binding specificity of GST-GABARAP, GST-LC3B, and GST-LC3A to the ULK1 LIR motif using a mutational peptide array scan (Fig. 3a, b and Supplementary Fig. 3d). Every position within the 24-mer ULK1 LIR peptide was mutated to all alternative aa, revealing the importance of each individual residue within the LIR motif. The binding pattern of GST-LC3B (Fig. 3b) and GST-LC3A (Supplementary Fig. 3d) showed that residues in position X₄, but also position X₋₃, X₂ seem unfavorable for LC3A/B binding. Mutation of P361^{ULK1} (position X₄) to almost any other aa increased LC3A/B binding. To confirm the importance of residues in position X₋₃, X₂, and X₄, we performed a peptide array analysis using peptides that carry single point mutations (Fig. 3c), which strongly increased LC3B binding in the mutational peptide array scan (Fig. 3b). Whereas T354E^{ULK1} resulted in a slight increase, both M359I^{ULK1} and P361D^{ULK1} point mutations strongly increased the binding of all three LC3 subfamily members (Fig. 3c). T354E/M359I/P361D^{ULK1} substitutions lead to the strongest increase in binding of LC3 proteins. Consistently, HA-ULK1 T354E/M359I/P361D and endogenous ULK complex members ATG13 and FIP200 show substantial increased binding to LC3B in GST pull-down experiments (Fig. 3d).

This underscores the importance of position X₋₃, X₂, and X₄ within the ULK1 LIR motif in regulating selective binding to GABARAP over LC3 subfamily proteins. Moreover, our data show that the C-terminal region of the ULK1 LIR is involved in directing binding specificity towards GABARAP subfamily proteins and LC3C.

Position X₂ in the PCMI core LIR motif inhibits LC3 binding.

Which residues in the PCMI LIR regulate binding specificity? Our structural analysis showed that the region C-terminal to the PCMI core LIR motif contributes to stabilizing GABARAP interaction. However, in contrast to the ULK1 LIR motif, deletion of the C-terminal region of PCMI did not increase interaction with LC3 subfamily members. To determine the role of the C-terminal region and/or the other regions of the PCMI LIR motif in mediating selective binding to GABARAP proteins, we tested chimera peptide sequences of PCMI and FYCO1 LIR motifs for GST-ATG8 protein binding in a peptide array experiment (Fig. 4a). We chose FYCO1 because it preferentially interacts with LC3A and LC3B and several structural studies showed that the C-terminal region stabilizes LC3 binding^{30–32}.

Replacing the PCMI LIR C-terminus by the corresponding FYCO1 C-terminus did not increase LC3A and LC3B binding. However, introduction of the FYCO1 core LIR into the PCMI LIR motif strongly increased LC3 subfamily binding. This interaction was further enhanced when both the FYCO1 core LIR and FYCO1 C-terminal region were present. On the other hand, the PCMI core LIR motif strongly inhibited binding of the FYCO1 chimera LIR motif to both LC3 and GABARAP subfamily members. In addition, the PCMI C-terminus strongly decreased binding to LC3A and LC3B.

The PCMI core LIR motif was most critical for inhibiting LC3 subfamily binding. Consequently, we mutated individual residues to the corresponding residue present in the FYCO1 core LIR motif and determined ATG8 binding affinities (Fig. 4c). As observed before, introduction of the FYCO1 core LIR motif (X_{1,2} and Γ₃) specifically activated LC3 subfamily binding. Whereas mutation of the GIM motif residues (X₁ and Γ₃) had opposing or only mild effects, mutation of the lysine in position X₂ (K1957^{PCMI}) dramatically increased both LC3 subfamily (LC3B: 95-fold; LC3A/LC3C: 75- and 47-fold, respectively) and GABARAP subfamily (~12-fold) binding. Mutations of residues N-terminal (X₋₂, -1) and C-terminal (X₄, X₇) did not enhance

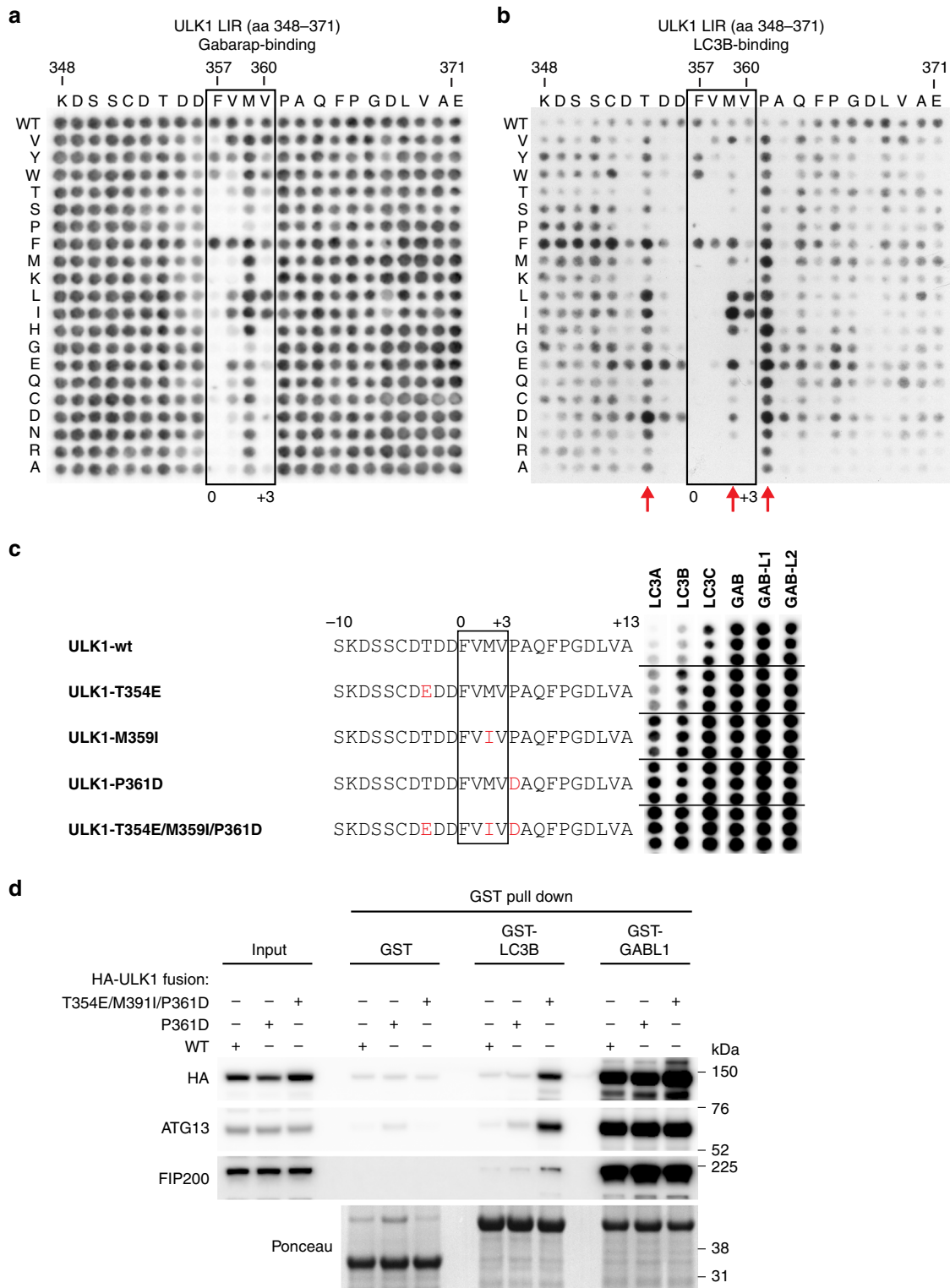
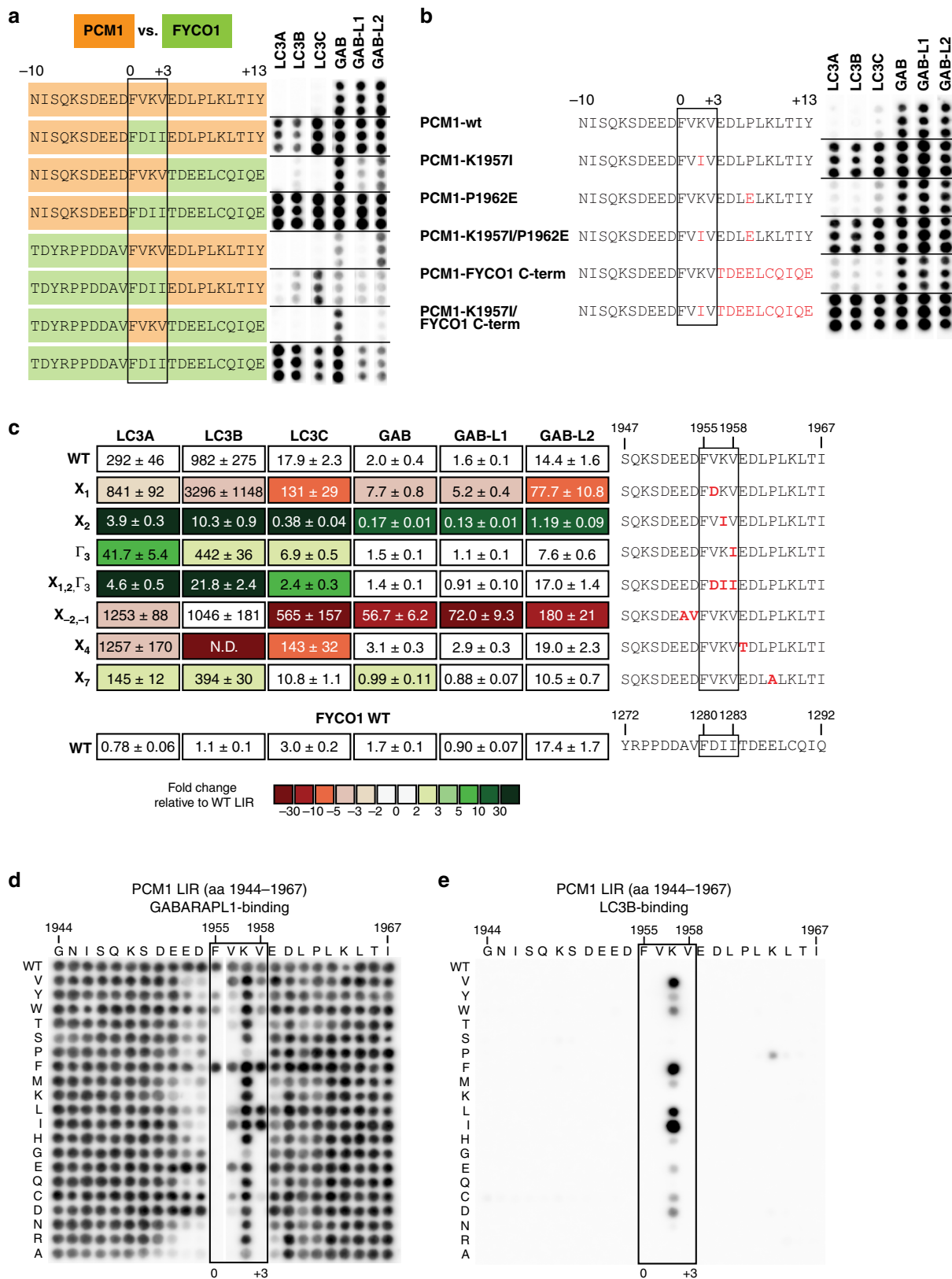


Fig. 3 The C-terminal region is critical for ULK1 LC3-interacting region (LIR) binding specificity. **a, b** Mutational peptide array of 24-mer ULK1 peptide covering LIR motif incubated with GST-GABARAP (**a**) or GST-LC3B (**b**) and immunoblotted with anti-GST. Each amino acid position was substituted for every other amino acid. **c** Twenty four-mer peptide array analysis of ULK1 LIR peptides containing point mutations incubated with indicated GST-ATG8 protein and immunoblotted with anti-GST. Each peptide is spotted in triplicates. Mutated residues are highlighted in red. **d** GST pull-down of HEK293A cells expressing indicated HA-ULK1 constructs and immunoblot with indicated antibodies



LC3 subfamily binding, indicating that LC3B binding was primarily regulated by K1957^{PCM1}.

A mutational peptide array scan (Fig. 4d, e) further corroborated that the lysine residue in position X₂ alone is sufficient in inhibiting LC3B binding. Single mutation of

K1957^{PCM1}, especially to aliphatic aa (isoleucine, leucine, and valine) and phenylalanine, strongly increased GST-LC3B association (Fig. 4e). Although GST-GABARAP²⁶ and GST-GABARAP-L1 (Fig. 4d) binding was slightly enhanced by mutations of position X₂, overall, GABARAP proteins seem to be able to

Fig. 4 Position X₂ in the pericentriolar material 1 (PCM1) core LC3-interacting region (LIR) motif inhibits LC3 binding. **a** Twenty four-mer peptide array analysis of PCM1-FYCO1 LIR chimera sequences incubated with indicated GST-ATG8 protein and immunoblotted with anti-GST. Each peptide is spotted in triplicates. PCM1 and FYCO1 peptide sequence are highlighted in orange and green, respectively. **b** Twenty four-mer peptide array analysis of PCM1 LIR containing point mutations incubated with GST-ATG8 protein and immunoblotted with anti-GST. Each peptide is represented in triplicates. Mutated residues are highlighted in red. **c** Affinities (K_d values) of LIR peptides to ATG8 proteins determined by bio-layer interferometry (BLI). Color code indicates fold changes relative to K_d value of PCM1 WT LIR peptide binding to the corresponding ATG8 protein. (data are mean \pm s.d., $n = 2$). **d, e** Mutational peptide array of 24-mer PCM1 peptide covering LIR motif incubated with GST-GABARAP (**d**) or GST-LC3B (**e**) and immunoblot. Each amino acid position was substituted for every other amino acid

tolerate almost any aa in that position of the PCM1 LIR motif, except for proline and glycine²⁶ (Fig. 4d). This intolerance for proline or glycine substitutions at X₂ was previously shown for ULK1 and ATG13 LIRs¹⁸.

Consequently, introduction of a lysine residue at X₂ reduced more LC3 than GABARAP subfamily binding to the ATG4B, FUNDC1, and FKBP8 LIR motifs (Supplementary Fig. 4d–f).

In the PCM1 LIR peptide array scan, GST-GABARAP binding was affected by mutation of the proline residue in position X₂²⁶, but neither affinity measurements (Fig. 4c) nor peptide array experiments (Fig. 4b–d) substantiated an important role of P196^{PCM1} in GABARAP subfamily binding. A point mutational peptide array clearly confirmed the regulatory function of K1957^{PCM1} (Fig. 4b). Moreover, in the presence of K1957I^{PCM1} the FYCO1 C terminus also contributed to ATG8 subfamily binding by further increasing binding.

Conforming to the PCM1 and ULK1 LIR motifs, positions X₂ and X₄ regulated LC3 subfamily binding to the FIP200 LIR motif. Both T704I^{FIP200} and P706D^{FIP200} mutation allowed LC3 subfamily binding but also enhanced interaction with GABARAPs (Supplementary Fig. 4a, b, g). Notably, mutation of position X₂ of the FIP200 LIR motif (T704I^{FIP200}) increases LC3 binding by 19- to 42-fold and GABARAP binding by 11- to 25-fold (Supplementary Fig. 4c).

In the case of the FYCO1 LIR motif (Supplementary Fig. 4h), introduction of a lysine in X₂, a proline in X₄, or the whole C terminus of either ULK1 or PCM1 weakened LC3A and LC3B interaction.

In summary, position X₂ and X_{4–13} of the C-terminal region of LIRs were critical in regulating subfamily-specific binding to ATG8 proteins.

Increased LC3 binding alters PCM1 dynamics in cells. The K1957I^{PCM1} mutation markedly changed the specificity of the LIR motif towards binding all ATG8s. Does this mutation affect PCM1 function in cells? GST pull-down experiments demonstrated that full-length GFP-PCM1 K1957I interacted strongly with both LC3 and GABARAP subfamily proteins (Fig. 5a). On the other hand, GFP-PCM1 wild type (WT) selectively interacted with GABARAPs and, to a lesser extent, with LC3C. GFP-PCM1 D1954A/F1955A/V1958A (PCM1 3xAla)²⁶ was deficient in ATG8 binding. The PCM1 LIR motif is required for recruiting peripheral PCM1-positive CS to sites of autophagosome formation, but not for PCM1 localization to the centrosome²⁶.

First, we tested whether the K1957I^{PCM1} mutation affected PCM1 localization to the centrosome (centriole and pericentriolar material (PCM) marked by γ -tubulin) in starved HEK293A cells. Loss of ATG8 binding resulted in a significant increase of centrosomal GFP-PCM1 (3xAla) relative to total GFP-PCM1 (Fig. 5b and Supplementary Fig. 5a, b), i.e. more PCM1 was retained on centrosomes. On the other hand, increased ATG8 protein binding (PCM1 K1957I) lead to a small reduction in the amount of GFP-PCM1 on the centrosome compared to WT. More strikingly, in the cell periphery PCM1 K1957I formed large cytosolic granules, which were partially positive for pericentrin and SSX2IP (Supplementary Fig. 5a, b), known PCM1 interactors

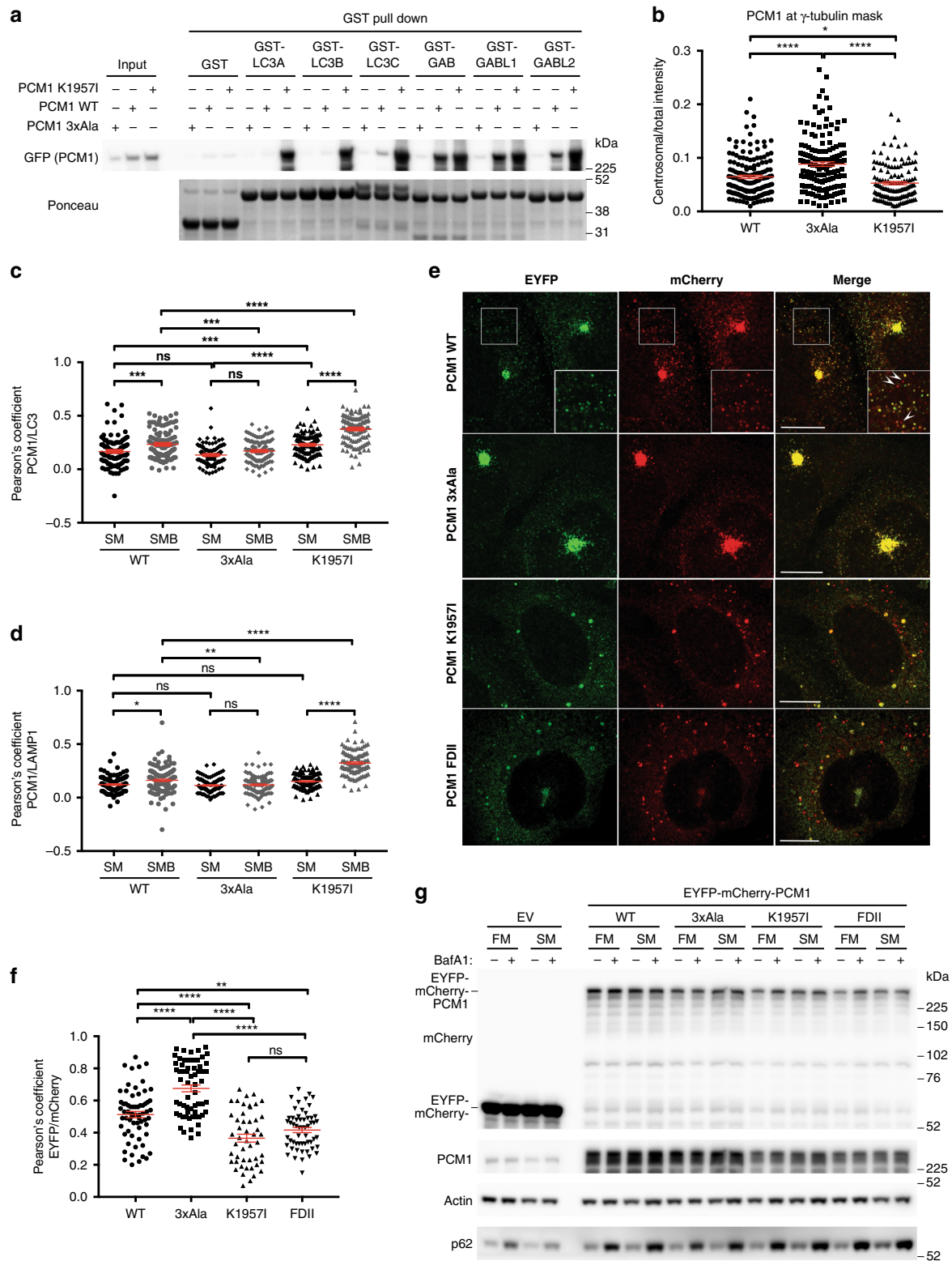
and CS and PCM markers³⁶. This suggests that increased ATG8 binding may alter CS integrity.

In response to starvation a subset of PCM1-positive CS colocalize with a range of autophagy markers, including LC3²⁶. Switching on LC3 subfamily binding and enhancing ATG8 binding overall may recruit more PCM1-positive CS to forming autophagosomes and increase PCM1 colocalization with LC3. Indeed, PCM1 K1957I colocalized significantly more with LC3 than PCM1 WT and PCM1 3xAla in starved cells (Fig. 5c and Supplementary Fig. 5c). Moreover, inhibition of lysosomal degradation by bafilomycin A1 (BafA1) enhanced LC3 colocalization of PCM1 K1957I compared to PCM1 WT. Colocalization between PCM1 3xAla and LC3 was not altered by BafA1 treatment. The responsiveness to BafA1 treatment suggested that activation of LC3 binding may target PCM1 K1957I to autophagosomes for lysosomal degradation. Hence, we also quantified PCM1 colocalization with the lysosomal marker LAMP1 (Fig. 5d and Supplementary Fig. 5c). PCM1 WT colocalization with LAMP1 was slightly increased by BafA1 treatment, PCM1 3xAla was unchanged, while PCM1 K1957I showed a significant increase. To resolve whether PCM1 K1957I is recruited into autophagosomes or associates with the outer membrane of autolysosomes, we expressed tandem fluorescence (EYFP-mCherry)-tagged PCM1 in HEK293A cells. The acidic pH of the lysosome quenches the EYFP signal faster than mCherry³⁷ and red-only puncta indicate autolysosomal PCM1. Compared to EYFP-mCherry PCM1 WT and 3xAla, PCM1 K1957I showed a significant increase in red-only puncta (Fig. 5e). To exclude any effects arising from increased affinity to GABARAPs, a PCM1 V1956D/K1957I/V1958I mutant (PCM1 FDII), where the PCM1 core LIR motif is replaced by the FYCO1 core LIR motif, was generated. BLI experiments showed the PCM1 FDII mutant had strongly increased affinities to LC3s without altering affinities to GABARAPs (Fig. 4c). Both PCM1 K1957I and PCM1 FDII formed red-only puncta in starved HEK293A cells (Fig. 5e), and colocalization between EYFP and mCherry was significantly reduced compared to PCM1 WT (Fig. 5f). Western blot analysis did not reveal any lysosomal turnover of overexpressed EYFP-mCherry-PCM1 K1957I and FDII (Fig. 5g), suggesting that only a small fraction of PCM1 is degraded by autophagy. This is in line with previous findings showing that PCM1 is not degraded by autophagy^{26,38}.

Interestingly, both enhanced binding of all ATG8s (PCM1 K1957I) or only LC3s (PCM1 FDII) resulted in formation of these larger PCM1 granules (Fig. 5e), suggesting that increased LC3 binding alters the dynamics of PCM1 in cells.

Non-conserved GABARAP residues key to selective LIR binding.

To fully understand the molecular mechanism underlying selective binding of ATG8 proteins to PCM1 and the ULK1 complex, we also addressed the role of the ATG8 protein sequence. Our structural analyses revealed several key residues within the LIR-GABARAP interface that were not conserved between the LC3 and GABARAP subfamilies (Figs. 1d, 2f). For instance, Q59^{GAB} is only conserved among GABARAPs and LC3C. Both LC3A and LC3B have a glutamate residue (E62^{LC3A/B}) instead, which cannot serve as a H-bond donor to main chain carbonyl residues in



position X_{7,8}. Thus, mutation of GABARAP Q59 to glutamate may affect PCM1 and ULK1 complex binding.

In Fig. 1d, asterisks denote the residues that we identified (based on our structures or previously published structures^{30–33,39}) and mutated in GABARAP and LC3B, making GABARAP more LC3B-like and vice versa. Changes in PCM1, ULK1, ATG13, FIP200, and p62 binding were determined by GFP-TRAP IP experiments (Fig. 6a–f and Supplementary Fig. 6) and/or BLI

affinity measurements (Fig. 6g). Q59E^{GAB} mutation only slightly affected PCM1 and ULK1 complex binding to GABARAP in IP experiments (Fig. 6a–e and Supplementary Fig. 6a, e), whereas F60L^{GAB} reduced interaction with PCM1 by ~90%. For ULK1, ATG13, and FIP200, we observed the strongest reduction in binding for K24Q/Y25H/Q59E/F60L^{GAB}.

Interestingly, the LC3B mutation E62Q^{LC3B}, corresponding to Q59E^{GAB} in GABARAP, led to an increase in both PCM1 and

Fig. 5 Increased LC3 binding alters pericentriolar material 1 (PCM1) dynamics in cells. **a** GST pull-down of HEK293A cells expressing indicated GFP-PCM1 constructs and immunoblot. **b** Quantification of GFP signal intensities at the centrosome (centriole and pericentriolar material marked by γ -tubulin) normalized to total GFP signal intensities of whole cells. Each measurement represents one cell; $n = 4$ independent experiments. **c, d** Quantification of GFP-PCM1 colocalization with LC3 (**c**) and LAMP1 (**d**); HEK293A cells expressing indicated GFP-PCM1 constructs starved for 2 h in Earle's balanced salt solution (EBSS) in the absence (SM) or presence of bafilomycin A1 (BafA1) (SMB), fixed and labeled with anti-LC3 and anti-LAMP1. See also Supplementary Fig. 5c. Pearson's coefficient was measured in 30 cells per condition per independent experiment; $n = 3$ independent experiments. **e** HEK293 cells expressing indicated EYFP-mCherry-PCM1 constructs starved for 6 h in EBSS and fixed for confocal microscopy. Scale bars represent 10 μm . **f** Quantification of EYFP colocalization with mCherry. HEK293 cells expressing indicated EYFP-mCherry-PCM1 constructs (shown in **e**) starved for 6 h in EBSS. Pearson's coefficient was measured in at least 50 cells per condition; $n = 3$ independent experiments. **g** HEK293 cells expressing indicated EYFP-mCherry-PCM1 constructs or empty vector (EV) were incubated in full medium (FM) or EBSS (SM) for 6 h in the presence or absence of BafA1 prior to immunoblotting. Statistical analysis using one-way analysis of variance (ANOVA) test; mean \pm s.e.m.; **** $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns not significant

ULK1 complex association (Fig. 6b, d, f and Supplementary Fig. 6b, f). The strongest increase in binding of PCM1 and the ULK1 complex was observed for Q26K/H27Y/E62Q^{LC3B}. The presence of the fourth mutation L63F^{LC3B} (corresponding to F60L^{GAB}) did not further improve binding. Affinity measurements further verified reduced binding of GABARAP K24Q/Y25H/Q59E/F60L^{GAB} and increased binding of LC3B Q26K/H27Y/E62Q^{LC3B} to PCM1 and ULK1 complex LIR peptides (Fig. 6g). Moreover, we could detect changes (two- to eight-fold) in binding of PCM1 and ULK1 complex LIR peptides to GABARAPs carrying single mutations of K24Q^{GAB}, Y25H^{GAB}, R28K^{GAB}, and Q59E^{GAB}, as well as to LC3Bs with single mutations of Q26K^{LC3B}, H27Y^{LC3B}, K30R^{LC3B}, and E62Q^{LC3B}. Triple mutations of K24Q/Y25H/R28K^{GAB} in GABARAP and Q26K/H27Y/K30R^{LC3B} in LC3B also lead to stronger changes in PCM1 and ULK1 LIR association. Single mutations of GABARAP residues L55V^{GAB}, F62K^{GAB}, and L63I^{GAB}, which form hydrophobic interactions with the C-terminal region of the PCM1 and ULK1 complex LIRs (Fig. 2f), had no effect or only a small effect on ULK1 LIR binding.

In our IP experiments, F60L^{GAB} interacted significantly less with p62 and this was rescued primarily by the K24Q^{GAB} mutation (Supplementary Fig. 6c, e). As previously reported, mutation of R10E/R11H^{LC3B} reduced LC3B binding to p62^{14,39} (Supplementary Fig. 6d, f). Interestingly, the presence of the E62Q^{LC3B} mutation partially rescued p62 binding in this mutant. No increase in PCM1 and ULK1 complex binding to LC3B was observed when mutating both R10E/R11H^{LC3B} and E62Q^{LC3B} (Fig. 6b–f and Supplementary Fig. 6b, f). In GABARAP, the corresponding E8R/H9R^{GAB} mutation (Fig. 6a–e and Supplementary Fig. 6a, c, e) increased PCM1, ULK1 complex, and p62 binding, suggesting that R10/R11^{LC3A/B} and R16/K17^{LC3C} are non-conserved residues critical for PCM1, ULK1 complex, and p62 binding by LC3 subfamily proteins.

Rendering GABARAP more LC3B-like impairs NDP52 degradation. Two recent studies showed that ATG8 proteins are dispensable for autophagosome formation, but essential for autophagic flux^{12,13}. Loss of all six ATG8 proteins results in p62 accumulation and defects in PINK1/Parkin-dependent clearance of mitochondria in HeLa cells. GABARAP proteins are crucial for autophagosome–lysosome fusion and the major drivers of PINK1/Parkin mitophagy and starvation induced autophagy.

To determine whether altering the binding specificity of GABARAP and LC3B affects their ability to rescue autophagic flux, i.e. p62 degradation, we generated stable inducible ATG8 hexa knockout (KO) HeLa¹² cell lines expressing either MYC-tagged WT GABARAP, GABARAP Q59E/F60L, GABARAP K24Q/Y25H/Q59E/F60L, WT LC3B, or LC3B Q26K/H27Y/E62Q. p62 degradation was significantly rescued by all five constructs (Fig. 7a, b). GABARAP WT, LC3B WT, and LC3B Q26K/H27Y/E62Q exhibited the strongest and comparable

activities. Mutations rendering GABARAP more LC3B-like notably affected its ability to rescue p62 turnover.

Interestingly, the levels of the autophagy receptor NDP52 were also elevated in hexa KO HeLa cells compared to control (Fig. 7a, c and Supplementary Fig. 7a). Both WT GABARAP and LC3B Q26K/H27Y/E62Q rescued NDP52 turnover, whereas no significant activity was observed for LC3B, GABARAP Q59E/F60L, and GABARAP K24Q/Y25H/Q59E/F60L.

Expression of MYC-tagged proteins and formation of puncta in response to starvation was also verified by immunofluorescence staining (Supplementary Fig. 7b). MYC-GABARAP K24Q/Y25H/Q59E/F60L formed fewer and smaller spots, suggesting that impaired ULK complex binding may affect its recruitment to forming autophagosomes.

In summary, rendering GABARAP more LC3B-like impaired its function in autophagic flux, while a more GABARAP-like LC3B, rescued selective degradation of NDP52.

Discussion

LIR-dependent interactions of autophagy adaptors and receptors with the LDS of mammalian ATG8 proteins are crucial in both non-selective and selective autophagy^{21,40}. The fact that many ATG8-interacting proteins preferentially bind to a specific subfamily indicates distinct, non-overlapping functions of LC3 and GABARAP subfamily proteins. Structural studies have been critical for understanding LIR-dependent interactions. However, the majority of analyses have focused on the core LIR and preceding N-terminal region. A potential role of residues C-terminal to the core LIR motif has been, apart from few exceptions (e.g., FYCO1^{30–32}, ALFY³³, AnkG/AnkB/FAM134B³⁴), largely not investigated.

In this study, we used N- and C-terminally extended LIR motifs in X-ray crystallography, affinity measurements, and peptide array binding experiments to elucidate molecular determinants mediating selective binding of the ULK1 complex and PCM1 to GABARAP subfamily proteins. Regarding the region N-terminal of the LIR our data is in line with previous findings²¹. Acidic residues in X₋₁ and X₋₂ are crucial for ULK1 and PCM1 LIR binding, as substitution (Figs. 3b, 4c, d) or removal of the whole N-terminal region of ULK1 (Supplementary Fig. 3b, c) reduced binding to all ATG8 proteins. More strikingly, we identified position X₂ within the core LIR motif as well as the adjacent C-terminal region (X_{4–10}) as key sites critical for selective binding of ULK1 complex members and PCM1 to GABARAPs.

There is still a lack of in-depth understanding of the molecular determinants in GABARAP and LC3 subfamily proteins mediating selective binding to LIRs. Our data show that binding of these C-terminally extended LIRs involves several non-conserved, GABARAP subfamily-specific residues, which are part of the LDS. Although the LDS is highly conserved among all ATG8 orthologs, these subfamily-specific residues create differences in

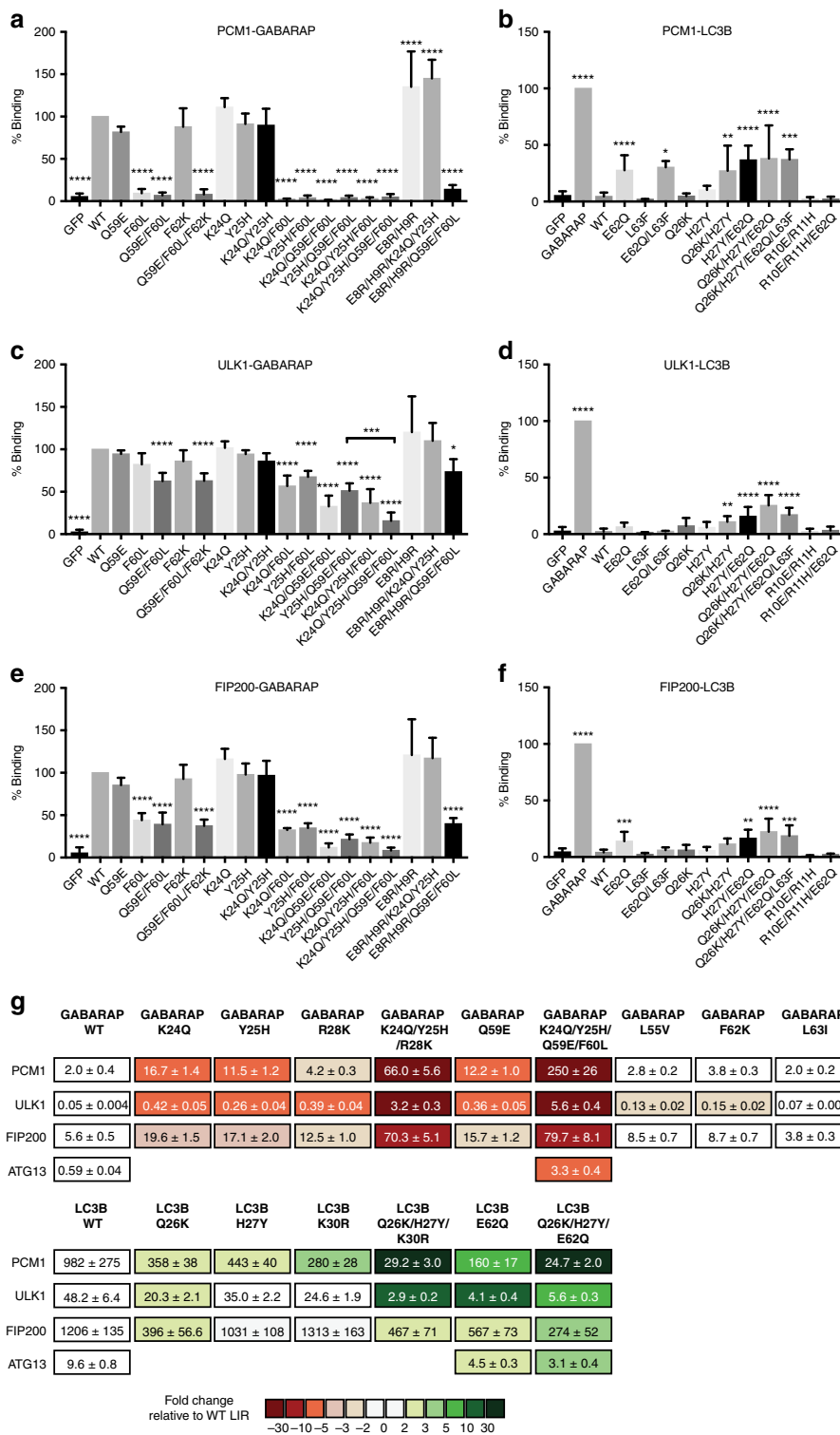
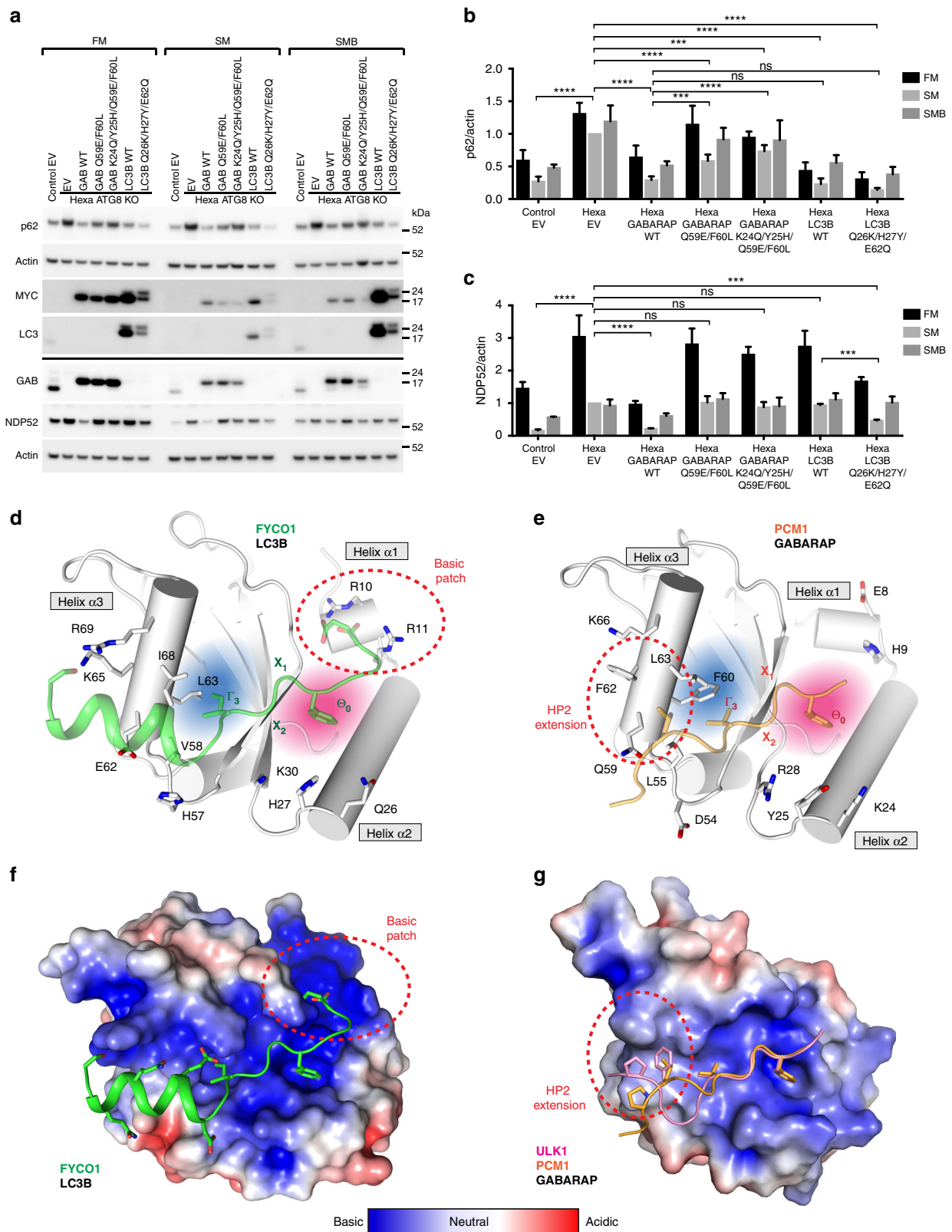


Fig. 6 Non-conserved GABARAP residues are key to selective LC3-interacting region (LIR) binding. **a, c, e** Quantification of endogenous pericentriolar material 1 (PCM1) (**a**), ULK1 (**c**), and FIP200 (**e**) binding to indicated GFP-GABARAP constructs expressed and immunoprecipitated by GFP-TRAP from HEK293A cells. Representative immunoblot of GFP-TRAP immunoprecipitation (IP) experiments is shown in Supplementary Fig. 6e. **b, d, f** Quantification of endogenous PCM1 (**b**), ULK1 (**d**), and FIP200 (**f**) binding to indicated GFP-LC3B constructs expressed and immunoprecipitated by GFP-TRAP from HEK293A cells. Representative immunoblot of GFP-TRAP IP experiments is shown in Supplementary Fig. 6f. Statistical analysis using one-way analysis of variance (ANOVA) test; mean ± s.d.; data from at least three independent experiments. *****p* ≤ 0.0001; ****p* ≤ 0.001; ***p* ≤ 0.01; **p* ≤ 0.05. **g** Affinities (*K_d* values) of LIR peptides to indicated ATG8 proteins measured by bio-layer interferometry (BLI). Color code represents fold changes relative to wild-type (WT) GABARAP and LC3B, respectively (data are mean ± s.d., *n* = 2–5)



the binding properties of the LDS, which ultimately direct the binding preference of the ULK1 complex and PCM1 LIR motifs towards GABARAPs and against LC3s (Fig. 7d–g).

In contrast to GABARAPs (E8/H9^{GAB} or D8/H9^{GABL1/2}), LC3s exhibit basic residues (R10/R11^{LC3A/B} or R16/K17^{LC3C}) on helix $\alpha 1$

at the edge of HP1 (Fig. 1d and Fig. 7d–g), which are crucial for FYCO1^{30–32} and p62^{21,39} (Supplementary Fig. 6d, f) binding, as they form electrostatic interactions with acidic residues in position X_{–2} to X_{–4}. Mutation of E8R/H9^{GAB} in GABARAP enhanced ULK1 complex and PCM1 binding (Fig. 6a, c, e and Supplementary Fig. 6a,

Fig. 7 Rendering GABARAP more LC3B-like impairs NDP52 degradation. **a** Control (wild-type) and hexa ATG8 CRISPR knockout (KO) HeLa cell lines stably expressing indicated MYC-ATG8 constructs or empty vector (EV) in full medium, Earle's balanced salt solution (EBSS) (SM) or EBSS + bafilomycin A1 (BafA1) (SMB) for 7 h prior to lysis and immunoblot with indicated antibodies. Expression of MYC-ATG8 constructs was induced by 1 μ g/ml doxycycline for 6 days. Note, immunoblot of non-induced cells showing equal p62 and NDP52 protein levels in the reconstituted hexa ATG8 CRISPR KO HeLa cell lines is included as Supplementary Fig. 7a. **b, c** Quantification of p62 (**b**) and NDP52 (**c**) protein levels (normalized to actin) shown in **a**. Statistical analysis of (SM) using one-way analysis of variance (ANOVA) test; mean \pm s.d.; data from four (p62) and three (NDP52) independent experiments. **** $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns, not significant. **d** Structure of FYCO1 LIR motif bound to LC3B (PDB ID 5WRD). Non-conserved LIR docking site (LDS) residues of LC3B are displayed in white sticks. LC3B is displayed in white cartoon and the FYCO1 LIR in green transparent cartoon. Hydrophobic pocket (HP) 1 and HP2 are indicated in pink and blue, respectively. **e** Structure of PCM1 LIR motif bound to GABARAP (P2₂,2₂). Non-conserved LDS residues of GABARAP are displayed in white sticks. GABARAP is displayed in white cartoon and the PCM1 LIR motif is shown in orange transparent cartoon. **f** Surface electrostatic potential of FYCO1:LC3B structure in the same orientation as shown in **d**. **g** Surface electrostatic potential of PCM1:GABARAP (P2₂,2₂) structure with ULK1 LIR superimposed (pink cartoon). **f, g** Projections shown are -5 (red) and $+5$ (blue) kT/e using pymol apbs plugin with red and blue representing negative and positive potential, respectively. Red dashed lines encircle basic patch in LC3B (**d, f**) and extension of HP2 in GABARAP (**e, f**)

c, e), whereas the R10E/R11H^{LC3B} mutation prevented increased ULK1 complex and PCM1 binding to the LC3B E62Q mutant (Fig. 6b, d, f and Supplementary Fig. 6b, d, f). Moreover, introducing the N-terminal region of the FYCO1 LIR (acidic residues only in X₋₃ and X₋₄) (Fig. 4a), as well as mutating the acidic residues in X₋₁ and X₋₂ (Fig. 4c) in the PCM1 LIR, weakened GABARAP subfamily binding to PCM1. This suggests that acidic residues in X₋₃ and X₋₄ promote specificity towards LC3 rather than GABARAP subfamily proteins. Consistent with previous results^{21,30–32,39}, R10/R11^{LC3A/B} (R16/K17^{LC3C}) are non-conserved residues critical for LC3 subfamily binding to the ULK1 complex and PCM1.

There is growing evidence that position X₇ plays a vital role in aiding ATG8 binding. A recent study showed that a short amphipathic α -helix (C-helix) C-terminal to the canonical LIR motifs of AnkG, AnkB, and FAM134B facilitates super-strong binding to ATG8 proteins³⁴. Similarly, a shorter C-helix is also critical for FYCO1 LIR binding to LC3A/B^{30,31} (Fig. 7d–f). A glutamate residue (in position X₇) at the beginning of all these C-helices forms salt bridges with R67^{GAB} or R70^{LC3B}, which are strictly conserved among all ATG8 proteins. Negatively charged glutamate or aspartate residues are highly prevalent in position X₇³¹ and involvement of the C-terminal region in stabilizing ATG8 binding may not be a unique feature of a few LIR sequences, but instead a more general mechanism facilitating LIR binding.

Within the C-terminal region of the PCM1, ATG13, and ULK1 LIR motifs, residues X_{5–10} are important for GABARAP binding. Position X₇ of the PCM1, ATG13, and ULK1 LIR is not an acidic residue and we did not detect C-helices. Instead, Q59^{GAB} and L55/F62/L63^{GAB} form hydrogen bonds and hydrophobic interactions with main chain carbonyl and side chain residues in position X_{5–10} (Fig. 2f and Fig. 7e–g). In GABARAPs these aromatic and aliphatic aa on helix α 3 expand slightly the hydrophobic surface of HP2, whereas in LC3 subfamily proteins (especially LC3A/B), the edge of HP2 is less hydrophobic and even charged due to V58/K65/I68^{LC3A/B} (Figs. 1d, 7d–g and Supplementary Fig. 2c, d). The single mutations of L55V^{GAB}, F62K^{GAB}, and L63I^{GAB} (Fig. 6g), producing small differences in the hydrophobic properties of HP2, had little or no effect, suggesting that contributions from these residues are not sufficient to alter LIR specificities. In contrast, Q59E^{GAB} mutation in GABARAP decreased (Fig. 6g) and E62Q^{LC3B} mutation in LC3B increased binding of ULK1 complex and PCM1 LIRs (Fig. 6b–g and Supplementary Fig. 6b, f). Q59E^{GAB} is only conserved in GABARAPs and LC3C and the corresponding residue in LC3A/B (E62^{LC3A/B}) is not able to function as hydrogen bond donor, indicating that E62^{LC3A/B} hinders binding to LC3A/B and is contributing to selectivity. Notably, both Q59^{GAB} and E62^{LC3B} stabilize binding of the AnkG/AnkB C-helix in position X₁₂³⁴. The LDS of GABARAPs can accommodate both C-helices and extended polypeptides, which indicates more plasticity and

dynamics in the interaction of the LDS with the C-terminal region of LIRs than anticipated.

Alongside positions X_{7–10}, a proline in position X₄ inhibits both ULK1 and FIP200 LIR binding to LC3 subfamily proteins (Fig. 3, Supplementary Fig. 4b, g). Due to the cyclic nature of the side chain, a proline in this position might induce geometric constraints on the LIR polypeptide chain unfavorable for LC3A/B interaction. Interestingly, E1959T^{PCM1} mutation in the PCM1 LIR (Fig. 4c) weakened only LC3 subfamily binding. In the mutational peptide array scan of the FIP200 LIR region, only hydrophobic, aromatic, and acidic aa (V, L, I, G, Y, W, F, E, D, and C) at X₄ improved LC3B binding, whereas mainly polar and basic aa did not (T, S, M, K, R, Q, N, P, H, and A) (Supplementary Fig. 4b). This suggests that apart from proline other residues in X₄ may also hinder LC3 subfamily binding. More structural analyses using C terminally extended LIR motifs will help to understand the underlying mechanism.

A recent study focusing on the core LIR motif defined a GIM, [W/F]-[V/I]-X₂-V, exhibiting preferential binding to GABARAP subfamily proteins²⁵. Mutation of both X₁ and X₂ residues modulated the binding specificity of the PLEKHM1 LIR towards different ATG8 proteins²⁵. Position X₂ in the LIR is not a strong determinant for GABARAP binding^{18,41} (Figs. 3a, 4d and Supplementary Fig. 4a) as only few substitutions (glycine, proline) disrupt the interaction. Although ULK1 and PCM1 have a valine at X₁, we found that position X₂ in the ULK1, FIP200, and PCM1 LIR motifs is hindering binding to LC3s. LIR binding to LC3 proteins is dramatically altered by substitutions of X₂: hydrophobic (I, L, V), and aromatic (F) amino acids strongly increase interaction of LC3B with ULK1, FIP200, and PCM1 LIR motifs (Fig. 3b–e and Supplementary Fig. 4b, c, g). Interestingly, the GABARAP-specific LIR of KBTBD6 exhibits an arginine (basic aa similar to lysine) in position X₂^{25,42}. Moreover, substitution of the X₂ residue with arginine renders the AnkG LIR highly selective for GABARAPs³⁴. Furthermore, by introducing a lysine in position X₂ (as found in the PCM1 LIR), we could effectively impair the binding of the ATG4B, FUNDC1, and FKBP8 LIR motifs to LC3s (Supplementary Fig. 4d–f), and alter the binding specificity of ATG4B towards GABARAPs. On the other hand, substitution of position X₂ in PCM1(K1957^{PCM1}) with isoleucine resulted in almost 100-fold increase in affinity to LC3B and \sim 12-fold increase to GABARAP (Fig. 4c). In cells, mutation of K1957^{PCM1} in PCM1 drastically switches on LC3 binding, enabling strong interaction with all six ATG8 proteins and altering PCM1 localization and dynamics (Fig. 5).

Why is the X₂ LIR residue of PCM1 affecting binding to LC3s more than GABARAPs? Both M359^{ULK1} and M446^{ATG13} (position X₂ in the ULK1 and ATG13 LIRs) form hydrophobic interactions with Y25^{GAB} and L50^{GAB}, whereas the charged and

longer side chain of K1957^{PCM1} only partially engages with the hydrophobic surface of GABARAP (Supplementary Figs. 2d, 7c). In the same way, the hydrophobic surface of LC3 subfamily proteins is too small to accommodate lysine or arginine side chains (Supplementary Fig. 7d). Moreover, lysine/arginine in X₂ clash with nearby positively charged R28^{GAB} and K30^{LC3A/B} and are generally not ideal for LIR binding to any ATG8 protein. Structural comparison of LIR-GABARAP complexes (this study and refs. 33,41,43) shows that the conformation of K24Q^{GAB}, Y25H^{GAB}, and R28K^{GAB} are well conserved in all structures (Supplementary Fig. 7c). R28^{GAB} is hydrogen bonding with the carbonyl of the LIR residue in position Γ₃ (except for PCM1 where it forms a salt bridge with the acidic residue in X₄). K24^{GAB} stabilizes the aliphatic part of Y25^{GAB}, which forms hydrophobic contacts with the aliphatic side chains of LIR residues in X₂. The conformations of the equivalent residues in LC3B are also well conserved across LIR-LC3B structures^{32,35,44–47} (Supplementary Fig. 7d). In contrast to R28^{GAB} in GABARAP, K30^{LC3B} of LC3B does not form a hydrogen bond with the carbonyl of LIR residues in Γ₃ and Q26^{LC3B} seems to not stabilize H27^{LC3B}, which is uncharged and has aromatic/hydrophobic properties at physiological pH. The single and triple mutations of K24Q^{GAB}, Y25H^{GAB}, and R28K^{GAB} in GABARAP reduced PCM1, ULK1, and FIP200 LIR binding (Fig. 6g), whereas triple mutation of the corresponding residues in LC3B (Q26K/H27Y/K30R^{LC3B}) increased binding. Thus, K24Q^{GAB}, Y25H^{GAB}, and R28K^{GAB} mediating binding of LIR residues in X₂ are critical for the PCM1 and ULK1 complex LIR binding specificity towards GABARAP.

Our study provides new mechanistic insight into the selective binding of LIR motifs to ATG8 proteins, highlighting an important role of the C-terminal region in addition to the core LIR motif and preceding acidic residues. Manipulation of the key sites in either the LIR motif, sequences flanking the LIR motif, or in the ATG8 proteins themselves can alter the binding specificity of proteins, including autophagy adaptors and receptors to ATG8 proteins. Degradation of NDP52, an autophagy receptor, depends on its specific interaction with LC3C¹⁵. We found that NDP52 degradation also seems to be facilitated by binding to GABARAP, as both GABARAP and LC3B rendered more GABARAP-like rescued NDP52 degradation (Fig. 7a–c). Our findings will be essential for future work to elucidate overlapping and distinct functions of LC3 and GABARAP subfamily proteins as well as the biology of LIR-dependent interactions with autophagy adaptors and receptors.

Finally, the intricate function of PCM1 and CS in autophagy is not well understood. CS are small, electron-dense granules around the centrosome that contain a variety of proteins involved in centrosome assembly/maintenance and ciliogenesis^{27,36}. CS traffic along microtubules and deliver centrosomal components to the centrosome in a dynein-dependent manner. Our recent study showed that CS regulate GABARAP-dependent autophagy²⁶. GABARAP is a novel component of a distinct CS subpopulation and is stabilized through interaction with PCM1, thereby preventing GABARAP degradation by the proteasome. Altering GABARAP-PCM1 binding may regulate PCM1 function. Loss of ATG8 binding (PCM1 3xAla) increases centrosomal PCM1 (Fig. 5b), suggesting that PCM1 trafficking from the centrosome may be affected. Moreover, both enhanced ATG8 binding (PCM1 K1957I) and LC3 binding (PCM1 FDII) alters CS integrity and causes PCM1 to form large cytosolic granules (Fig. 5e and Supplementary Fig. 5). Interestingly, deletion of the PCM1 C-terminus likewise induces the formation of large cytosolic PCM1 aggregates^{48,49}. The C-terminal region inhibits PCM1 self-aggregation, thereby regulating the size of PCM1-positive CS⁴⁸. Strong association of both LC3s and GABARAPs with the C-terminal PCM1 LIR might promote PCM1 self-aggregation and impair proper CS formation and function. Whether ATG8

proteins and/or autophagy regulate PCM1-dependent CS trafficking, assembly, and size needs to be addressed by future research.

In line with previous findings^{26,38} PCM1 is not appreciably degraded by autophagy. Interestingly, increased LC3 binding targeted some PCM1 K1957I and PCM1 FDII into autophagosomes for lysosomal degradation (Fig. 5e, f). Whether binding specificity to GABARAPs protects autophagy adaptors like PCM1 from autophagic degradation is an interesting question for future studies. Moreover, it is becoming apparent in recent years that the centrosome, ciliogenesis machinery and autophagy proteins are intimately linked^{24,26,38,50}. Understanding how these pathways are co-regulated is a fascinating direction for future research.

Methods

Antibodies. The following primary antibodies were used: horse radish peroxidase (HRP)-conjugated anti-GST (GE Healthcare, #RPN1236, 1:5000); anti-PCM1 (for western blot (WB) Atlas antibodies, #AMAb90565, 1:500; for immunofluorescence (IF), Sigma, #SAB1406228, 1:200, Cell signaling, #5213, 1:250); anti-ULK1 (Santa Cruz, #sc-33182, 1:250); anti-ATG13⁵¹ (1:1000); anti-FIP200 (Bethyl Labs, #A301-536A-1, 1:2000); anti-p62 (Abnova, #H00008878-M0, 1:500); anti-MYC (Cancer Research UK (raised in-house), clone 9E10, 1:500 for WB, 1:250 for IF; Abcam, #ab9106, 1:1000); anti-HA (Covance, clone 16B12, #MMS-101R-500, 1:1000); anti-GFP (Cancer Research UK (raised in-house), clone 3E1, 1:1000; Santa Cruz, #sc-8334, 1:1000); anti-γ-tubulin ascites (Sigma, Clone GTU-88, #T6557, 1:5000); anti-SSX2IP (ThermoFisher, #PA5-18258, 1:250); anti-pericentrin (Abcam, #ab4448, 1:250); anti-LC3 (Abcam, #ab48394, 1:1000 for WB, 1:2500 for IF); anti-LAMP1 (BD Biosciences, #34201A, 1:250); anti-actin (Abcam, #ab8227, 1:1000); anti-GABARAP (Abgent, #AP1821a, 1:250); anti-NDP52/CALCOCO2 (Abcam, #ab68588, 1:1000); anti-mCherry (Abcam, #ab167453, 1:1000).

Secondary antibodies for IF were all from Life Technologies and diluted 1:500: anti-rabbit immunoglobulin G (IgG) Alexa Fluor 488 (#A11034), 555 (#A31772), and 647 (#A31573); anti-mouse IgG Alexa Fluor 488 (#A21202), 555 (#A31570), 647 (#A31571); anti-goat IgG Alexa Fluor 594 (#A21468). HRP-conjugated secondary antibodies used for WB were from GE Healthcare (#NA931 (anti-mouse IgG), #NA934 (anti-rabbit IgG), 1:4000).

Plasmids. EGFP-PCM1 (NP_001302436) (pEGFP-C2)⁵² was a gift from Takashi Toda (Hiroshima University, Japan). EGFP-PCM1 (pEGFP-C2) 3xAla (D1954A/F1955A/V1958A) was generated by us previously²⁶. EGFP-PCM1 (pEGFP-C2) K1957I and pDNOR221 PCM1 FDII (V1956D/K1957I/V1958I) were generated using Q5 Site-Directed Mutagenesis Kit (New England Biolabs). EYFP-mCherry-PCM1 WT, 3xAla (D1954A/F1955A/V1958A), K1957I, and FDII (V1956D/K1957I/V1958I) were generated by using the Gateway recombination system from Invitrogen. BP reactions into pDNOR221 and LR reactions into pDEST-EYFP-mCherry were performed according to the manufacturer's instructions. pDEST-EGFP-ATG8 homologs, pDEST-MYC-ATG8 homologs (human), and pDEST-EYFP-mCherry were generated by Terje Johansen (UiT, The Arctic University of Norway, Tromsø)^{18,53}. pDEST-EGFP-GABARAP and LC3B mutants were generated by QuickChange Multi Site-Directed Mutagenesis Kit (Agilent) and Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

HA-ULK1 (human ULK1) in pcDNA3.1 was generated by us previously⁵⁴. HA-ULK1 P361D and ULK1 T354E/M391I/P361D (pcDNA3.1) were generated using Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

The sequences of human GABARAP WT, Q59E/F60L or K24Q/Y25/Q59E/F60L and LC3B WT or Q26K/H27Y/E62Q were inserted together with a N-terminal MYC-tag in the pLVX-TetOne-Puro vector (Clontech) using *AgeI/BamHI* restriction sites.

For recombinant protein expression in bacteria, a modified plasmid of pGEX-6P2 (GE Healthcare) (referred to as pAL) containing an N-terminal glutathione S tag followed by a 3C protease cleavage site was used. *BamHI* and *NotI* restriction sites were used for insertion of full-length human GABARAP, GABARAP-L1, LC3A, and LC3C, and *EcoRI* and *NotI* restriction sites for cloning of LC3B and GABARAP-L2. For crystallization, LIR sequences of PCM1 (aa 1951–1964), ATG13 (aa 441–454), and ULK1 (aa 354–366) were N terminally fused with a Gly-Ser linker to full-length GABARAP (GST-GABARAP (pAL)) using *NcoI* and *BamHI* sites. pAL-GST-GABARAP and LC3B mutants were generated using Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

All plasmid constructs generated in this study were verified by DNA sequencing.

See Supplementary Table 1 for primers used in this study.

Cell culture and DNA transfection. HEK293A cells (provided by Cell Services of the Francis Crick Institute) were grown in a humidified incubator at 37 °C in 10% CO₂ in full medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 4 mM L-glutamine). Control and ATG8 hexa KO HeLa cells¹² were a gift from Michael Lazarou (Monash University, Australia). For

reconstitution of ATG8 hexa KO HeLa cell lines with TetOn-inducible MYC-tagged GABARAP and LC3B proteins cells were transfected with pLVX-TetOne-Puro-MYC-GABARAP/LC3B vector DNA using jetPrime transfection reagent (Polyplus-transfection) according to the manufacturer's instructions. Transduced cells were selected with 2 µg/ml puromycin. Following single-cell sorting, protein expression was induced with 1 µg/ml doxycycline for 4–6 days and colonies were screened for MYC-GABARAP/LC3B expression by western blotting. Verified clones were maintained in full medium containing 1 µg/ml puromycin. For rescue experiments, MYC-GABARAP/LC3B expression was induced with 1 µg/ml doxycycline for 6 days. To induce autophagy by starvation, cells were washed two times with (137 mM NaCl, 3.4 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2) (PBSA) and once with Earle's balanced salt solution (EBSS) and then incubated in EBSS for 2 h (7 h for p62 and NDP52 degradation assays). Where indicated, cells were treated with 100 nM Baf A1 (Calbiochem).

Lipofectamine 2000 (Life Technologies) was used for transient transfection of cells according to the manufacturer's instructions. DNA plasmids were used at a concentration of 1 µg/ml of transfection mix. Cells were harvested or fixed after 24–48 h.

Western blotting. Cells were lysed in ice-cold TNTE buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% w/v Triton X-100, 10% v/v glycerol, 5 mM EDTA) containing EDTA-free Complete Protease Inhibitor cocktail (Roche) and PhosSTOP (Roche). Lysates were cleared by centrifugation and resolved on NuPAGE Bis-Tris 4–12% gels (Life Technologies) followed by transfer onto a PVDF membrane (Millipore). After incubation with primary and secondary antibodies, the blots were developed by chemiluminescence using Immobilon Classic Western HRP substrate (Merck Millipore). Densitometry was performed with ImageJ software. For western blotting of weak signal antibodies, primary antibody was diluted with SignalBoost Immunoreaction Enhancer Kit (Merck Millipore, 407207) and blots were developed with Luminata Crescendo Western HRP substrate (Merck Millipore).

Immunoprecipitation. Cells were lysed in ice-cold TNTE buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% w/v Triton X-100, 10% v/v glycerol, 1× Complete protease inhibitor (Roche), 1× PhosSTOP (Roche)). Lysates were pre-cleared with control agarose beads (ChromoTek) for 1 h at 4 °C. GFP-tagged proteins were immunoprecipitated using GFP-TRAP beads (ChromoTek) and MYC-tagged proteins with MYC-Trap beads (ChromoTek) for 2 h at 4 °C. Beads were washed three times with TNTE (w/o PhosSTOP) and bound protein was eluted with 2× Laemmli buffer at 100 °C for 10 min before resolving by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–12% Bis-Tris NuPAGE gels, Life Technologies) and western blotting.

Peptide array and GST overlay assay. GST or GST-ATG8 proteins were expressed (from GST-ATG8 (pAL) plasmids) in *Escherichia coli* BL21 (DE3) pLysS cells (Agilent, #200132) in LB medium supplemented with 50 µg/ml kanamycin. Expression was induced by the addition of 0.5 mM IPTG at OD₆₀₀ = 0.6 and cells were incubated at 25 °C overnight or at 37 °C for 5 h. Harvested cells were lysed in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 0.4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride HCl (AEBSF), and 15 µg/ml benzamidine. Fusion protein was batch adsorbed onto Glutathione-Sepharose 4B beads (GE Healthcare). After five washes with wash buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 0.4 mM AEBSF, and 15 µg/ml benzamidine), fusion proteins were eluted in 50 mM Tris, pH 8.0, 2 mM L-glutathione reduced, 0.4 mM AEBSF, and 15 µg/ml benzamidine.

A MultiPep or ResPep SL automated synthesizer (INTAVIS Bioanalytical Instruments AG, Germany) was used for SPOT synthesis of peptide arrays on cellulose membranes⁵⁵. After blocking membranes in TBST with 5% nonfat dry milk, peptide interactions with GST or GST fusion proteins were tested by overlaying the membranes with either 1 µg/ml (mutational peptide array scan) or 2 µg/ml of recombinant protein (all other peptide arrays) for 2 h at room temperature. Membranes were washed in TBST, and bound proteins were detected with HRP-conjugated anti-GST antibody (1:5000, GE Healthcare, RPN1236).

GST pulldowns. Per reaction 50 µg GST-tagged protein was bound to 30 µl Glutathione-Sepharose 4B beads for 2 h at 4 °C. After several washes with PBSA, HEK293A lysate was added and beads were incubated at 4 °C overnight. Beads were washed three times with TNTE lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% w/v Triton X-100, 10% v/v glycerol, 1× Complete protease inhibitor (Roche)) before SDS-PAGE and western blotting.

Protein expression and purification for crystallization. GST-LIR-GABARAP chimera proteins (pAL) were expressed in *E. coli* Rosetta (DE3) pLysS (Merck, #70956) at 25 °C overnight. Bacteria were harvested by centrifugation and lysed in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% TX-100, 0.5 mM Tris (2-carboxyethyl) phosphine (TCEP), 0.4 mM AEBSF, and 15 µg/ml benzamidine. The fusion protein was batch adsorbed onto Glutathione-Sepharose 4B affinity matrix (GE Healthcare) and recovered by cleavage with 3C protease at 4 °C overnight in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5 mM TCEP. The protein was then

further purified by size exclusion chromatography using a Superdex 200 26/60 column (GE Healthcare) equilibrated and run in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5 mM TCEP.

Crystallization and data processing. GABARAP chimera proteins were crystallized at 20 °C using the sitting-drop vapor diffusion method with a protein concentration of 10–20 mg/ml. Initial crystallization trial was performed using Qiagen (JCSG core 1–4, AM504), Molecular dimension (PACT, Wizard 1–4), and Jena Bioscience (PIPEG). In all cases the drop included 0.5 µl of protein and 0.5 µl of mother liquor. For PCM1^{1951–1964}-GABARAP chimeras, crystals grew in 20% w/v PEG 6 K, 0.2 M CaCl₂, 0.1 M HEPES, pH 7 (P_{2,2,2,2}) and 25% w/v PEG 1500 and 0.1 M SPG pH 8.5 (P_{4,3}). For ATG13^{441–454}-GABARAP chimera, crystals grew in 0.2 M NaCl, 10% PEG 8000, and 0.1 M Na K phosphate, pH 6.2. For ULK1^{354–366}-GABARAP chimera, crystals grew in 0.2 M MgCl₂, 0.1 M Na cacodylate, pH 6.5, and 20% PEG1000. Crystals were flash frozen in liquid nitrogen, and X-ray data sets were collected at 100 K at the I04, I04-1 beamline (mx13775) of the Diamond Light Source Synchrotron (Oxford, UK). Data collection and refinement statistics are summarized in Table 1. The data sets were indexed and scaled with xia2⁵⁶. Molecular replacement was achieved by using the atomic coordinates of the peptide-free GABARAP (PDB code: 1GNU) in PHASER⁵⁷. Refinement was carried out using Phenix⁵⁸. Model building was carried out in COOT⁵⁹. Model validation used PROCHECK⁶⁰, and figures were prepared using the graphics program PYMOL (<http://www.pymol.org>).

BLI assay. BLI is an optical analytical technique for measuring kinetics of interactions in real time. The biosensor tip surface immobilized with a ligand is incubated with an analyte in solution, resulting in an increase in optical thickness at the biosensor tip and a wavelength shift, which is a direct measure of the change in thickness. BLI analyses of ATG8s binding to immobilized biotinylated LIR peptides were performed using an Octet Red 96 (ForteBio). Biotinylated LIR peptide (50 µg/ml) was immobilized on streptavidin-coated biosensor (SA, ForteBio) and the typical immobilization levels were above 0.3 nm. Ligand-loaded SA biosensors were then incubated with different concentrations of ATG8. All binding experiments were performed in solid-black 96-well plates containing 200 µl of solution (25 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 0.1% Tween-20, 1 mg/ml bovine serum albumin (BSA)) in each well at 25 °C with an agitation speed of 1000 rpm. Each measurement was repeated two to five times. Dissociation constants for LIR-ATG8 interactions were determined from plotting the increase in BLI response as a function of the protein concentration and fitting using non-linear regression of ForteBio 7.1 data analysis and GraphPad Prism 7 software.

Peptides were synthesized using FMOC (fluorenylmethoxycarbonyl) solid-phase peptide chemistry by the Francis Crick Peptide Chemistry Technology Platform and are listed in Supplementary Table 2.

Immunostaining and confocal microscopy. Cells were grown on coverslips, and then fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min before permeabilization with methanol at room temperature for 5 min. Coverslips were then blocked in 5% BSA (Roche) in PBS for 20 min. Coverslips were incubated with primary antibody in 1% BSA in PBS 1 h at room temperature. Coverslips were washed and incubated with secondary antibody in 1% BSA for 1 h. After final washing with PBS and water, coverslips were mounted in mowiol. Images were acquired using a Zeiss LSM 710 confocal microscope (×63 oil-immersion lens), or a Zeiss LSM 880 Airyscan Confocal microscope (×63 oil-immersion lens) and Zeiss ZEN imaging software.

Quantifications and statistical analysis. Centrosomal and total intensity of GFP-PCM1 was quantified using ImageJ software. Region of interest was created by manually selecting the expressing cells and γ-tubulin signal was used to identify the centrosomal area in the cells. Pearson's correlation coefficients were calculated using ImageJ software.

The statistical details of all experiments are reported in the figure legends and figures, including statistical analysis performed, error bars, statistical significance, and exact n numbers. Statistics were performed using GraphPad Prism 7 software, as detailed in figure legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Atomic coordinates and crystallographic structure factors have been deposited in the Protein Data Bank under accession codes PDB 6HYL (PCM1^{1951–1964}-GABARAP, P_{2,2,2,2}), PDB 6HYM (PCM1^{1951–1964}-GABARAP, P_{4,3}), PDB 6HYN (ATG13^{441–454}-GABARAP), and PDB 6HYO (ULK1^{354–366}-GABARAP). The source data underlying Figs. 1e, 3d, 5a, g, 7a and Supplementary Figs. 6e, f, 7a are provided in Supplementary Fig. 8. The source data underlying Figs. 1g, 4c, 5b–d, 5f, 6, 7b, c and Supplementary Figs. 3c, 4c, 6a–d are provided as a Source Data file. All other data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Author contributions

M.W. and M.R. performed cell and biochemical studies. S.M., M.W., W.Z. and L.N. performed structural studies. D.J. and N.O'R synthesized peptides and peptide arrays. M.W., S.M., S.A.T. and T.J. wrote the manuscript. All authors discussed the results and commented on the manuscript. M.W., S.M. and S.A.T. supervised the work. M.W. coordinated the project.

Additional information

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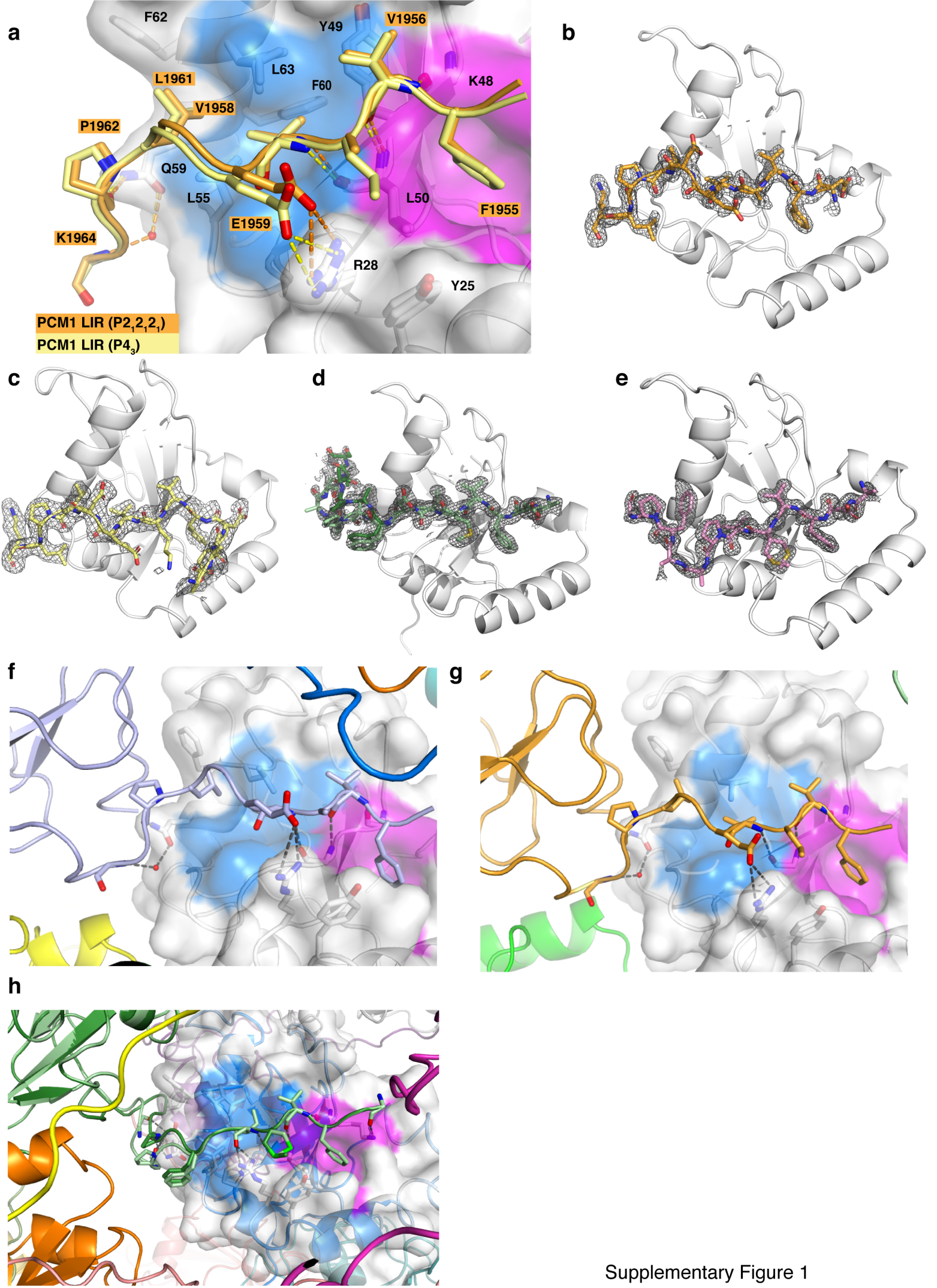
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Supplementary Information

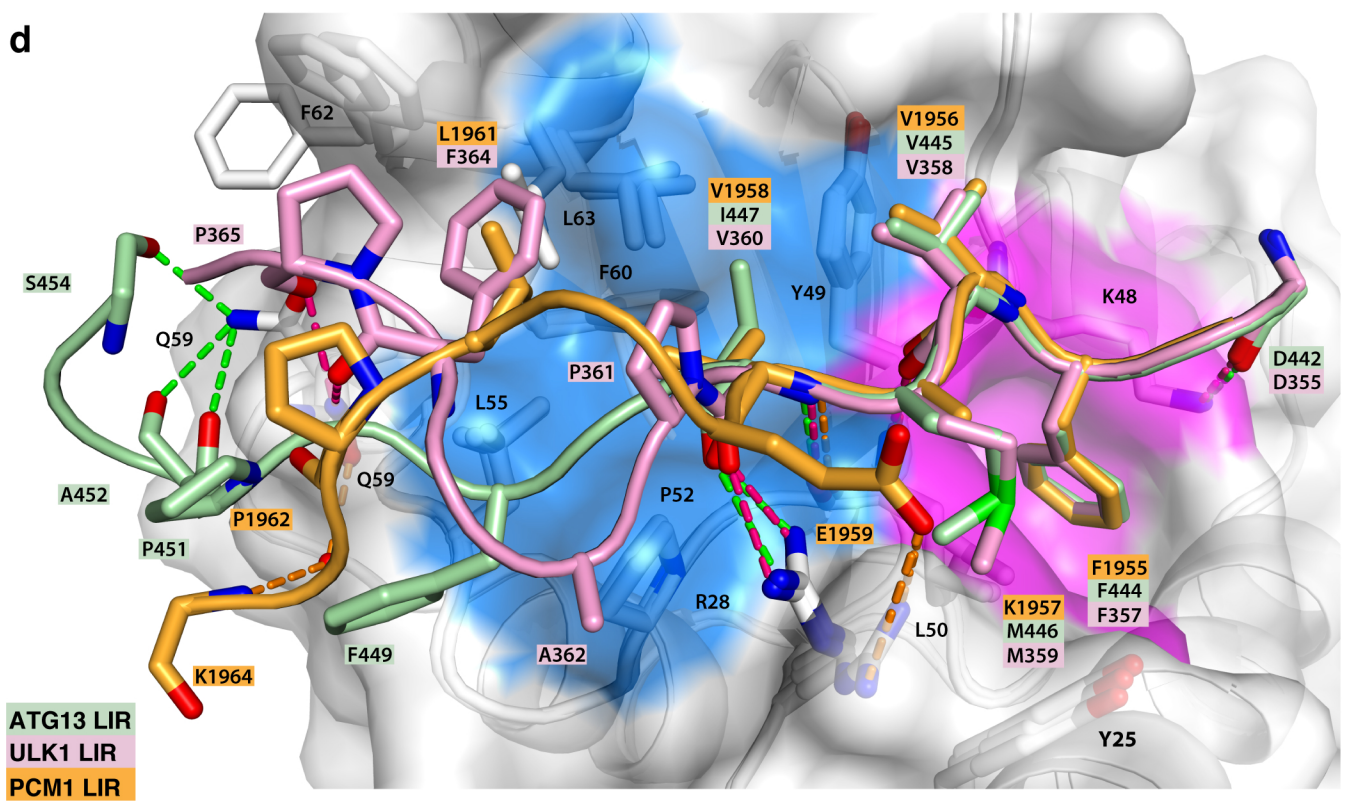
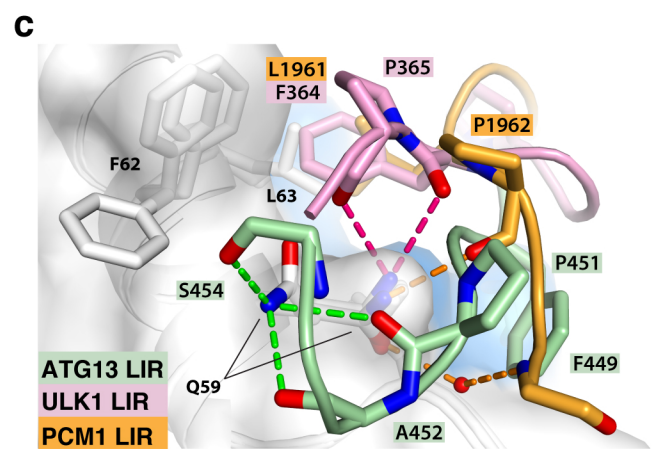
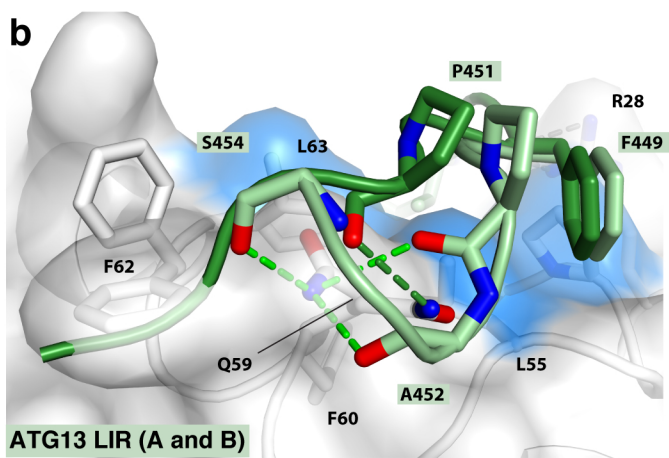
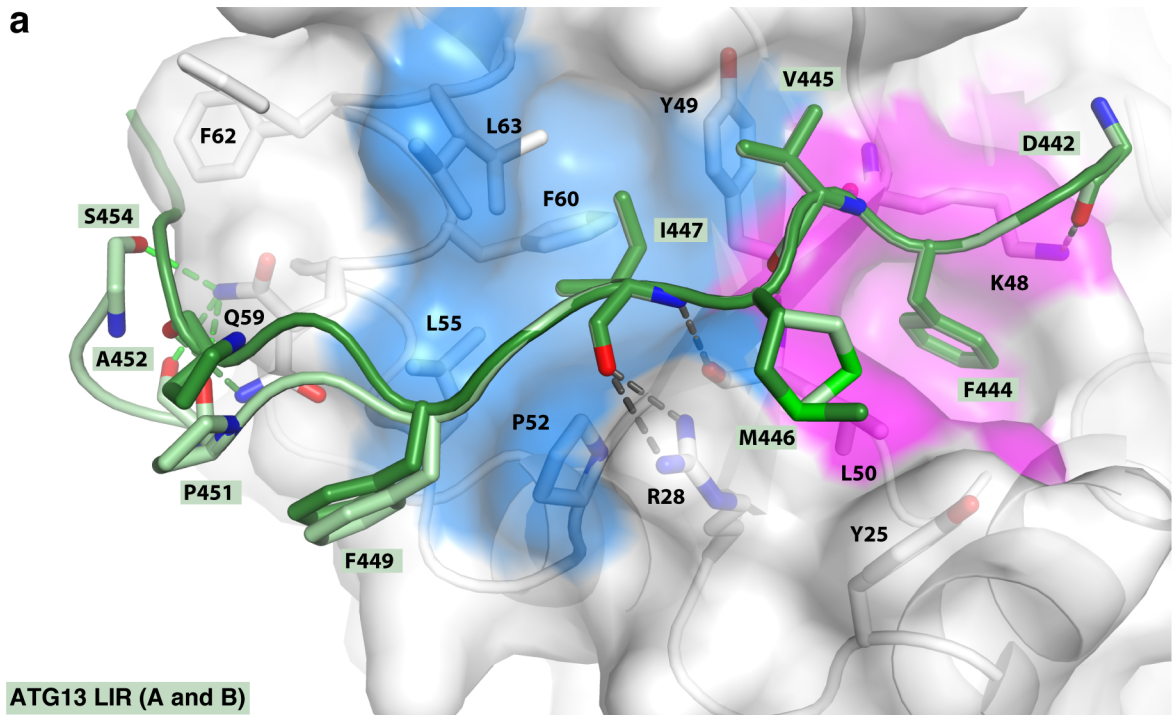
Molecular determinants regulating selective binding of autophagy adaptors and receptors to ATG8 proteins

M. Wirth et al.



Supplementary Fig. 1: PCM1 binds to GABARAP via a C-terminally extended LIR motif.

(a) Superposition of the two PCM1¹⁹⁵¹⁻¹⁹⁶⁴ LIR-GABARAP structures solved in space group P2₁2₁2₁ and P4₃. The PCM1 LIR sequence is shown in orange (P2₁2₁2₁) and yellow (P4₃) cartoon with interacting residues depicted as sticks. GABARAP is displayed in white cartoon and transparent surface with hydrophobic pocket 1 and 2 colored in pink and blue surfaces, respectively. (b) to (e) Electron density map of PCM1 LIR P2₁2₁2₁ (b), PCM1 LIR P4₃ (c), ATG13 LIR (d), and ULK1 LIR (e) bound to GABARAP. The Fo-Fc omit map of the LIR motifs is contoured at 3.0 σ . GABARAP is displayed in white cartoon and the LIR motif in cartoon and sticks. (f) Crystal packing around the PCM1 LIR P2₁2₁2₁ structure. All the symmetry related molecules within 12 Å are displayed in different colors. (g) Crystal packing around the PCM1 LIR P4₃ structure. All the symmetry related molecules within 12 Å are displayed in different colors. (h) Crystal packing around the ATG13 LIR structure. All the symmetry related molecules within 12 Å are displayed in different colors.



Supplementary Figure 2

Supplementary Fig. 2: ATG13 and ULK1 bind GABARAP via extended LIR motifs.

(a) Superposition of the two alternate conformations of ATG13⁴⁴¹⁻⁴⁵⁴ LIR bound to GABARAP. The ATG13 LIR sequences are shown in light green (alternate conformation A) and dark green (alternate conformation B) cartoon with interacting residues depicted as sticks. GABARAP is displayed in white cartoon and transparent surface with hydrophobic pocket 1 (pink) and 2 (blue). (b) Close up view of superposed conformations displaying interactions formed by ATG13 LIR residues in position X₅₋₁₀. (c) Close up view of superposed structures of GABARAP bound to ATG13 (light green), ULK1 (pink) and PCM1 (orange) showing interactions of Q59^{GAB}, F62^{GAB} and L63^{GAB} with LIR residues in position X₇₋₁₀. (d) Superposition of structures of GABARAP bound to ATG13 (light green), ULK1 (pink) and PCM1 (orange). In (c) and (d) only the surface of GABARAP from the PCM1:GABARAP structure is displayed in white transparency with HP1 and HP2 colored in pink and blue, respectively.

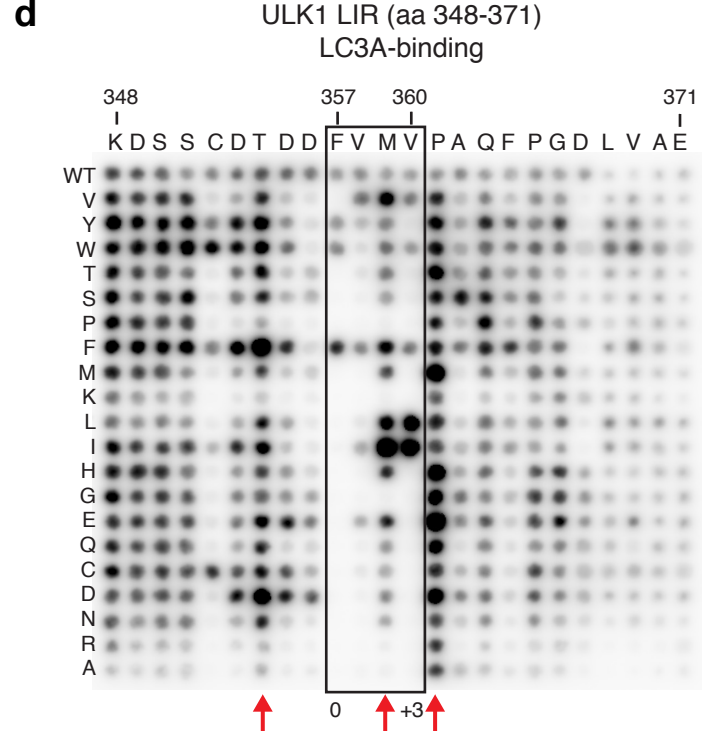
a

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 Mouse PCM1 1954 NISQKSDEEDFVKVEDLPLKLTIV 1977
 X.laevis PCM1 1956 NLSQKSDEEDFVKVEDLPLKLSLP 1979
 D.rerio PCM1 1944 NTSQRSDEEDFVKVEDLPIQMSVL 1967

Human ULK1 347 SKDSSCDTDDFVMVPAQFPGDLVA 370
 Mouse ULK1 347 SSKDSCDTDDFVMVPAQFPGDLVA 370
 D.rerio ULK1B 347 SKNSSCDTDDFVMVPAHFPSLTC 370
 DmATG1B 381 SCSSHEDSDDFVLVFNKLPEDQRQ 404
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 Human ULK2 343 SKNSSCDTDDFVLVPHNISSDHSC 366

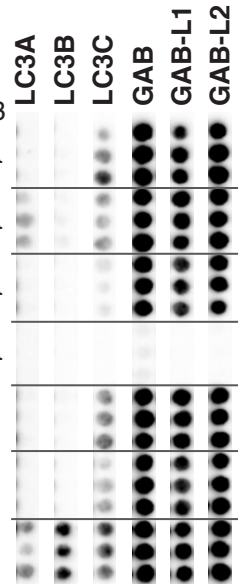
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 X.laevis ATG13 426 CSSSETPQDDFVMIDFRPAFSKDD 449
 D.rerio ATG13 420 SHSGGQPNDDFVMVDFKPAFSKDD 443

Human FIP200 692 PDSIDAHTFDFTETIHPNIEQTIH 715
 Mouse FIP200 691 PDSIDAHTFDFTETISHPNTEQPVH 714
 X.laevis FIP200 686 PDSIDAHTFDFTETIAHPSTEQSLK 709
 D.rerio FIP200 695 PDSIDAQTDFETIIGHPSMDPVLQ 718
 DmFIP200 620 SDCETDTETEFEKLSSTTSKCVATS 643



b

ULK1 (aa 347-370) SKDSSCDTDDFVMVPAQFPGDLVA
 ULK1 (aa 353-370) -----DTDDFVMVPAQFPGDLVA
 ULK1 (aa 355-370) -----DDFVMVPAQFPGDLVA
 ULK1 (aa 357-370) -----FVMVPAQFPGDLVA
 ULK1 (aa 347-364) SKDSSCDTDDFVMVPAQF-----
 ULK1 (aa 347-362) SKDSSCDTDDFVMVPA-----
 ULK1 (aa 347-360) SKDSSCDTDDFVMV-----



c

	LC3A	LC3B	LC3C	GAB	GAB-L1	GAB-L2	
ULK1 (aa 349-369)	5.9 ± 0.1	48.2 ± 6.4	2.5 ± 0.3	0.050 ± 0.004	0.048 ± 0.004	0.53 ± 0.05	DSSCDTDDFVMVPAQFPGDLV
ULK1 (aa 353-369)	7.7 ± 1.0	82.0 ± 12.9	1.9 ± 0.2	0.116 ± 0.009	0.102 ± 0.008	0.58 ± 0.06	----DTDDFVMVPAQFPGDLV
ULK1 (aa 355-369)	407 ± 64.7	494 ± 64.1	7.8 ± 1.4	0.288 ± 0.038	0.243 ± 0.034	2.2 ± 0.3	-----DDFVMVPAQFPGDLV
ULK1 (aa 357-369)	3726 ± 907	11247 ± 10315	1156 ± 781	169 ± 42.4	861 ± 153	1499 ± 251	-----FVMVPAQFPGDLV
ULK1 (aa 349-364)	8.4 ± 0.7	68.6 ± 9.4	2.2 ± 0.2	0.166 ± 0.008	0.167 ± 0.009	0.54 ± 0.03	DSSCDTDDFVMVPAQF-----
ULK1 (aa 349-362)	9.2 ± 1.0	76.9 ± 13.1	2.8 ± 0.2	0.174 ± 0.014	0.148 ± 0.013	0.71 ± 0.05	DSSCDTDDFVMVPA-----
ULK1 (aa 349-360)	2.9 ± 0.3	8.5 ± 0.9	2.2 ± 0.2	0.218 ± 0.020	0.220 ± 0.022	1.1 ± 0.1	DSSCDTDDFVMV-----

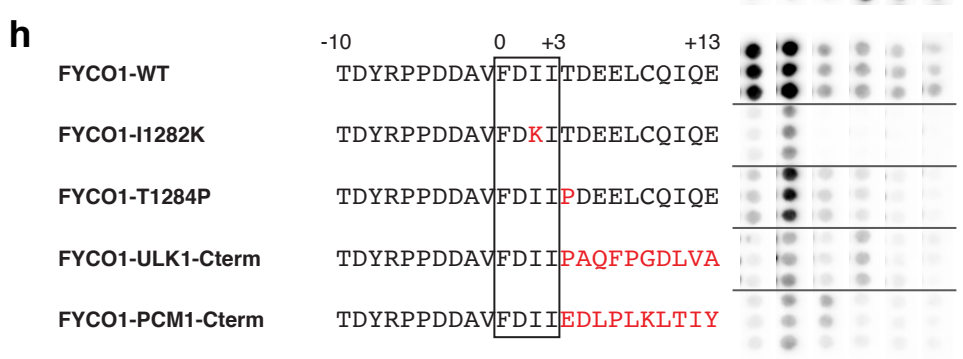
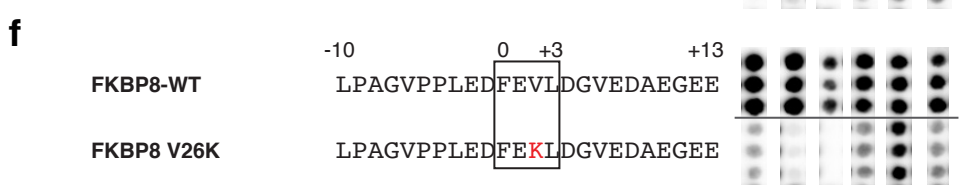
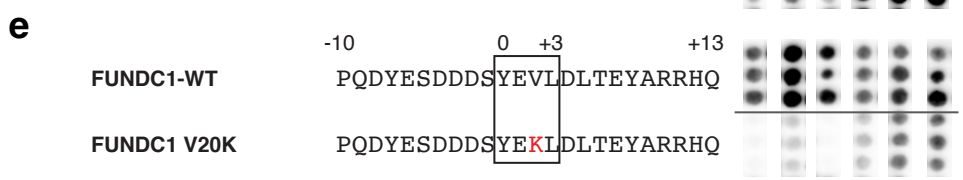
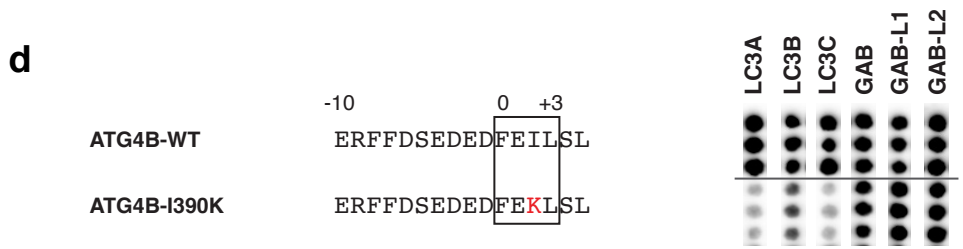
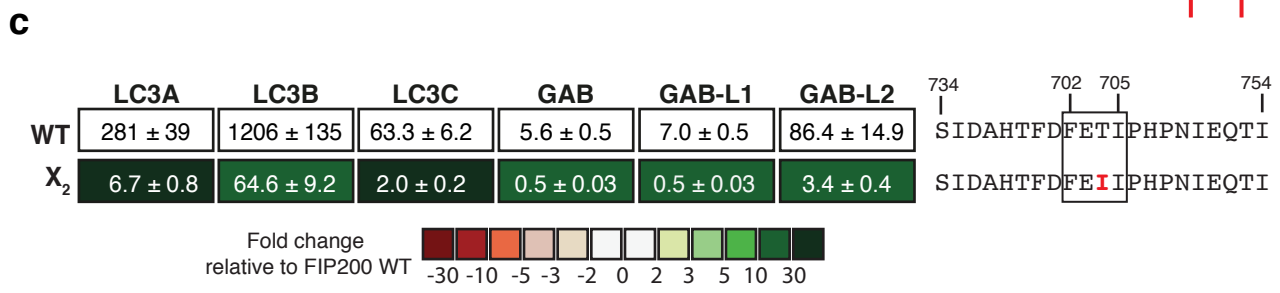
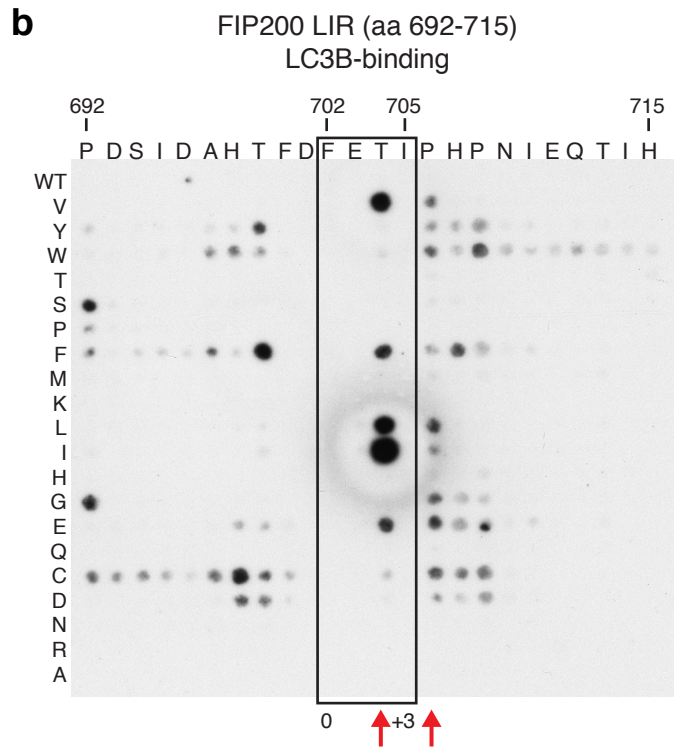
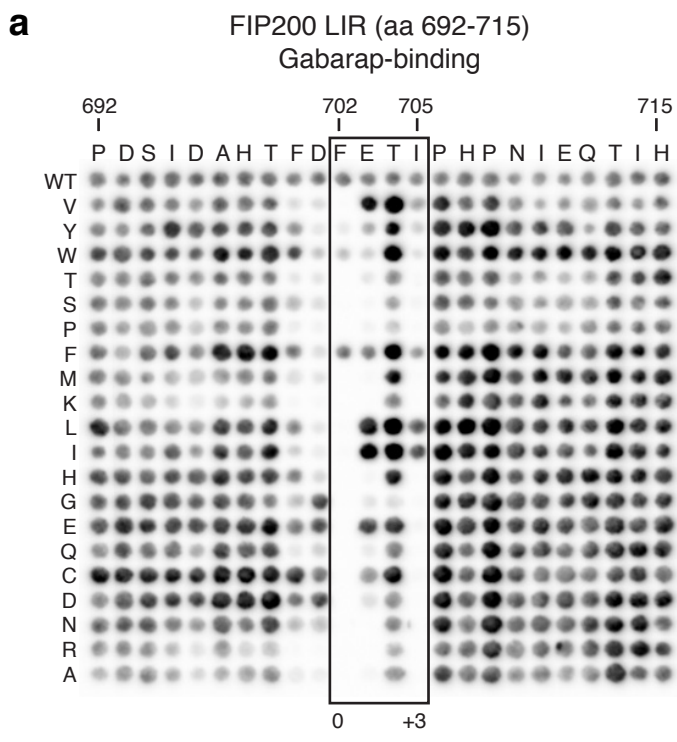
Fold change relative to ULK1 WT

-30 -10 -5 -3 -2 0 2 3 5 10 30

Supplementary Figure 3

Supplementary Fig. 3. The C-terminal region is critical for ULK1 LIR binding specificity.

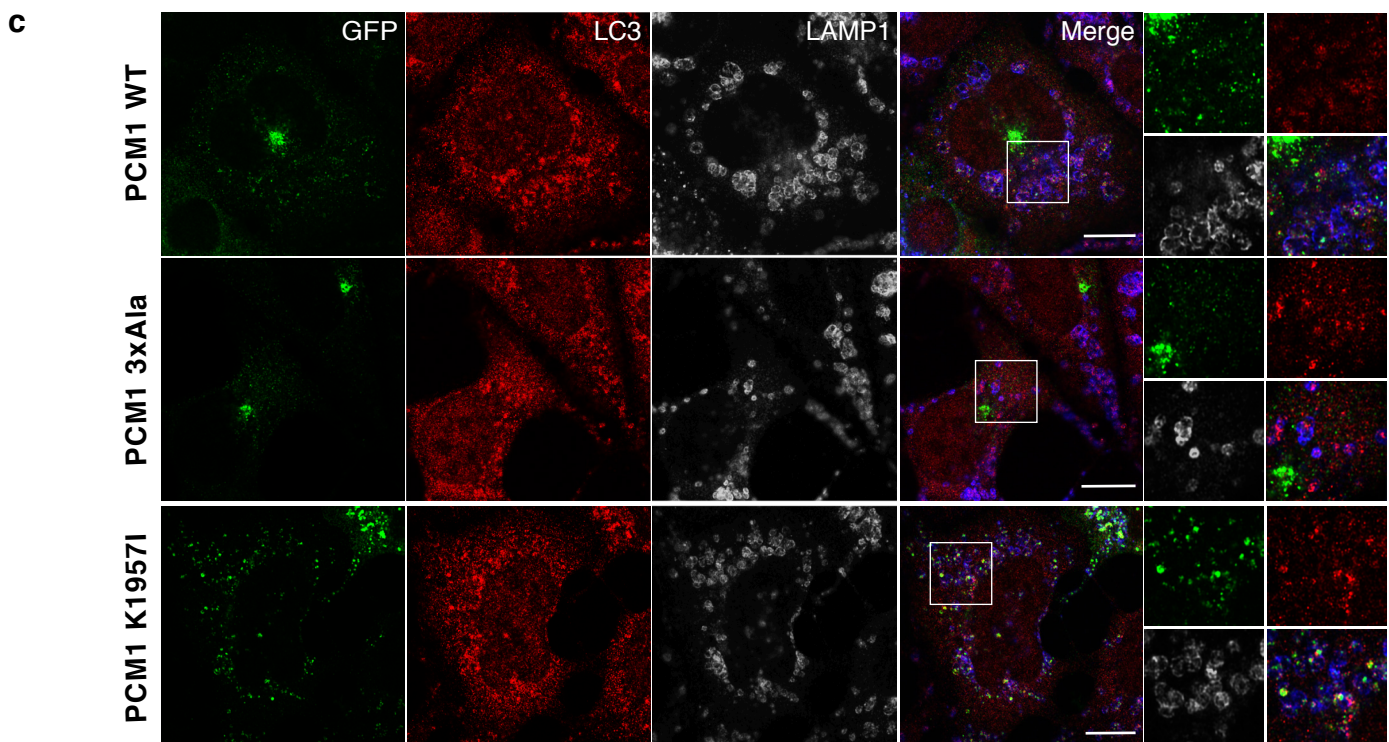
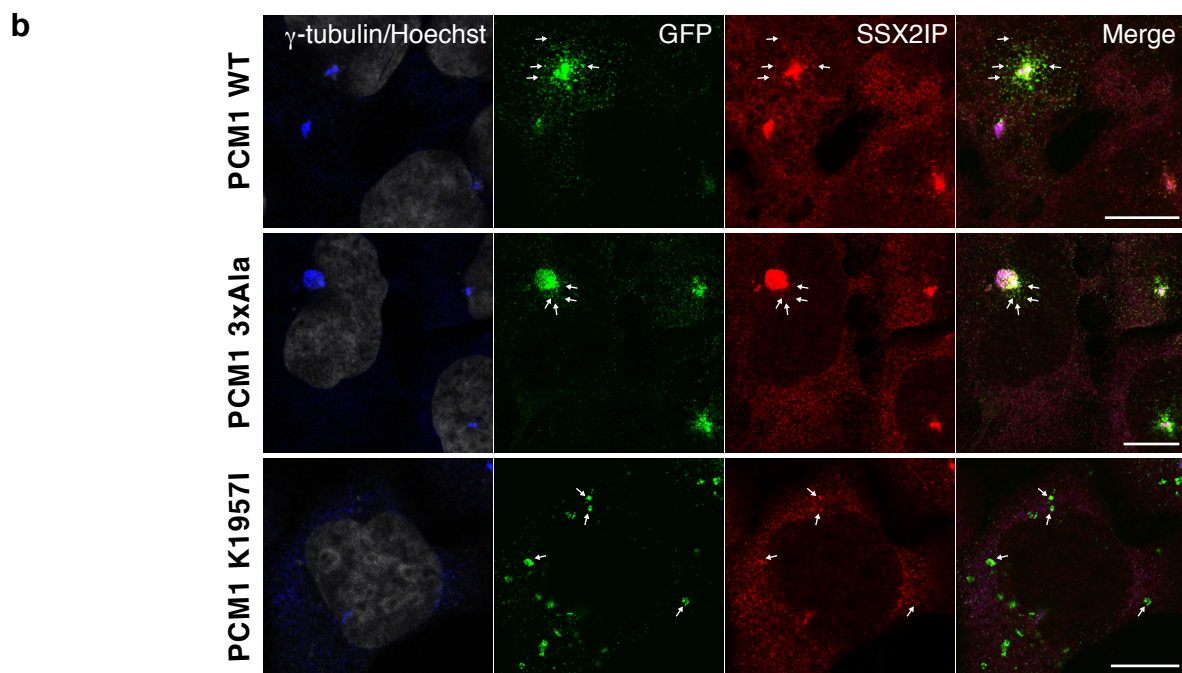
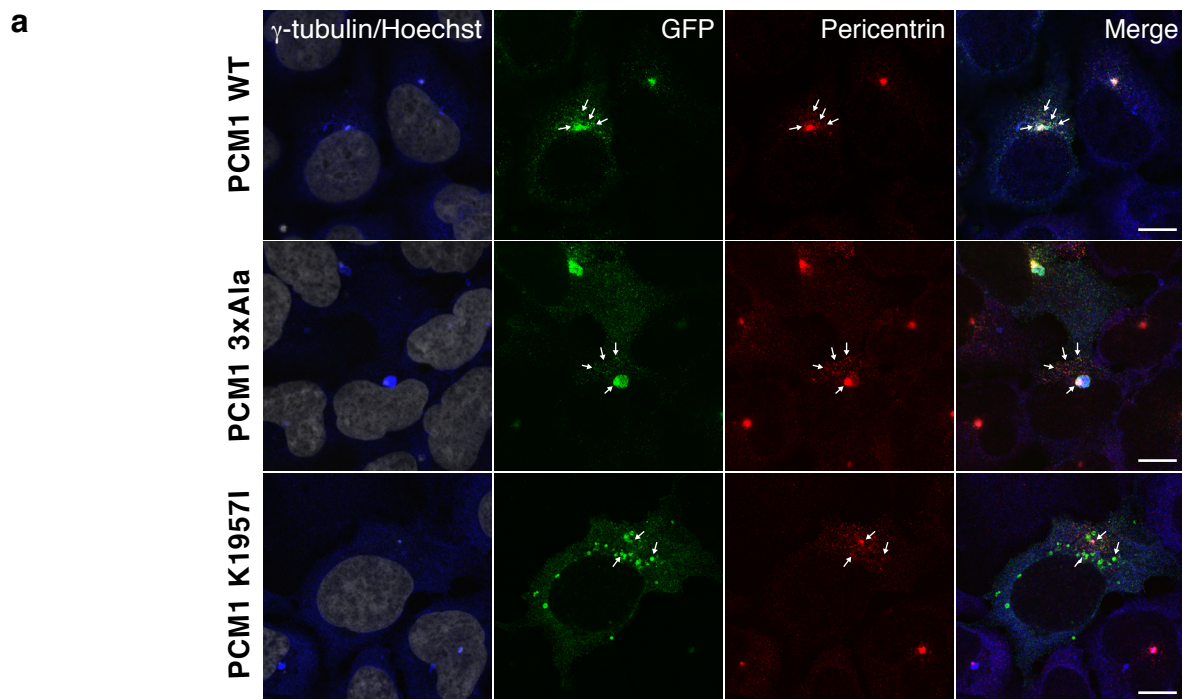
(a) Sequence alignment of PCM1, ULK1, ATG13 and FIP200 LIR motif sequences from various species (X. laevis: *Xenopus laevis*, D. rerio: *Danio rerio*, Dm: *Drosophila melanogaster*, Ce: *Caenorhabditis elegans*). The core LIR motif is boxed and aromatic and hydrophobic residues in position Θ_0 and Γ_3 depicted in blue. Conserved proline residues are shown in yellow, acidic residues in red. (b) Peptide array of truncated ULK1 LIR peptides incubated with indicated GST-ATG8 protein and immunoblotted with anti-GST. Each peptide is spotted in triplicates. (c) Affinities (Kd values) of truncated ULK1 LIR peptides to ATG8 proteins determined by Bio-Layer Interferometry (BLI). Colour code indicates fold-changes relative to Kd value of ULK1 WT LIR peptide binding to the corresponding ATG8 protein. (data are mean \pm s.d., n=2). (d) Mutational peptide array of 24-mer ULK1 peptide covering LIR motif incubated with GST-LC3A and immunoblotted with anti-GST. Each amino acid position was substituted for every other amino acid.



Supplementary Figure 4

Supplementary Fig. 4.: Modulation of X₂ residues changes LIR binding specificity.

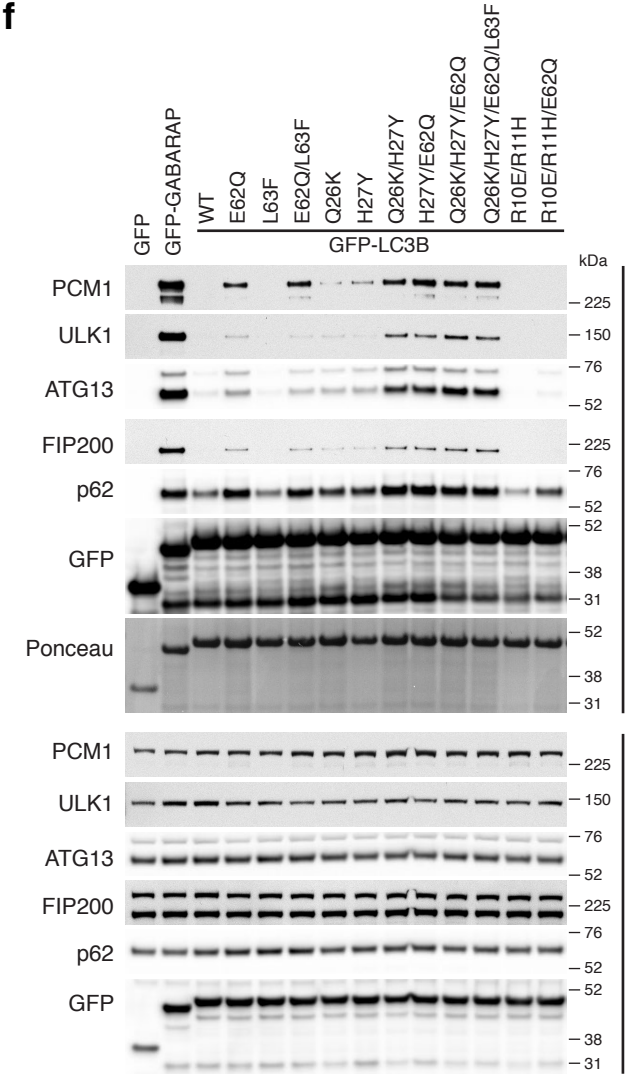
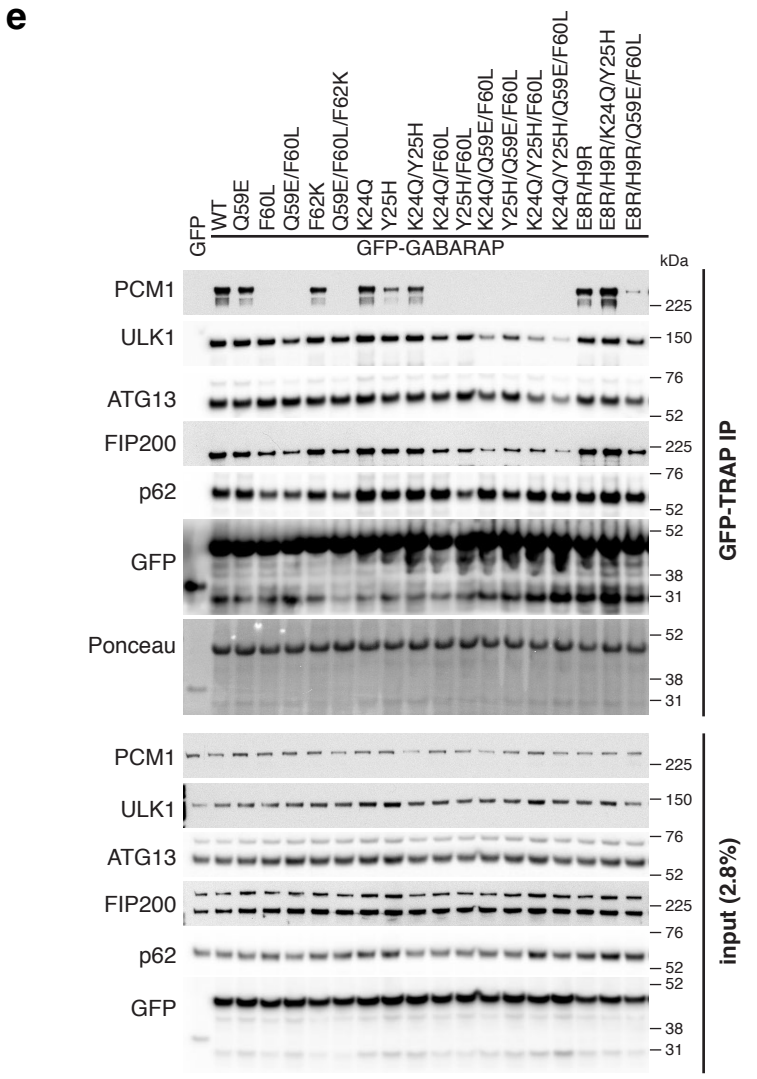
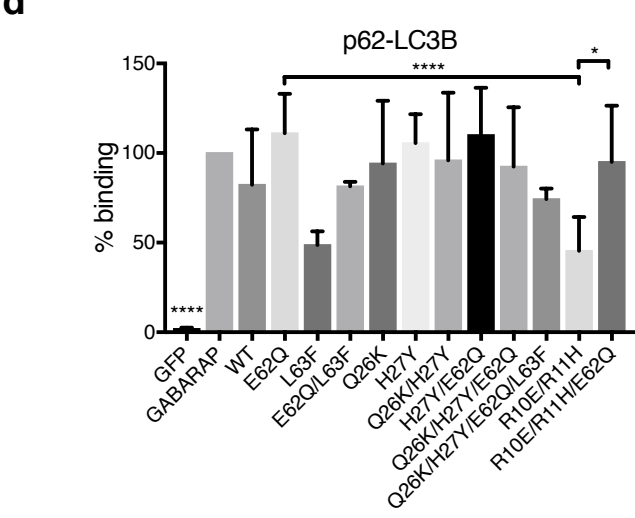
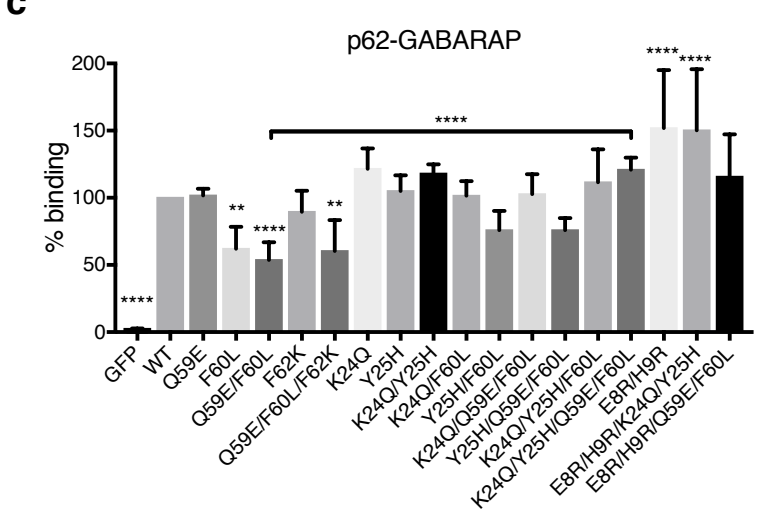
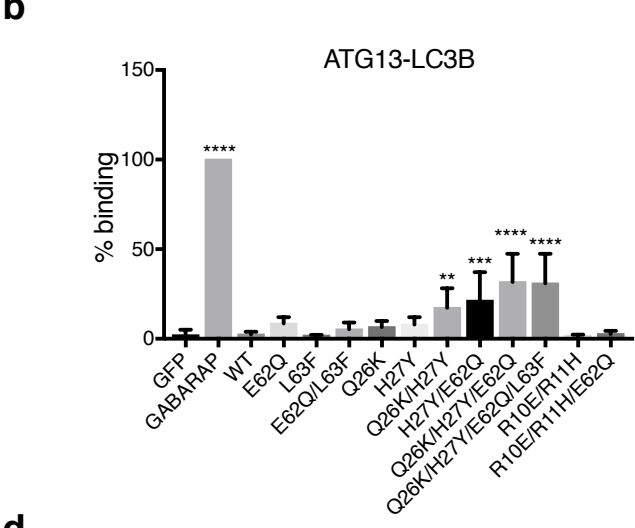
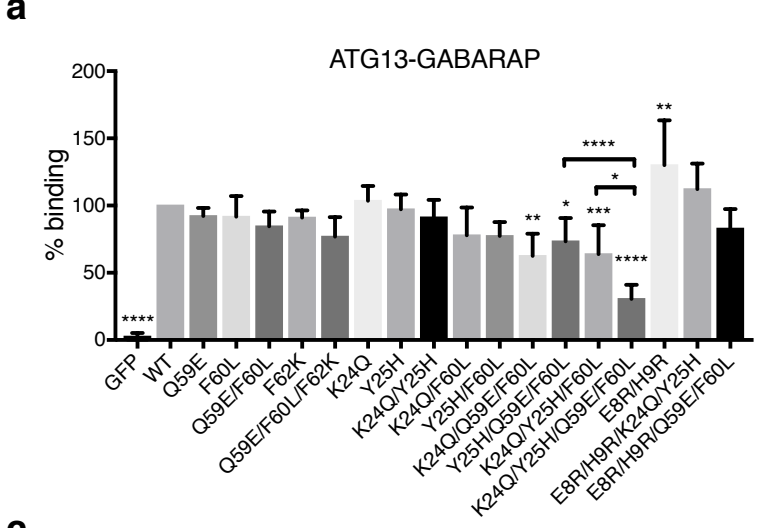
(a) and (b) Mutational peptide array of 24-mer FIP200 peptide covering LIR motif incubated with GST-GABARAP (a) or GST-LC3B (b) and immunoblotted with anti-GST. Each amino acid position was substituted for every other amino acid. (c) Affinities (K_d values) of FIP200 WT and FIP200 T704I LIR peptides to ATG8 proteins determined by Bio-Layer Interferometry (BLI). Color code indicates fold-changes relative to K_d value of FIP200 WT LIR peptide binding to the corresponding ATG8 protein. (data are mean ± s.d., n=2) (d) to (h) 24-mer peptide array analyses of ATG4B (d), FUNDC1 (e) and FKBP8 (f) FIP200 (g), and FYCO1 (h) LIR peptides containing point mutations incubated with indicated GST-ATG8 protein and immunoblotted with anti-GST. Each peptide is spotted in triplicates. Mutated residues are highlighted in red.



Supplementary Figure 5

Supplementary Fig. 5: PCM1 colocalization with pericentrin, SSX2IP, LC3 and LAMP1.

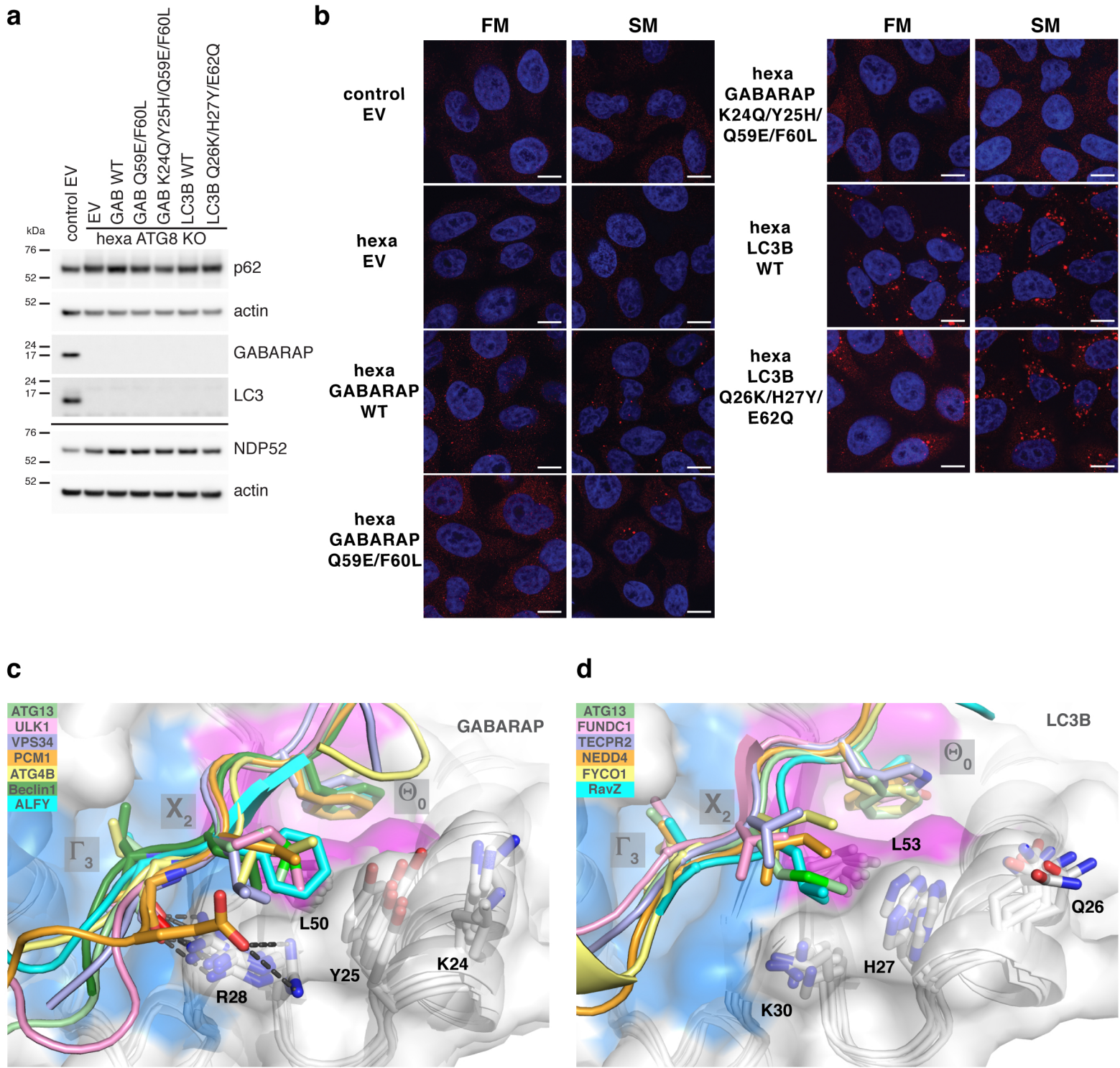
(a) HEK293A cells expressing indicated GFP-PCM1 constructs starved for 2 h in EBSS, fixed and labelled with anti- γ -tubulin, anti-pericentrin and Hoechst. Scale bars represent 10 μ m. (b) HEK293A cells expressing indicated GFP-PCM1 constructs starved for 2 h in EBSS, fixed and labelled with anti- γ -tubulin, anti-SSX2IP and Hoechst. Scale bars represent 10 μ m. (c) HEK293A cells expressing indicated GFP-PCM1 constructs starved for 2 h in EBSS in the presence of BafA1, fixed and labelled with anti-LC3 and anti-LAMP1. Scale bars represent 10 μ m.



Supplementary Figure 6

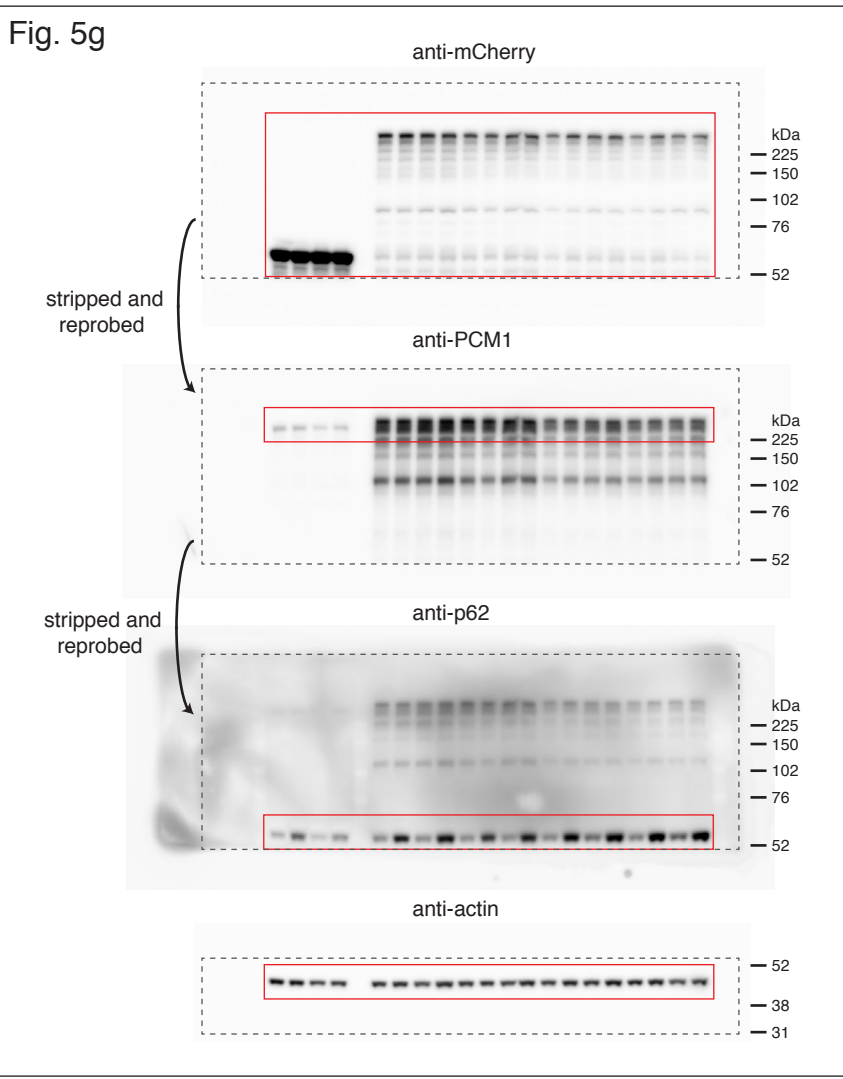
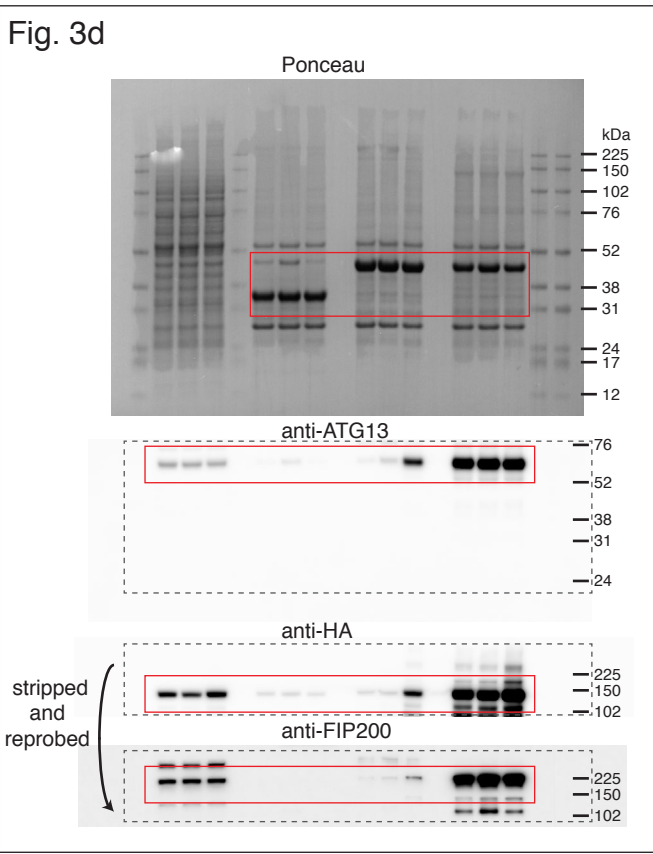
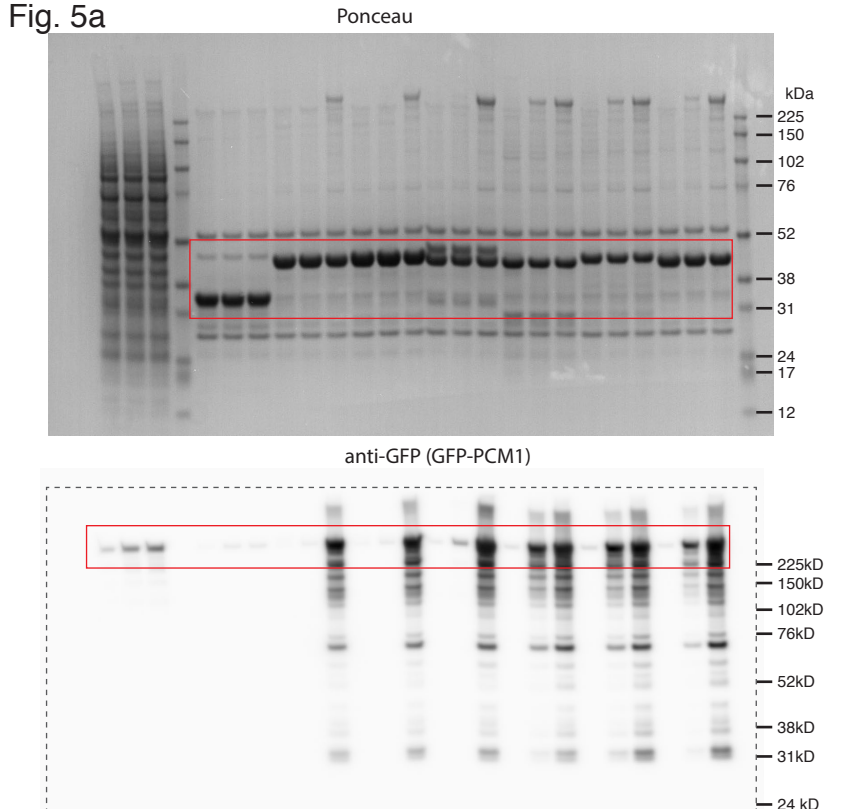
Supplementary Fig. 6: Non-conserved GABARAP residues key to selective LIR binding.

(a) and (c) Quantification of endogenous ATG13 (a) and p62 (c) binding to indicated GFP-GABARAP constructs expressed and immunoprecipitated by GFP-TRAP from HEK293A cells. (e) Representative immunoblots of GFP-TRAP IP experiments. (b) and (d) Quantification of endogenous ATG13 (b) and p62 (d) binding to indicated GFP-LC3B constructs expressed and immunoprecipitated by GFP-TRAP from HEK293A cells. (f) Representative immunoblots of GFP-TRAP IP experiments. Statistical analysis using One-Way ANOVA test; mean \pm s.d.; data from at least three independent experiments. **** $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$.



Supplementary Figure 7

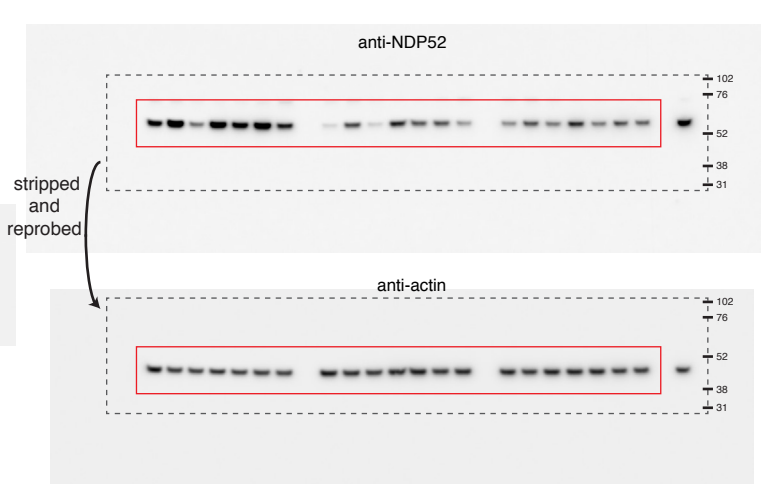
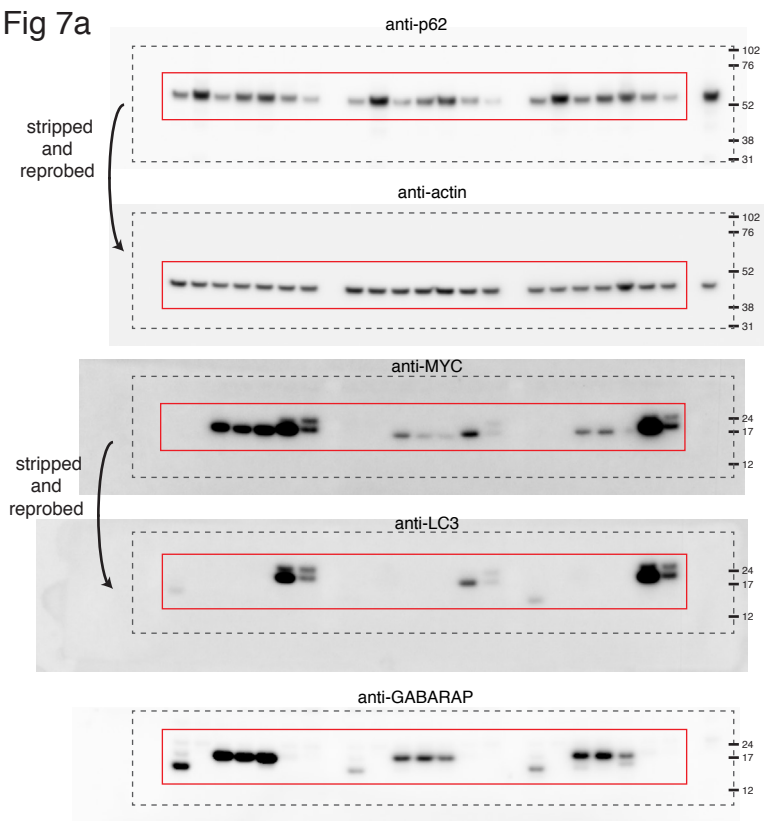
Supplementary Fig. 7: Rendering GABARAP more LC3B-like impairs NDP52 degradation. (a) ATG8 CRISPR KO HeLa cell lines stably expressing indicated MYC-ATG8 constructs were grown in full medium without doxycycline prior to lysis and immunoblot. No differences in p62 and NDP52 protein levels between different ATG8 hexa KO HeLa cell lines when MYC-ATG8 construct expression was not induced. (b) ATG8 CRISPR KO HeLa cell lines stably expressing indicated MYC-ATG8 constructs starved for 2h in EBSS, fixed and labelled with anti-MYC and Hoechst. Expression of MYC-ATG8 constructs was induced by 1 μ g/ml doxycycline for 6 days. Scale bars represent 10 μ m. (c) Structure of the ATG13 LIR bound to GABARAP. The ATG13 LIR sequence is shown in pale green cartoon with residues in position Θ_0 , X_2 and Γ_3 depicted as sticks. GABARAP is displayed in white cartoon and transparent surface with hydrophobic pocket 1 and 2 colored in pink and blue surfaces, respectively. GABARAP residues K24^{GAB}, Y25^{GAB}, R28^{GAB} and L50^{GAB} are shown in white stick. The structures of various LIR motifs bound to GABARAP and containing a hydrophobic residue (except PCM1) in position X_2 were superposed to the ATG13:GABARAP structure for comparison. ULK1 LIR (pink), VPS34 LIR (purple, PDB 6HOG), PCM1 LIR (orange), ATG4B LIR (yellow, PDB 5LXH), Beclin1 LIR (dark green, PDB 6HOJ) and ALFY LIR (cyan, PDB 3WIN). (d) Structure of FYCO1 LIR bound to LC3B (PDB:5WRD). The FYCO1 LIR sequence is shown in pale yellow cartoon with residues in position Θ_0 , X_2 and Γ_3 depicted as sticks. LC3B is displayed in white cartoon and transparent surface with hydrophobic pocket 1 and 2 colored in pink and blue surfaces, respectively. LC3B residues Q26^{LC3B}, H27^{LC3B}, K30^{LC3B} and L53^{LC3B} are shown in white stick. The structures of various LIR motif bound to LC3B and containing a hydrophobic residue in position X_2 were superposed to the FYCO1:LC3B structure for comparison. ATG13 LIR (green, PDB 3WAO), FUNDC1 LIR (pink, PDB 5GMV), TECPR2 LIR (purple, PDB 5DCN), NEDD4 LIR (orange, PDB 5V4K) and RavZ LIR (blue, PDB 5MS6).



Supplementary Figure 8

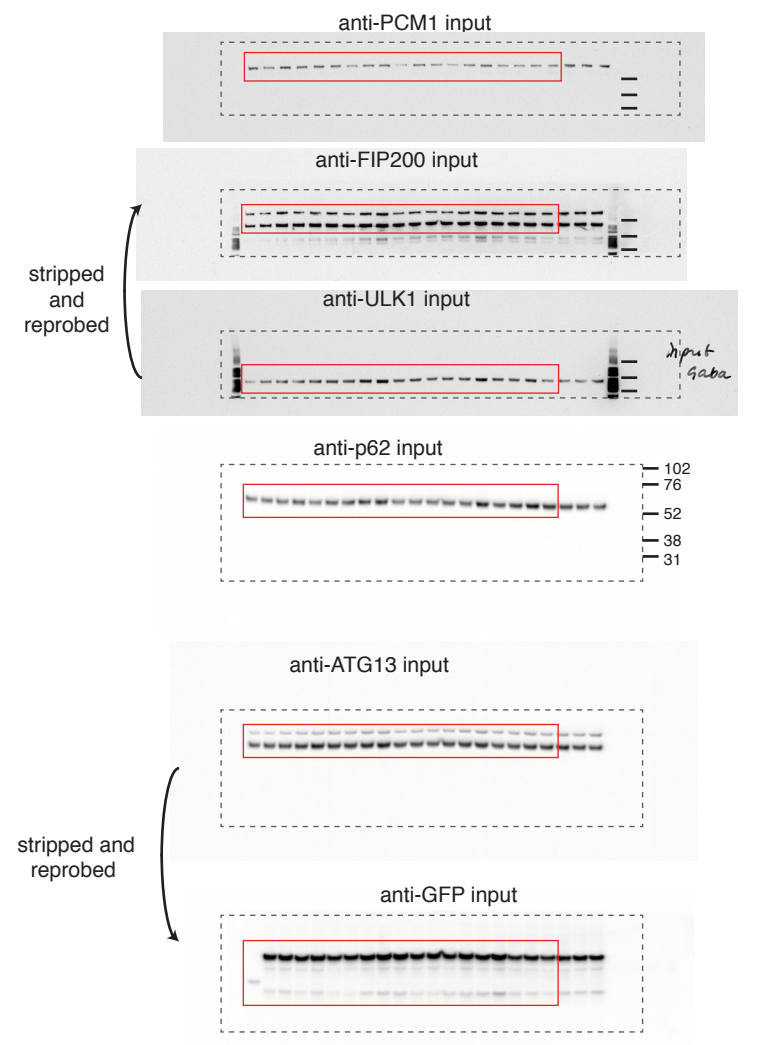
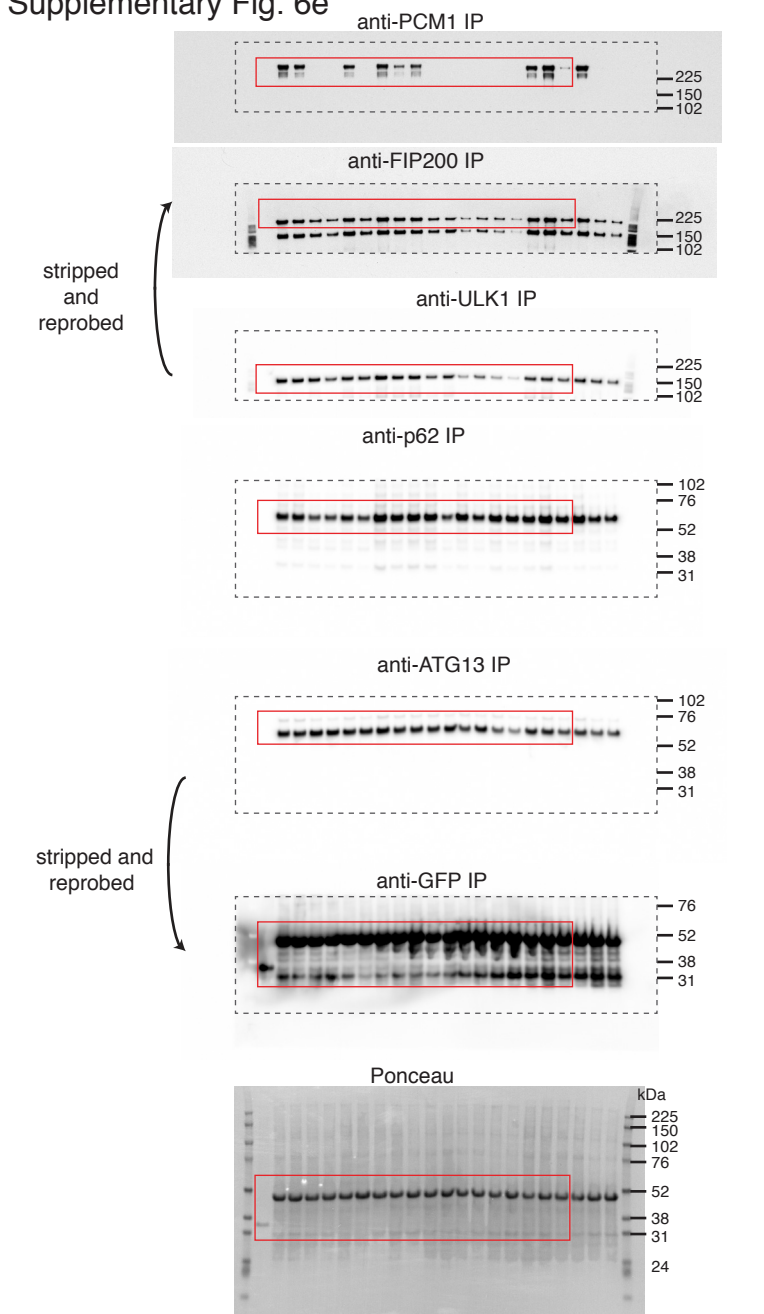
Supplementary Fig. 8: Uncropped Western blots.
Full blot is indicated by dashed grey lines, red box shows cropped region.

Fig 7a



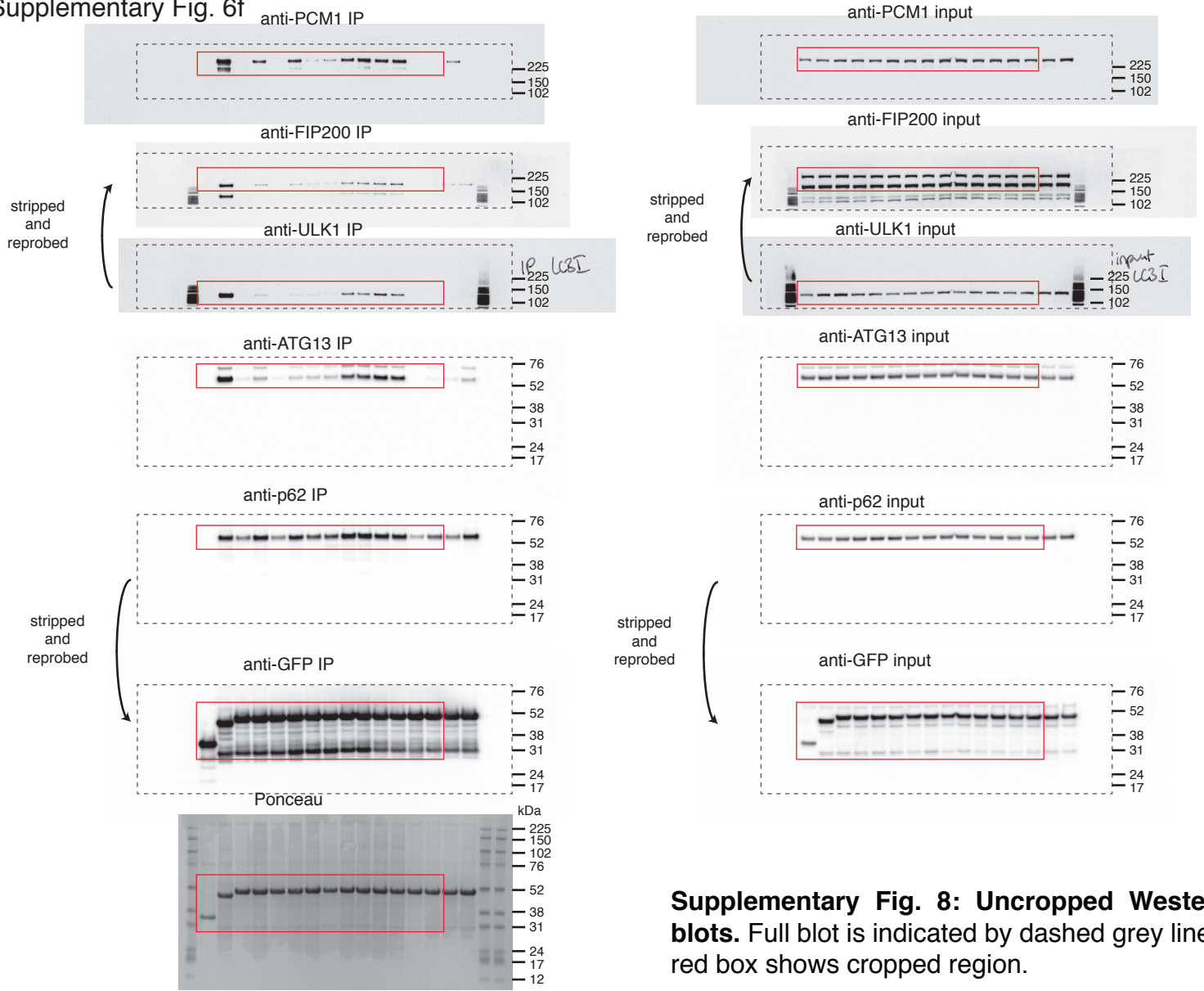
Supplementary Fig. 8: Uncropped Western blots. Full blot is indicated by dashed grey lines, red box shows cropped region.

Supplementary Fig. 6e



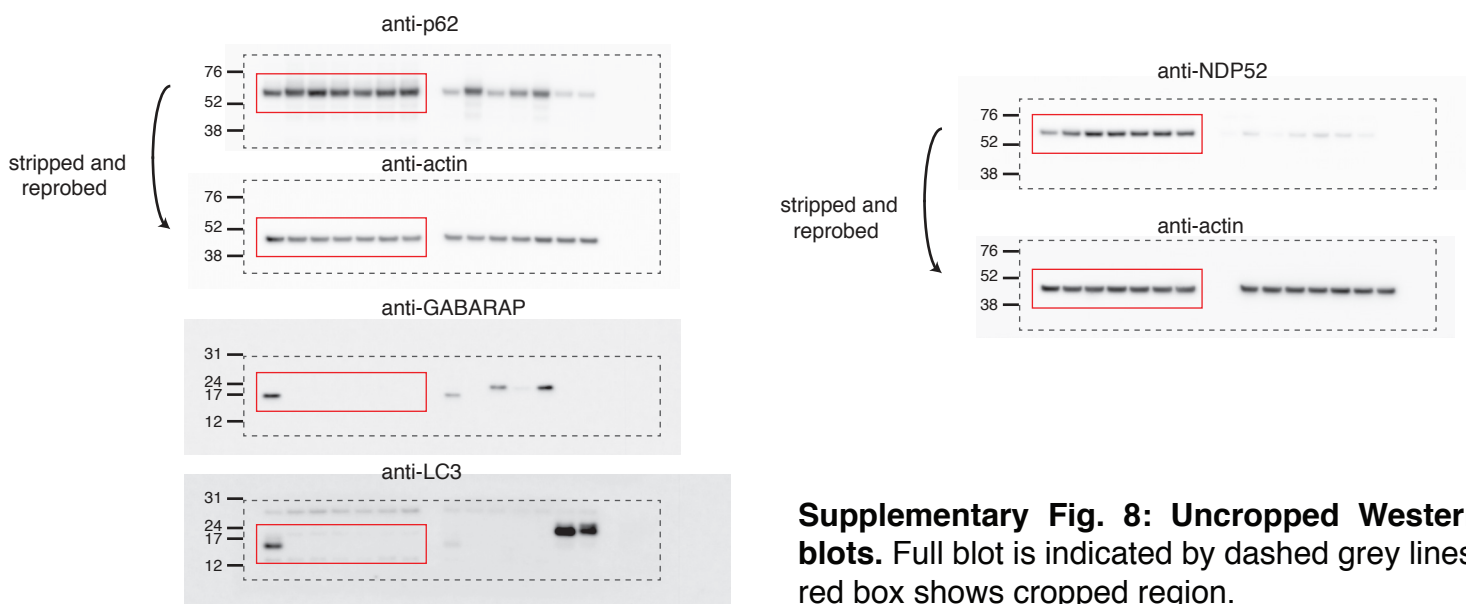
Supplementary Fig. 8: Uncropped Western blots. Full blot is indicated by dashed grey lines, red box shows cropped region.

Supplementary Fig. 6f



Supplementary Fig. 8: Uncropped Western blots. Full blot is indicated by dashed grey lines, red box shows cropped region.

Supplementary Fig. 7a



Supplementary Fig. 8: Uncropped Western blots. Full blot is indicated by dashed grey lines, red box shows cropped region.

Supplementary Table 1: Primers used in this study.

Mutagenesis PCR of PCM1 in pEGFP-C2 and pDNOR221 vectors	
Primer	Sequence
EGFP-PCM1 K1957I (pEGFP-C2) forward	GATTTTGTAAATAGTTGAAGATTTACCAC
EGFP-PCM1 K1957I (pEGFP-C2) reverse	TTCTTCATCAGACTTTTGAC
PCM1 FDII (V1956D/K1957I/V1958I) (pDNOR221) forward	GAAGATTTTGACATAATTGAAGATTTACCACTG
PCM1 FDII (V1956D/K1957I/V1958I) (pDNOR221) reverse	TTCATCAGACTTTTGACTTATATTAC
Gateway cloning of PCM1 WT, 3xAla (D1954A/F1955A/V1958A) or K1957I into pDNOR221 vector	
Primer	Sequence
PCM1 forward	GGGGACA AGT TTG TAC AAA AAA GCA GGC TTGatggccacaggaggagggtccc
PCM1 reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTAtcatcatataactctgggctccc acc
Mutagenesis PCR of pDEST-EGFP-GABARAP and/or pAL-GST-3C-GABARAP	
Primer	Sequence
GABARAP Q59E forward	CACAGTTGGTgAGTTCTACTTC
GABARAP Q59E reverse	AGATCAGAAGGCACCAGG
GABARAP F60L forward	AGTTGGTCAGcTCTACTTCTTG
GABARAP F60L reverse	GTGAGATCAGAAGGCACC
GABARAP Q59E/F60L forward	CACAGTTGGTgagcTCTACTTCTTG
GABARAP Q59E/F60L reverse	AGATCAGAAGGCACCAGG
GABARAP F62K forward	TCAGTTCTACaagTTGATCCGGAAGC
GABARAP F62K reverse	CCAACTGTGAGATCAGAAG
GABARAP Q59E/F60L/F62K forward	TGAGCTCTACAAGTTGATCCGGAAGC
GABARAP Q59E/F60L/F62K reverse	CCAACTGTGAGATCAGAAG
GABARAP K24Q forward	GATCCGAAAGCAATACCCGGACCG
GABARAP K24Q reverse	TTCTCGCCCTCAGAGCGG
GABARAP Y25H forward	CCGAAAGAAACACCCGGACCG
GABARAP Y25H reverse	ATCTTCTCGCCCTCAGAG

GABARAP K24Q/Y24H forward	GATCCGAAAGCAACACCCGGACCG
GABARAP K24Q/Y24H reverse	TTCTCGCCCTCAGAGCGG
GABARAP E8R/H9R forward	GAAGTTCGTGTACAAAGAAcgGCgTCCGTTTCGAGAAGCGCCGC
GABARAP R28K forward	ATACCCGGACAAGGTGCCGGTG
GABARAP R28K reverse	TTCTTTCCGATCTTCTCGC
GABARAP K24Q/Y25H/R28K forward	CGGACAAGGTGCCGGTGATAGTAGA
GABARAP K24Q/Y25H/R28K reverse	GGTGTGCTTTCCGATCTTCTCGCC
GABARAP L55V forward	GCCTTCTGATGTCACAGTTGG
GABARAP L55V reverse	ACCAGGTATTTCTTTTTGTC
GABARAP L63I forward	GTTCTACTTCATCATCCGGAAGC
GABARAP L63I reverse	TGACCAACTGTGAGATCAG
Mutagenesis PCR of pDEST-EGFP-LC3B and/or pAL-GST-3C-LC3B	
Primer	Sequence
LC3B E62Q forward	CAACATGAGTcAGCTCATCAAG
LC3B E62Q reverse	ACATGGTCAGGTACAAG
LC3B L63F forward	CATGAGTGAGTTCATCAAGATAATTAG
LC3B L63F reverse	TTGACATGGTCAGGTACAAG
LC3B E62Q/L63F forward	CATGAGTCAGTTCATCAAGATAATTAG
LC3B E62Q/L63F reverse	TTGACATGGTCAGGTACAAG
LC3B Q26K forward	TATTCGAGAGAAGCATCCAAC
LC3B Q26K reverse	AGTCGGACATCTTCTACTC
LC3B H27Y forward	TCGAGAGCAGTATCCAACCAA
LC3B H27Y reverse	ATAAGTCGGACATCTTCTAC
LC3B Q26K/H27Y forward	TATTCGAGAGAAGTATCCAACCAAATC
LC3B Q26K/H27Y reverse	AGTCGGACATCTTCTACTC
LC3B K30R forward	CATCCAACCAGAATCCCGGTG
LC3B K30R reverse	CTGCTCTCGAATAAGTCG
LC3B Q26K/H27Y/K30R forward	CAACCAGAATCCCGGTGATAATAGAAC

LC3B Q26K/H27Y/K30R reverse	GATACTTCTCTCGAATAAGTCGGAC
LC3B R10E/R11H forward	CTTCAAGCAGGAGCACACCTTCGAACAAAGAGTAG
LC3B R10E/R11H reverse	GTCTTCTCCGACGGCATG
Mutagenesis of HA-ULK1 (pCDNA3.1)	
Primer	Sequence
ULK1 P361D forward	CGTCATGGTGCACGCGCAGTTTC
ULK1 P361D reverse	AAGTCGTCTGTGTCACAG
ULK1 T354E/M391I/P361D forward	GTCATCGTCGACGCGCAGTTTCCAGGTGA
ULK1 T354E/M391I/P361D reverse	GAAGTCGTGTCGTACAGGAAGAGTCCTTGC
Cloning of MYC-GABARAP WT, Q59E/F60L or K24Q/Y25H/Q59E/F60L and MYC-LC3B WT or Q26K/H27Y/E62Q into pLVX-TetOne-Puro vector	
Primer	Sequence
GABARAP AgeI forward	GctgcaggACCGGTGCCGCCACCATGGAACAAAACTCATCTCAGA AGAGGATCTGTCTggcATGAAGTTCGTGTACAAAGAAGAGC
GABARAP BamHI reverse	CcatgGGATCCTCATCACAGACCGTAGACACTTTCG
LC3B AgeI forward	GctgcaggACCGGTGCCGCCACCATGGAACAAAACTCATCTCAGA AGAGGATCTGTCTggcATGCCGTCGGAGAAGACCTTC
LC3B BamHI reverse	CcatgGGATCCTCATTACTGACAATTTTCATCCCGAACG
Cloning of GST-3C-ATG8 proteins into pAL (pGEX-6P2) vector for recombinant protein expression in bacteria	
Primer	Sequence
LC3A BamHI forward	CcatgggatccATGCCCTCAGACCGGCCTTTC
LC3A NotI reverse	ATAAGAATGCGGCCCGCCTTATCAGAAGCCGAAGGTTTCCTGG
LC3B EcoRI forward	GctgcagggaattcATGCCGTCGGAGAAGACCTTCAAG
LC3B NotI reverse	ATAAGAATGCGGCCCGCCTTACTACTGACAATTTTCATCCCGAACGTC
LC3C BamHI forward	CcatgggatccATGCCGCTCCACAGAAAATCC
LC3C NotI reverse	ATAAGAATGCGGCCCGCCTTACTAGAGAGGATTGCAGGGTCTGTC CT
GABARAP BamHI forward	CcatgggatccATGAAGTTCGTGTACAAAGAAGAGCATC
GABARAP NotI reverse	ATAAGAATGCGGCCCGCCTTATCACAGACCGTAGACACTTTCGTCA C
GABARAPL1 BamHI forward	CcatgggatccATGAAGTTCAGTACAAGGAGGACCAT
GABARAPL1 NotI reverse	ATAAGAATGCGGCCCGCCTTATCATTTCATAGACTCTCATCA CTG

GABARAPL2 EcoRI forward	GctgcaggggaattcATGAAGTGGATGTTCAAGGAGGACCAC
GABARAPL2 NotI reverse	ATAAGAATGCGGCCCGCCTTAGAAGCCAAAAGTGTCTCTCCGCTG
Cloning of chimera constructs (pAL-GST-3C-LIR motif-GABARAP) for crystallisation	
Primer	Sequence
PCM1 LIR NcoI forward	catgggggatgaagaagattttgtaaagttgaagatttaccactgaaag
PCM1 LIR BamHI reverse	gatccttcagtggtaaatcttcaactttacaaaatcttctcatcccc
ULK1 LIR NcoI forward	CatggggACAGACGACTTCGTCATGGTCCCCGCGCAGTTTCCAGGTg
ULK1 LIR BamHI reverse	gatccACCTGGAAACTGCGCGGGGACCATGACGAAGTCGTCTGTcc
ATG13 LIR NcoI forward	CatggggCATGATGACTTTGTTATGATAGACTTTAAACCAGCTTTTTCTg
ATG13 LIR BamHI reverse	gatccAGAAAAAGCTGGTTTAAAGTCTATCATAACAAAGTCATCATGccc

Supplementary Table 2: Peptides used for BLI affinity measurements.

Peptide	Sequence
ATG13 WT	Biotin-Linker-SSGNTHDDE <u>F</u> VMI DFKPAFSKD-Amide
ULK1 WT (aa 349-369)	Biotin-Linker-DSSCDTDD <u>F</u> VMV PAQFPGDLV-Amide
ULK1 (aa 353-369)	Biotin-Linker-DTDDE <u>F</u> VMV PAQFPGDLV-Amide
ULK1 (aa 355-369)	Biotin-Linker-DDE <u>F</u> VMV PAQFPGDLV-Amide
ULK1 (aa 357-369)	Biotin-Linker- <u>F</u> VMV PAQFPGDLV-Amide
ULK1 (aa 349-364)	Biotin-Linker-DSSCDTDD <u>F</u> VMV PAQF-Amide
ULK1 (aa 349-362)	Biotin-Linker-DSSCDTDD <u>F</u> VMV PA-Amide
ULK1 (aa 349-360)	Biotin-Linker-DSSCDTDD <u>F</u> VMV-Amide
FIP200 WT	Biotin-Linker-SIDAHTFD <u>F</u> ETI PHPNIEQTI-Amide
FIP200-T704I	Biotin-Linker-SIDAHTFD <u>F</u> EII PHPNIEQTI-Amide
PCM1 WT	Biotin-Linker-SQKSDEED <u>F</u> VK VEDLPLKLTl-Amide
PCM1 V1956D	Biotin-Linker-SQKSDEED <u>F</u> DK VEDLPLKLTl-Amide
PCM1 K1957I	Biotin-Linker-SQKSDEED <u>F</u> VIV VEDLPLKLTl-Amide
PCM1 V1958I	Biotin-Linker-SQKSDEED <u>F</u> VKI EDLPLKLTl-Amide
PCM1 V1956D/ K1957I/ V1958I	Biotin-Linker-SQKSDEED <u>F</u> DI EDLPLKLTl-Amide
PCM1 E1953A/D1954V	Biotin-Linker-SQKSDE <u>A</u> V <u>F</u> VK VEDLPLKLTl-Amide
PCM1 E1959T	Biotin-Linker-SQKSDEED <u>F</u> VKV TDLPLKLTl-Amide
PCM1 P1962A	Biotin-Linker-SQKSDEED <u>F</u> VKV EDLALPLKLTl-Amide
FYCO1 WT	Biotin-Linker-YRPPDDA <u>V</u> EDII TDEELCQIQ-Amide
p62-WT	Biotin-Linker-NCSGGDD <u>D</u> W THLSSKEVDPST-Amide