Supplementary Materials to:

Pharmacological modulators of autophagy activate a parallel noncanonical pathway driving unconventional LC3 lipidation

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Inventory of Supplementary Materials.

Supplementary Tables 1-2

Table 1. Chemical properties of drugs used in the study.
Table 2. CRISPR/Cas9 gRNAs and plasmids used in this study

Supplementary Figures 1-7

Figure 1. Analysis and validation of ATG13⁻/⁻ cells generated by CRISPR/Cas9.

Figure 2. Repeat western blots of drug induced LC3 lipidation in wild type and ATG13⁻/⁻ HEK293 cells.

Figure 3. Non-canonical autophagy induction in ATG13⁻/⁻ MCF10A cells.

Figure 4. Effect of ionophores and lysosomotropic drugs on WIPI2 puncta.

Figure 5. GABARAPL2 relocalization during non-canonical autophagy.

Figure 6. Validation of non-canonical autophagy mechanism in drug induced LC3 lipidation.

Figure 7. Effect of ionophore and lysosomotropic drugs on endomembrane damage assessed by Galectin 3 localization.
Table S1. Chemical properties of drugs used in the study.

| Drug                          | Clinical indications | Mechanism of action | pKa | Properties                          | Action on autophagy                                      | References |
|-------------------------------|----------------------|---------------------|-----|-------------------------------------|----------------------------------------------------------|------------|
| Amiodarone                    | Arrhythmia           | Ca²⁺ channel blocker | 9.37| Lysosomotropic                     | Inducer (MTOR dependent and independent)                | 1-5        |
| Ammonium chloride (NH₄Cl)     | Hypochloremic states, metabolic alkalosis | Converted to HCl and NH₃ by oxidation in the liver | 9.25| Lysosomotropic                     | Flux Inhibitor                                            | 1-5        |
| Betahistine dihydrochloride (BH) | Vertigo             | Histamine H1 receptor agonist and H3 receptor antagonist | 9.75| Lysosomotropic                     | Flux Inhibitor                                            | 6          |
| CCCP                          | *                    | Protonophore, proton uncoupler | 6   | Ionophore                           | Mitophagy inducer, Flux inhibitor                        | 7, 10      |
| Choloroquine (CQ)             | Malaria, cancer      | Weak base, protonated and trapped in acidic compartments | 10.47| Lysosomotropic                     | Flux Inhibitor                                            | 13         |
| Hydroxychloroquine (HCQ)     | Malaria, cancer      | Weak base, protonated and trapped in acidic compartments | 9.91| Lysosomotropic                     | Flux Inhibitor                                            | 7, 11, 12  |
| Lidocaine hydrochloride (LH)  | Local anaesthesia    | Na⁺ channel blocker  | 7.96| Lysosomotropic                     | Flux Inhibitor                                            | 14         |
| Monensin (Mon)                | Veterinary use, antibiotics | Na⁺/H⁺ exchange | 13.03| Ionophore                           | Flux Inhibitor                                            | 7, 13, 14  |
| Nigericin (Nig)               | Veterinary use, antibiotics | K⁺/H⁺ exchange | 4.43| Ionophore                           | Flux Inhibitor                                            | 9, 10      |
| Procainamide (ProA)           | Arrhythmia           | Na⁺ channel blocker  | 9.09| Lysosomotropic                     | Flux Inhibitor                                            | 12         |

pKa values calculated from [http://www.ebi.ac.uk/chembl/](http://www.ebi.ac.uk/chembl/).

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Table S2. Nucleotide sequences of the gRNA and references of the plasmids used to deplete ATG13 or ATG16L1 genes in HEK293 GFP-LC3 and MCF10A GFP-LC3 cells.

| Oligonucleotide | Sequence (5’ - 3’) | Cloning plasmid | Cell line |
|-----------------|-------------------|-----------------|-----------|
| ATG13gRNA_Fw1   | TTTCTTGGCTTTATATATCTTG GAAAGGACGAAACACCAGCAGCTGCC TGCAGTCG GGA | gRNA Cloning Vector (Addgene, 41824) | HEK293 GFP-LC3 |
| ATG13gRNA_Rev1  | GACTAGCCTTTATTTTTACTTGCTAT TTCTAGCTCTAAACCCCCACTGCA GGCAGCTGTC | gRNA Cloning Vector (Addgene, 41824) | HEK293 GFP-LC3 |
| ATG13gRNA_Fw2   | CACCGGACAGCTGCCTGCAGTCGG | pSpCas9(BB)-2A-GFP (Addgene, 48138) | MCF10A GFP-LC3 |
| ATG13gRNA_Rev2  | AAACCCCCGACTGCAGGACGTGC | pSpCas9(BB)-2A-GFP (Addgene, 48138) | MCF10A GFP-LC3 |
| ATG16gRNA_Fw    | CACCGGTGGATCTCCTGTTTC | pSpCas9(BB)-2A-GFP (Addgene, 48138) | MCF10A GFP-LC3 |
| ATG16gRNA_Rev   | AAACGAACCAGGATGATCCACC | pSpCas9(BB)-2A-GFP (Addgene, 48138) | MCF10A GFP-LC3 |
Supplementary Figure 1

**Figure S1.** Analysis and validation of ATG13−/− cells generated by CRISPR/Cas9. (A) Representative western blots of ATG13, LC3 and GAPDH in wild type and ATG13−/− HEK293 cells starved with HBSS or treated with PP242 (1 µM) for 1 h. (B) Confocal imaging of GFP-LC3 in wild-type and ATG13−/− HEK293 cells transiently transfected or not with an mCherry-ATG13 expression plasmid. Cells were starved with HBSS for 1 h. Bar: 10 µm for all images. (C) Representative western blots of ATG13, LC3 and GAPDH in wild-type and ATG13−/− MCF10A cells treated with starved PP242 (1 µM) or bafilomycin A1 (Baf, 100 nM) for 1 h or starved with HBSS for 2 h in presence of Baf (100 nM) during the last hour (Baf + HBSS). (D) Confocal imaging of GFP-LC3 in wild-type and ATG13−/− MCF10A cells transiently transfected or not with an mCherry-ATG13 expression plasmid. Cells were starved with HBSS for 2 h in the presence of Baf (100 nM) during the last hour (Baf + HBSS). Bar: 10 µm for all images.
Figure S2. Repeat western blots of drug-induced LC3 lipation in wild-type and ATG13−/− HEK293 cells. Repeat representative western blots for LC3 and GAPDH in (A) wild-type and (B) ATG13−/− HEK293 cells treated with drugs at the indicated concentration for 2 h. Ratios of lipidated LC3-II:nonlipidated LC3-I were quantified and graphed.
Figure S3. Noncanonical autophagy induction in ATG13<sup>−/−</sup> MCF10A cells. (A) Confocal images of GFP-LC3 and endogenous LAMP1 immunostaining in ATG13<sup>−/−</sup> MCF10A cells treated with bafilomycin A<sub>1</sub> (Baf, 100 nM), monensin (Mon, 50 µM), nigericin (Nig, 50 µM), chloroquine (CQ, 100 µM), hydroxychloroquine (HCQ, 100 µM), NH<sub>4</sub>Cl (10 mM), procainamide (ProA, 5 mM) or betahistine (BH, 5 mM) for 2 h. Inserts show zoomed regions highlighting colocalization of the GFP-LC3 and LAMP1 signal. Bar: 5 µm for all images. (B) Confocal images of GFP-LC3 and endogenous LAMP1 immunostaining on entotic corpse vacuoles in ATG13<sup>−/−</sup> MCF10A cells treated with CCCP (100 µM) for 2 h. Arrow indicates lipidated LC3 on vacuoles. Bar: 5 µm for all images.
**Figure S4.** Effect of ionophores and lysosomotropic drugs on WIPI2 puncta. Confocal images of endogenous WIPI2 immunostaining in wild-type, *atg13<sup>−/−</sup>* and *atg9<sup>−/−</sup>* MEFs treated with (A) PP242 (1 µM), (B) bafilomycin A<sub>1</sub> (Baf, 100 nM), monensin (Mon, 50 µM), nigericin (Nig, 10 µM), chloroquine (CQ, 100 µM), hydroxychloroquine (HCQ, 100 µM), NH<sub>4</sub>Cl (10 mM), betahistine (BH, 5 mM), procainamide (ProA, 5 mM) or lidocaine hydrochloride (LH, 2.5 mM) for 2 h were analyzed. The average number of WIPI2 puncta per cell (100 cells/condition) was quantified and graphed.
Figure S5. GABARAPL2 relocalization during noncanonical autophagy. Confocal images of GFP-GABARAPL2 and endogenous LAMP1 immunostaining in atg9−/− MEF cells treated with bafilomycin A1 (Baf, 100 nM), chloroquine (CQ, 100 µM) or CCCP (100 µM). Inserts show zoomed regions highlighting colocalization of the GFP-GABARAPL2 and LAMP1 signal. Scale bar: 15 µm, for all images.
Supplementary Figure 6

Figure S6. Validation of noncanonical autophagy mechanism in drug-induced LC3 lipidation. (A) Representative western blots of ATG16L1, LC3 and GAPDH in wild-type and ATG16L1−/− MCF10A GFP-LC3 cells treated with PP242 (1 µM) or Baf (100 nM) for 1 h. (B) Representative western blots of LC3 and GAPDH in wild-type and ATG16L1−/− MCF10A GFP-LC3 cells treated with the ionophore CCCP (100 µM) or the lysosomotropic drugs CQ (100 µM),...
ProA (5 mM), Lido (2.5 mM) or LH (2.5 mM) for 2 h. (C) Representative confocal images of GFP-LC3<sup>G120A</sup> following treatment with CQ (100 µM), ProA (5 mM) or CCCP (100 µM) for 2 h. Bar: 10 µm for all images. (D) Representative confocal images of GFP-LC3 and endogenous ATG12 staining in ATG13<sup>−/−</sup> MCF10A GFP-LC3 cells treated with CQ (100 µM), ProA (5 mM) or CCCP (100 µM) for 2 h. Bar: 10 µm for all images. (E) Representative confocal images of GFP-LC3 and endogenous ATP6V0D1 staining in ATG13<sup>−/−</sup> MCF10A GFP-LC3 cells treated with CQ (100 µM), ProA (5 mM) or CCCP (100 µM) for 2 h. Bar: 5 µm for all images.
**Supplementary Figure 7**

**Figure S7.** Effect of ionophore and lysosomotropic drugs on endomembrane damage assessed by LGALS3 localization. (A) Confocal live cell imaging mCherry-LGALS3 (mCherry-Gal3) transiently expressed in ATG13<sup>−/−</sup> MCF10A cells treated with glycyl-L-phenylalanine 2-naphthylamide (GPN, 500 μM) for 30 min or with monensin (50 μM), nigericin (10 μM), CCCP (100 μM), chloroquine (100 μM), hydroxychloroquine (100 μM), NH₄Cl (10 mM), amiodarone (50 μM), lidocaine hydrochloride (2.5 mM), procainamide (5 mM) or betahistine (5 mM) for 2 h. Bar: 5 μm for all images. (B) Representative confocal images of GFP-LC3 and mCherry-Gal3 in ATG13<sup>−/−</sup> MCF10A cells treated with procainamide (5 mM) for 2 h showing the absence of mCherry-Gal3 recruitment onto GFP-LC3-positive vacuoles. Bar: 5 μm for all images.