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Supplemental Material to:

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LC3 fluorescent puncta in autophagosomes or in protein aggregates can be distinguished by FRAP analysis in living cells

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Supporting Information

Fig. S1. Measurement of the crosstalk between the GFP-LC3 and mCherry/mRFP fluorescent signals using confocal microscopy.

Fig. S2. FRAP analysis of the GFP-ZFYVE1^{+}/mRFP-LC3^{+} vesicles.

Fig. S3. Puromycin induces the formation of protein aggregate-associated LC3/LC3^{G120A} puncta in HeLa cells.

Fig. S4. The puromycin-induced LC3/LC3^{G120A} puncta were IBs that were positive for mono- or poly-ubiquitin.

Fig. S5. Rapid dynamic exchange of GFP-LC3^{G120A} occurs in protein aggregates induced by LY294002, wortmannin, and MG-132.

Fig. S6. Rapamycin- or CQ- induced GFP-LC3 puncta showed no fluorescence recovery after photobleaching.

Fig. S7. Both membrane-free and membrane-trapped LC3^{G120A} puncta exist in *atg5*$^{-/-}$ MEFs.

Fig. S8. GFP-ATG16L1 localized adjacent to the aggregate-associated LC3 puncta in both wild-type and *atg5*$^{-/-}$ MEFs.

Fig. S9. FRAP analysis of the membrane-free mRFP-SQSTM1 dots in living cells.

Fig. S10. More mCherry-LC3^{G120A} puncta than GFP-LC3^{G120A} puncta were trapped in acidic structures.

Fig. S11. GFP-LC3^{G120A} puncta induced by NH_4Cl were trapped in autophagosomal

and lysosomal structures.

Supplemental Table S1. Oligonucleotides used in this work.

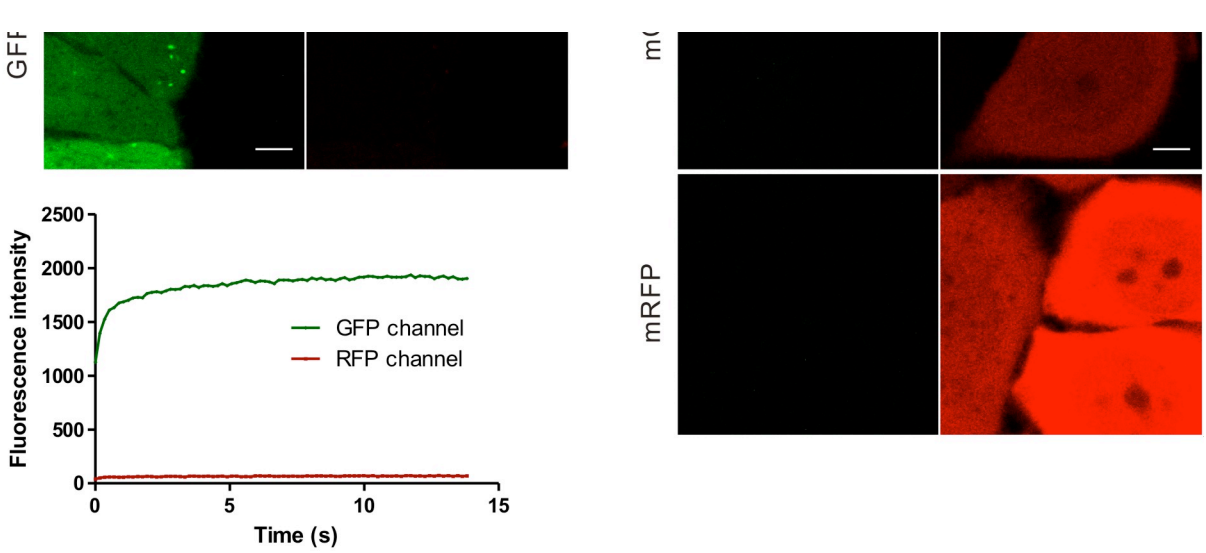

Fig. S1. Measurement of the crosstalk between the GFP-LC3 and mCherry/mRFP fluorescent signals using confocal microscopy. (**A**) The fluorescent signals of HeLa cells expressing GFP-LC3 alone were collected using the same imaging parameters as the two-color FRAP imaging period. The leakage of the GFP signal into the RFP channel was very low (< 3%). A typical FRAP curve is shown in the lower graph. (**B**) Confocal imaging of mCherry- or mRFP-expressing HeLa cells using the same imaging parameters as the two-color FRAP imaging period. There was no leakage of the RFP (mCherry or mRFP) signal into the GFP channel. Scale bars: 5 μm.

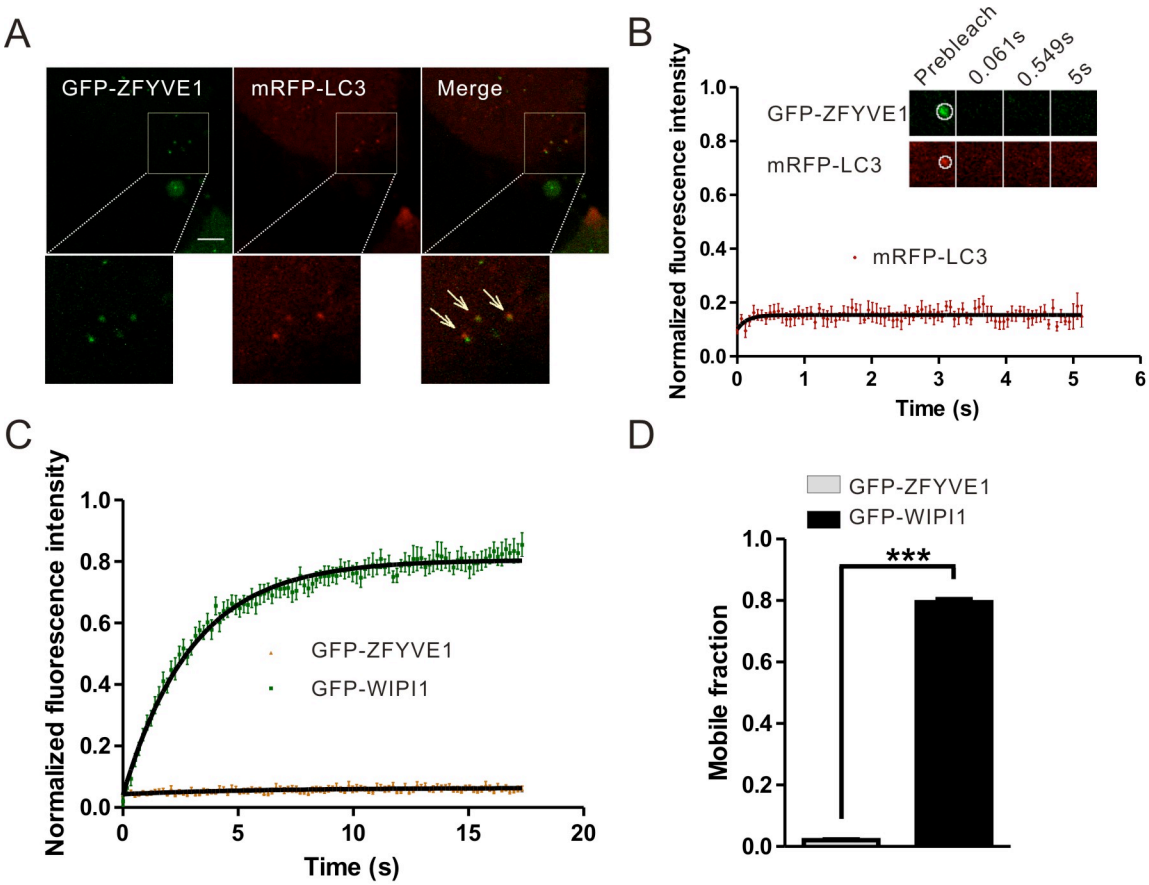

Fig. S2. FRAP analysis of the GFP-ZFYVE1^{+}/mRFP-LC3^{+} vesicles. (**A-B**) HeLa cells coexpressing GFP-ZFYVE1 and mRFP-LC3 were starved in HBSS for 30 min. The GFP-ZFYVE1^{+}/mRFP-LC3^{+} vesicles were subjected to two-color FRAP analysis in GFP-ZFYVE1 low-expressing cells (arrows). For FRAP analysis, the puncta were outlined (white circle) and the intensities were extracted. The recovery kinetics of mRFP-LC3 in the autophagosomes are shown in the graph (**B**). Error bars: s.e.m. with n=11. (**C-D**) FRAP analysis of GFP-WIPI1 and GFP-ZFYVE1 puncta. The recovery kinetics were shown in (**C**) as indicated. Error bars: s.e.m. with n=20. The mobile fraction for the two kinds of puncta were shown in (**D**). Data showed the mean ± SD. Scale bar: 5 μm. Photobleaching box size: 2.691 μm × 2.691 μm.

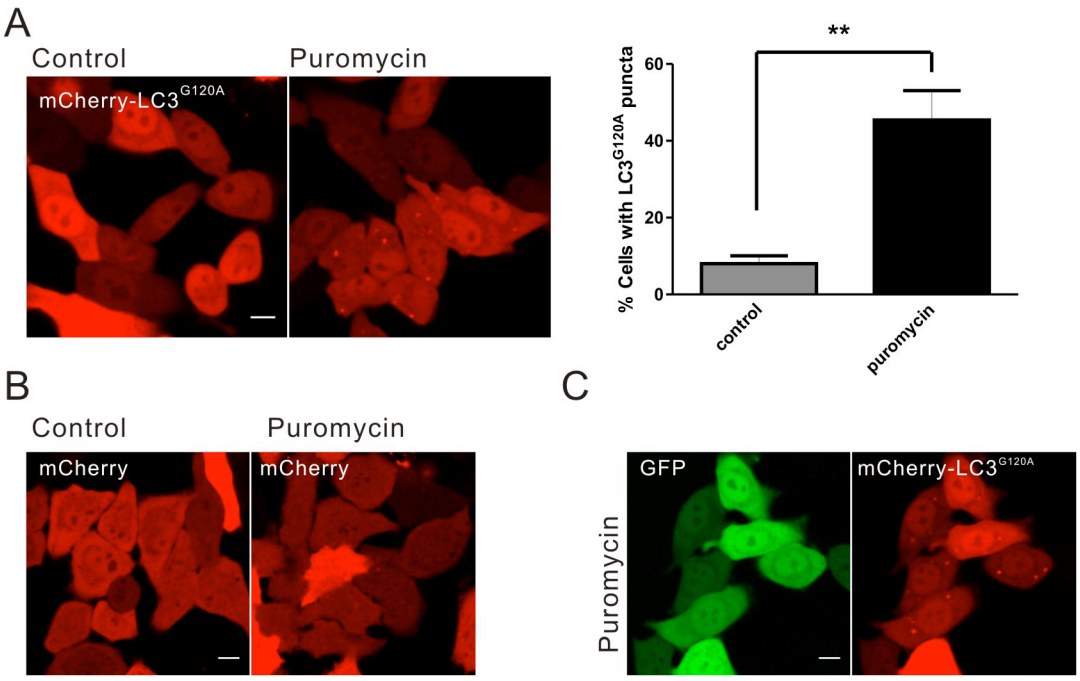

Fig. S3. Puromycin induces the formation of protein aggregate-associated LC3/LC3^{G120A} puncta in HeLa cells. (**A**) HeLa cells transiently expressing mCherry-LC3^{G120A} were treated with or without puromycin (5 μg/ml) for 2.5 h and then subjected to confocal imaging. The representative images are shown, as indicated. The quantitation of the percentage of cells with LC3^{G120A} puncta is shown in the right graph. Totally 270 cells were counted for each from three independent experiments. ** denotes $p<0.01$. Error bars represent s.e.m.. Scale bar: 10 μm. (**B**) HeLa cells transiently expressing mCherry were treated with or without puromycin for 2.5 h. Next, the live cells were subjected to confocal analysis, and the representative images are shown. (**C**) HeLa cells transiently coexpressing GFP and mCherry-LC3^{G120A} were treated with puromycin for 2.5 h and then subjected to confocal imaging. The representative images are shown. Scale bar: 10 μm.

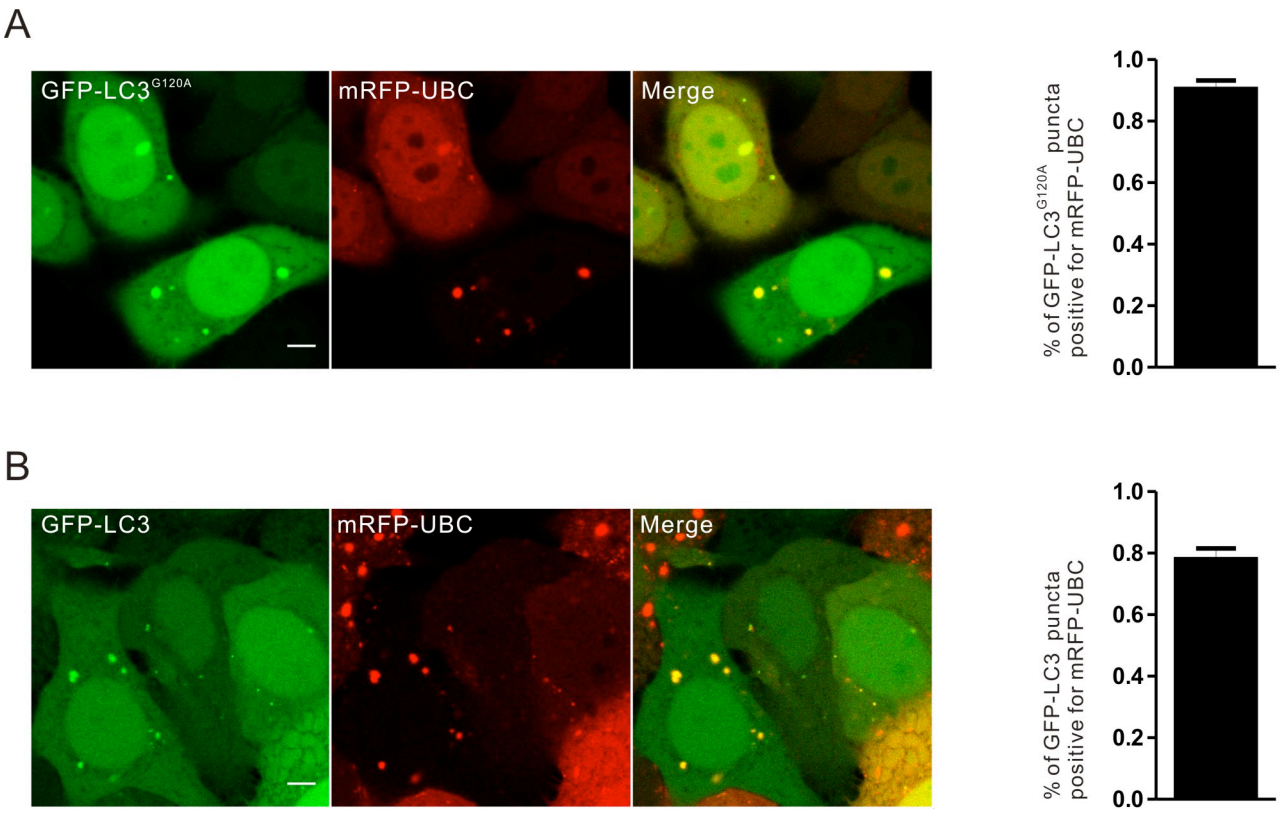

Fig. S4. The puromycin-induced LC3/LC3^{G120A} puncta were IBs that were positive for mono- or poly-ubiquitin. (**A-B**) HeLa cells transiently coexpressing GFP-LC3^{G120A}/LC3 and mRFP-UBC were treated with puromycin for 2.5 h and then imaged. Puncta with a diameter > 0.75 μm (8 pixels in images) were analyzed. Green and red puncta with the same geometric center, similar shape and comparative fluorescent intensity were used as criteria for the colocalization analysis. The percentages of GFP-LC3/LC3^{G120A} puncta positive for mRFP-UBC are quantified in the right graph. Error bars: s.e.m. with n=90 cells for GFP-LC3^{G120A}/mRFP-UBC and n=104 cells for GFP-LC3/mRFP-UBC. Scale bars: 5 μm.

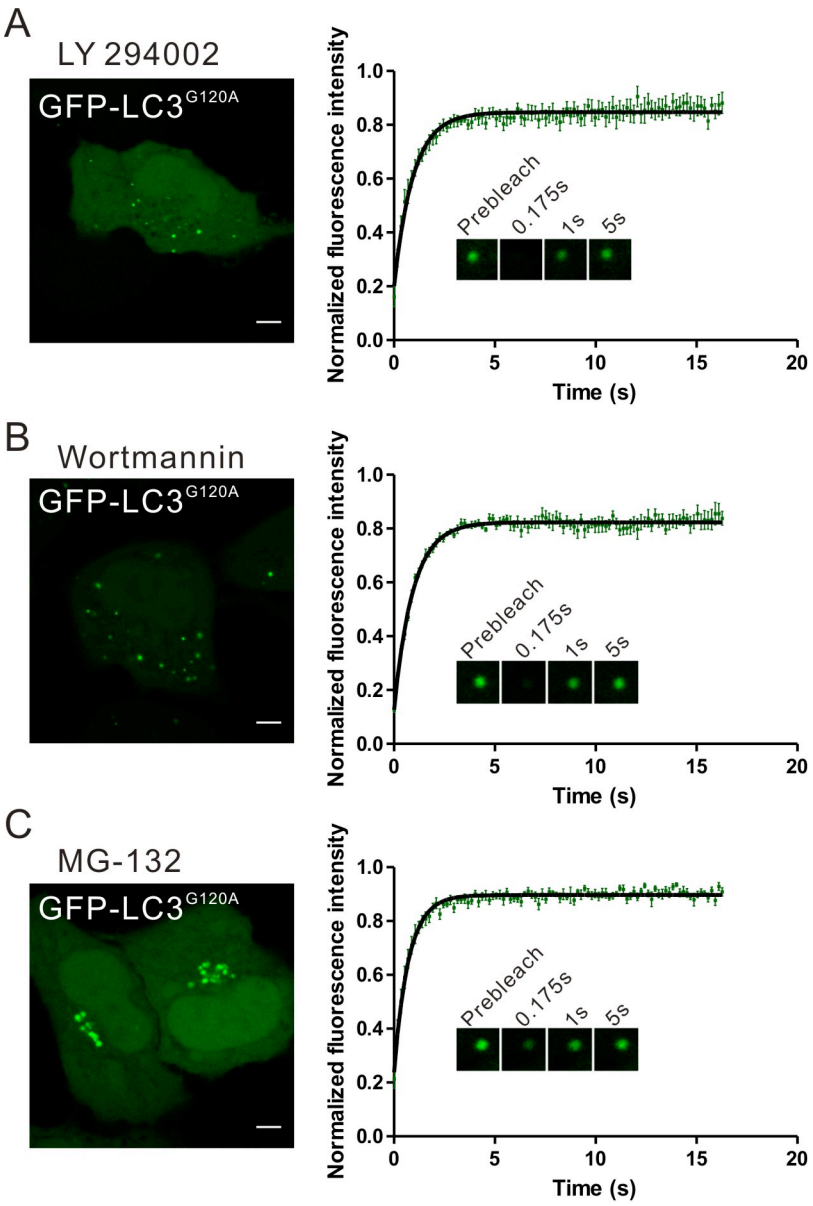

Fig. S5. Rapid dynamic exchange of GFP-LC3^{G120A} occurs in protein aggregates induced by LY294002, wortmannin, and MG-132. (**A-C**) HeLa cells that were transiently transfected with GFP-LC3^{G120A} for 24 h were treated with 50 μM LY294002 for 6 h (**A**), 200 nM wortmannin for 6 h (**B**) or 10 μM MG-132 for 9 h (**C**), and then imaged using confocal microscopy. Representative images are shown. The induced LC3^{G120A} puncta were subjected to FRAP analysis, and the quantitative and normalized fluorescence recovery kinetics of GFP-LC3^{G120A} after photobleaching are shown in the right graph. Error bars: s.e.m. with n=8 (**A**), 8 (**B**), 9 (**C**). Inset: representative FRAP images. Scale bars: 5 μm. Photobleaching box size: 2.691μm × 2.691 μm.

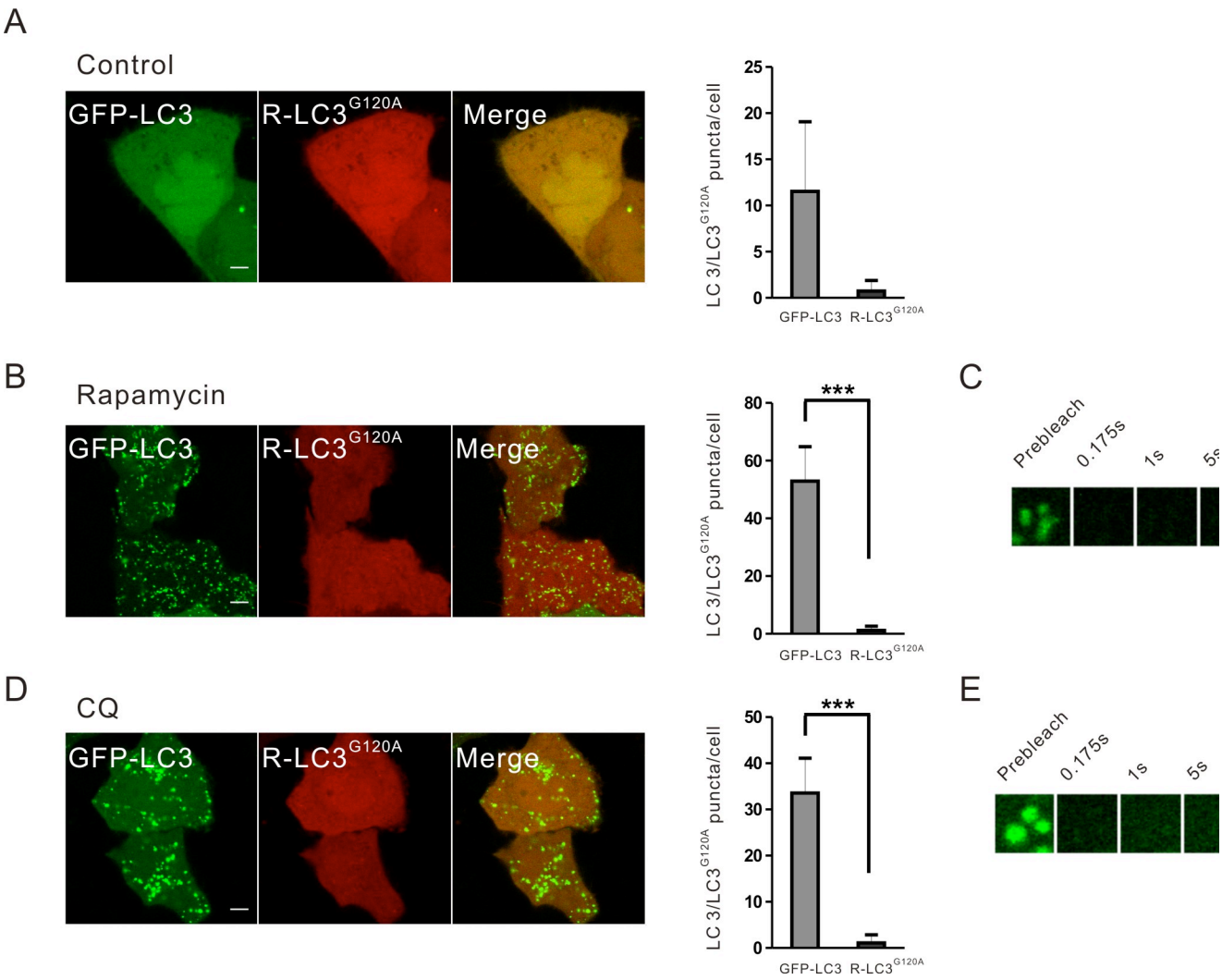

Fig. S6. Rapamycin- or CQ- induced GFP-LC3 puncta showed no fluorescence recovery after photobleaching. (**A-E**) Rapamycin or CQ induced true autophagosomes. HeLa cells that were transiently transfected with GFP-LC3 and mCherry-LC3^{G120A} for 20 h were left untreated (**A**), treated with 1 μM rapamycin for 6 h (**B**) or 50 μM CQ for 6 h (**D**). Representative images are shown. The LC3/LC3^{G120A} puncta per cell are shown in the right graph. (**C**) and (**E**) FRAP analysis of the induced puncta demonstrated that no fluorescence recovery of GFP-LC3. LC3 or LC3^{G120A} puncta (diameter > 0.5 μm) were measured using ImageJ software from a pool of at least 30 cells. Error bars: s.d. R-LC3^{G120A}, mCherry-LC3^{G120A}. Scale bars: 5 μm. Photobleaching box size: 2.691 μm × 2.691 μm.

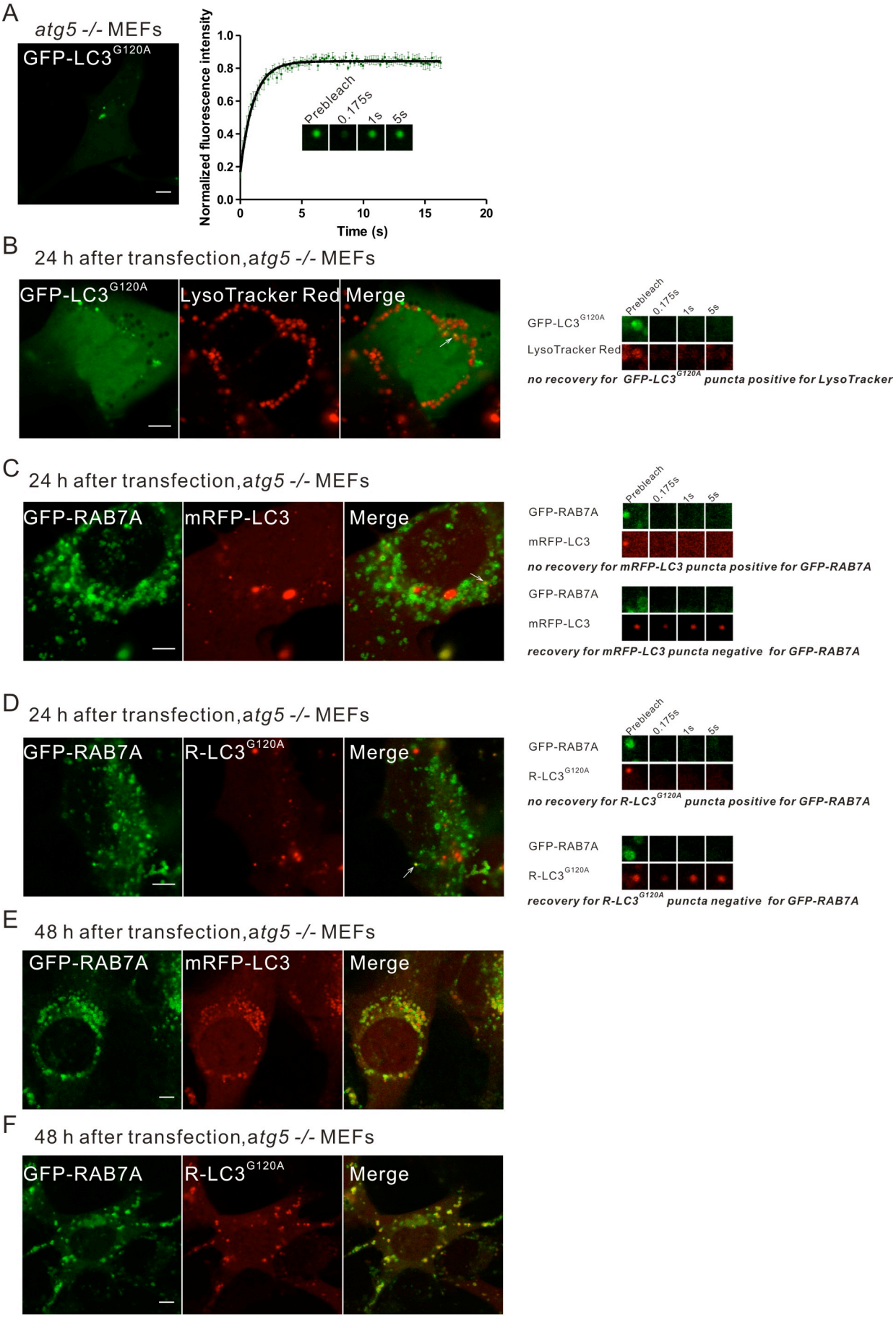
A
atg5 -/- MEFs
GFP-LC3G120A
Normalized fluorescence intensity
Time (s)
Prebleach
0.175s
1s
5s
B
24 h after transfection,atg5 -/- MEFs
GFP-LC3G120A
LysoTracker Red
Merge
GFP-LC3G120A
LysoTracker Red
no recovery for GFP-LC3G120A puncta positive for LysoTracker
C
24 h after transfection,atg5 -/- MEFs
GFP-RAB7A
mRFP-LC3
Merge
GFP-RAB7A
mRFP-LC3
no recovery for mRFP-LC3 puncta positive for GFP-RAB7A
recovery for mRFP-LC3 puncta negative for GFP-RAB7A
D
24 h after transfection,atg5 -/- MEFs
GFP-RAB7A
R-LC3G120A
Merge
no recovery for R-LC3G120A puncta positive for GFP-RAB7A
recovery for R-LC3G120A puncta negative for GFP-RAB7A
E
48 h after transfection,atg5 -/- MEFs
GFP-RAB7A
mRFP-LC3
Merge
F
48 h after transfection,atg5 -/- MEFs
GFP-RAB7A
R-LC3G120A
Merge

Fig. S7. Both membrane-free and membrane-trapped LC3^{G120A} puncta exist in *atg5*$^{-/-}$ MEFs. (**A**) *atg5*$^{-/-}$ MEFs were transiently transfected with GFP-LC3^{G120A} for 20 h. The GFP-LC3^{G120A} puncta were subjected to FRAP analysis, and the quantitative and normalized fluorescence recovery kinetics are shown in the right graph. Error bars: s.e.m. with n=8. Inset: representative FRAP images. (**B**) *atg5*$^{-/-}$ MEFs that were transfected with GFP-LC3^{G120A} for 24 h were stained with 100 nM LysoTracker Red for 30 min before imaging, and representative images are shown. The arrow indicates a GFP-LC3^{G120A} punctum colocalized with LysoTracker Green. An example of FRAP analysis of the colocalized puncta are shown in the right images. (**C-D**) *atg5*$^{-/-}$ MEFs cotransfected either with GFP-RAB7A and mRFP-LC3 (**C**) or with GFP-RAB7A and mCherry-LC3^{G120A} (D) for 24 h were subjected to confocal imaging. The arrows indicate GFP^{+}/mRFP^{+} puncta. Examples of FRAP analysis of the GFP^{+}/mRFP^{+} or GFP^{-}/mRFP^{+} puncta are shown in the right images. (**E-F**) *atg5*$^{-/-}$ MEFs cotransfected with either GFP-RAB7A and mRFP-LC3 (**E**) or with GFP-RAB7A and mCherry-LC3^{G120A} (**F**) for 48 h. Representative confocal images are shown. R-LC3^{G120A}, mCherry-LC3^{G120A}. Scale bars: 5 μm. Photobleaching box size: 2.691μm × 2.691 μm.

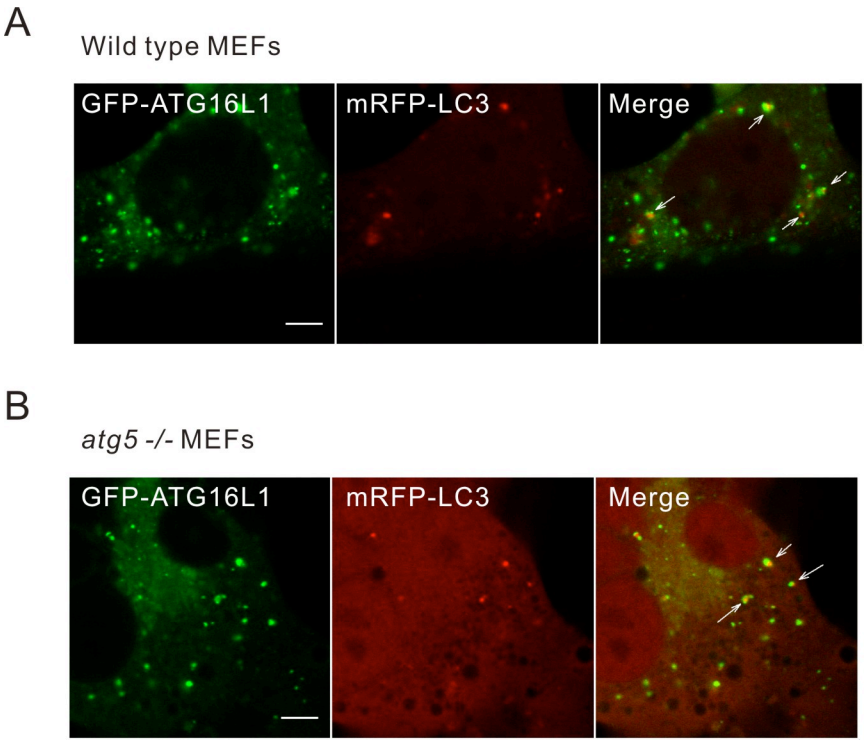

Fig. S8. GFP-ATG16L1 localized adjacent to the aggregate-associated LC3 puncta in both wild-type and *atg5*$^{-/-}$ MEFs. (**A-B**) Wild-type MEFs (**A**) and *atg5*$^{-/-}$ MEFs (**B**) transiently coexpressing GFP-ATG16L1 and mRFP-LC3 were starved in HBSS for 1 h and representative images are shown. The mRFP-LC3 puncta that localized adjacent to the GFP-ATG16L1 puncta are indicated by arrows. Scale bars: 5 μm.

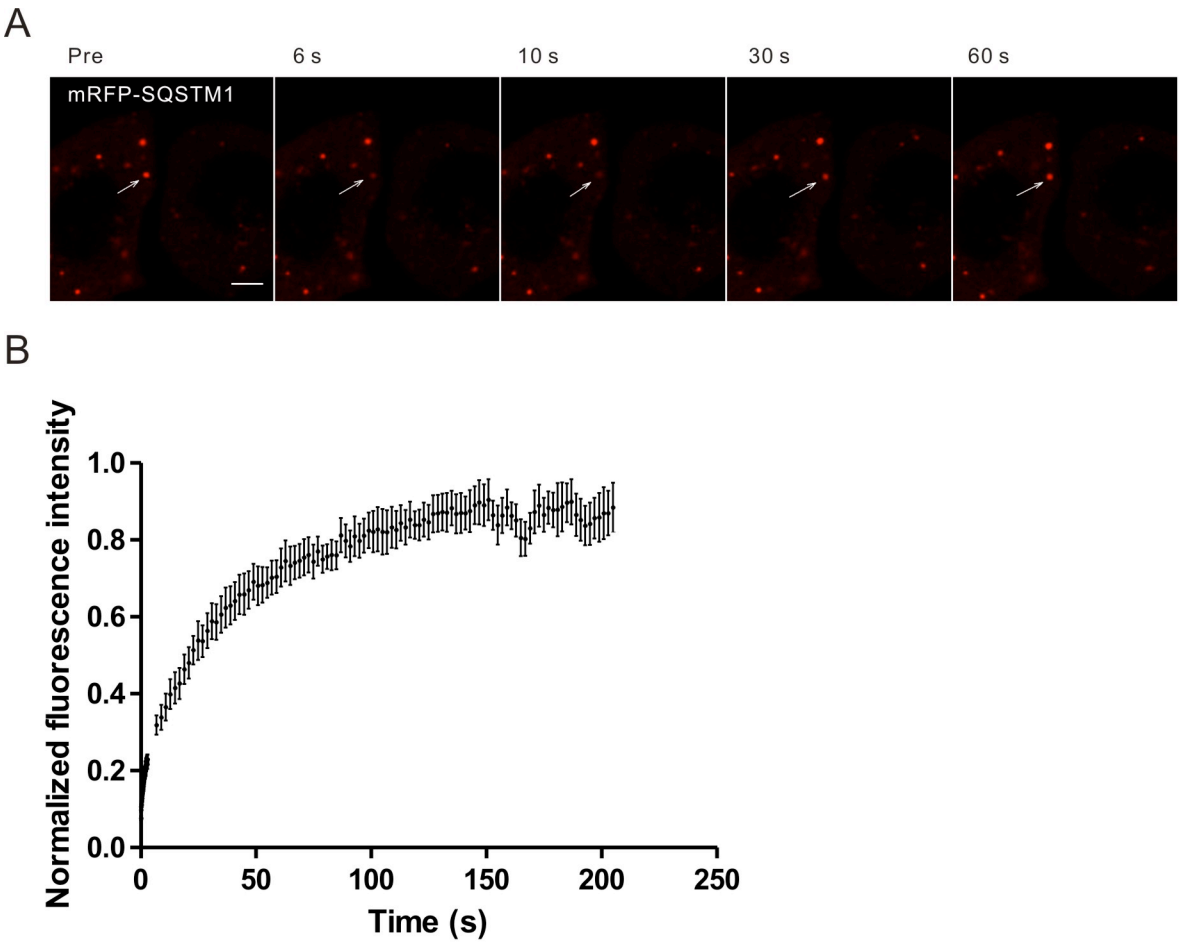

Fig. S9. FRAP analysis of the membrane-free mRFP-SQSTM1 dots in living cells. (**A-B**) HeLa cells were transfected with mRFP-SQSTM1 for 24 h and the mRFP-SQSTM1 dots were subjected to FRAP analysis (arrow). The normalized fluorescence recovery kinetics were shown. Error bars: s.e.m. with n=6. Scale bar: 5 μm. Photobleaching size: 2.691 μm × 2.691 μm.

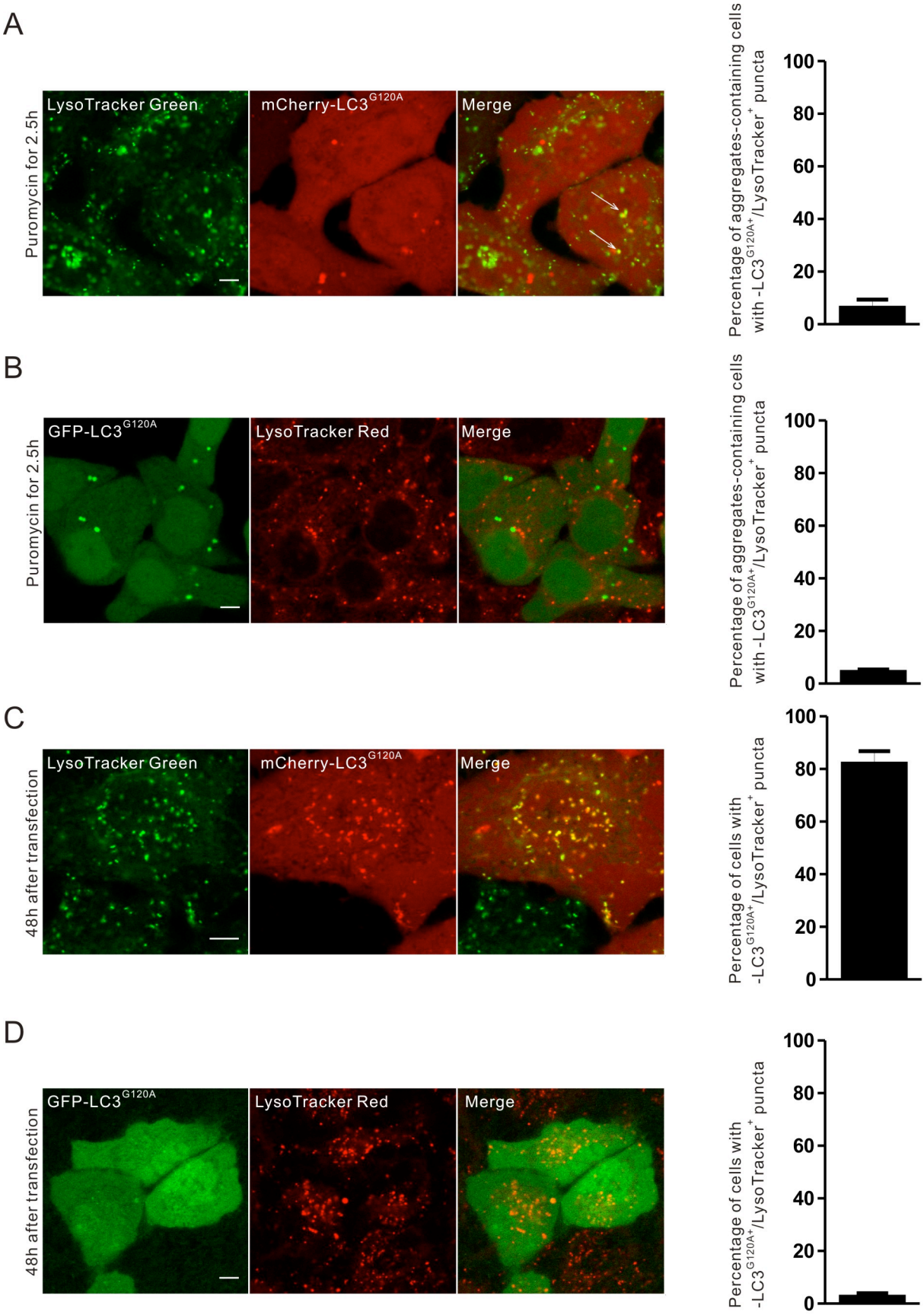

Fig. S10. More mCherry-LC3^{G120A} puncta than GFP-LC3^{G120A} puncta were trapped in

acidic structures. (**A-B**) HeLa cells transiently expressing either mCherry-LC3^{G120A} (**A**) or GFP-LC3^{G120A} (**B**) were treated with puromycin for 2.5 h. Next, the cells were stained with 100 nM of either LysoTracker Green (**A**) or LysoTracker Red (**B**) for 30 min before imaging, and representative images are shown. The arrows indicate mCherry-LC3^{G120A} colocalized with LysoTracker Green. The percentages of cells in which mCherry-LC3^{G120A} puncta (**A**) or GFP-LC3^{G120A} puncta (**B**) were positive for LysoTracker are shown in the right graph. Error bar represents s.e.m.. Totally 151 cells (**A**) or 198 cells (**B**) were analyzed. (**C-D**) HeLa cells transiently expressing either mCherry-LC3^{G120A} (**C**) or GFP-LC3^{G120A} (**D**) for 48 h were incubated with 100 nM LysoTracker Green (**C**) or LysoTracker Red (**D**) for 30 min. The representative confocal images are shown. The percentages of cells in which mCherry-LC3^{G120A} puncta (**C**) or GFP-LC3^{G120A} puncta (**D**) were positive for LysoTracker are shown in the right graph. Error bar represents s.e.m.. Totally 276 cells (**C**) or 241 cells (**D**) were analyzed. Scale bars: 5 μm.

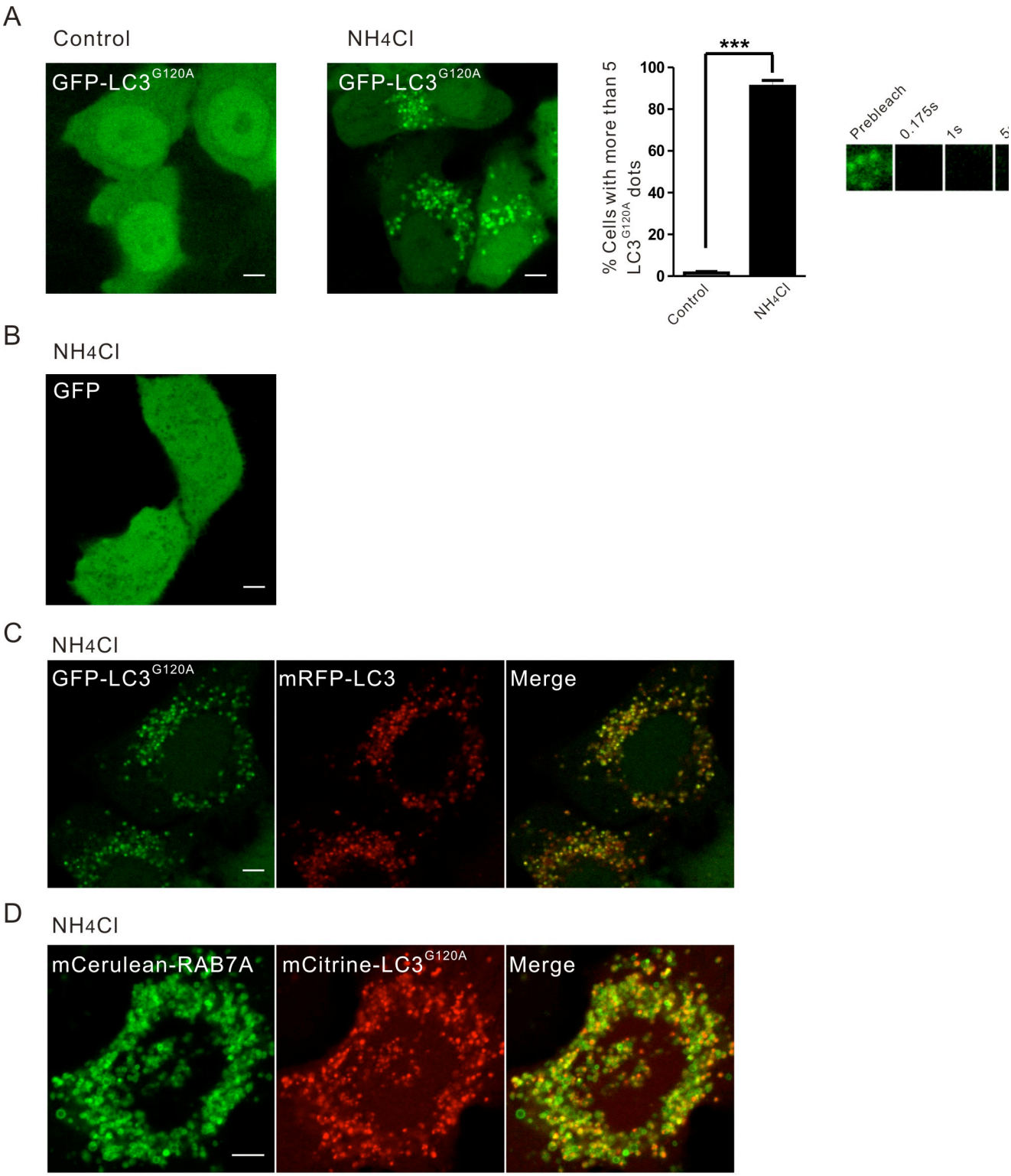

Fig. S11. GFP-LC3^{G120A} puncta induced by NH_4Cl were trapped in autophagosomal and lysosomal structures. (**A**) HeLa cells that were transiently transfected with GFP-LC3^{G120A} for 20 h were treated with 50 mM NH_4Cl for 24 h or left untreated. Representative images are shown. The percentages of cells with more than 5 GFP-LC3^{G120A} puncta are shown in the right graph. Error bars represent s.e.m.. A

total of 341 cells (control) or 344 cells (NH_4Cl treatment) were analyzed. An example of FRAP analysis of these puncta induced by NH_4Cl is shown in the right images. (**B**) HeLa cells that were transiently transfected with GFP for 20 h were treated with 50 mM NH_4Cl for 24 h. Representative images are shown. (**C-D**) HeLa cells were transiently cotransfected with either GFP-LC3^{G120A} and mRFP-LC3 (**C**) or mCerulean-RAB7A and mCitrine-LC3^{G120A} (**D**) for 20 h and then treated with 50 mM NH_4Cl for 24 h. Representative images are shown. Scale bars: 5 μm. Photobleaching box size: 2.691 μm × 2.691 μm.

Supplemental Table S1. Oligonucleotides used in this work.

Primers	Oligo Sequence
LC3B-EcoRI-up	AAAAGAATTCTATGCCGTCGGAGAAGACCTTCAAGCAGCGCCGCACC
LC3B-BamHI-down	AAAAGGATCCTTACACTGACAATTTCATGCCGAAGGTTTCCTGGGAGGCG
WIPI1-XhoI-F	AAAACTCGAGCTATGGAGGCCGAGGCCGCGG
WIPI1-EcoRI-R	AAAAGAATTCTCATGACTGCTTCGTTTTGCCCTTCTG
ATG16L-XhoI-up	AAAACTCGAGCTATGTCGTCGGGCCTCCGCGC
ATG16L-EcoRI-down	AAAAGAATTCTCAGTACTGTGCCCACAGCACAGCT
EGFP-RAB7A-EcoRI-F	AAAAGAATTCTATGACCTCTAGGAAGAAAGTGTTGCTG
EGFP-RAB7A-BamHI-R	AAAAGGATCCTCAGCAACTGCAGCTTTCTGCCGAGGC
$LC3B^{G120A}$-BamHI-F	ACGCGGATCCGGAGGCTCAATGCCGTCGGAGAAGACCTTCAAGC
$LC3B^{G120A}$-EcoRI-R	ACCGGAATTCTTACACTGACAATTTCATGGCGAAGGTTTCCTGGG
NheI-dendra2-F	ATCCGCTAGCGCCACCATGAACACCCCGGGAATTAACCTGATC
XhoI-dendra2-R	ACCGCTCGAGATCTGAGTCCGGACCACACCTGGCTGGGCAGGGGG
UBC-BamHI-F	AAAAGGATCCATGCAGATCTTCGTGAAGACTCTGACTGG
UBC-EcoRI-R	AAAAGAATTCTCACCCACCTCTGAGACGGAGCACCAG
SQSTM1-EcoRI-F	AAAAGAATTCTATGGCGTCGCTCACCGTGAAGGCC

SQSTM1-XhoI-R	AAAACTCGAGTCACAACGGCGGGGGATGCTTTGAATAC
GFP^{G120A}-EcoRI-F	AAAAGAATTCTATGCCGTCGGAGAAGACCTTCAAGC
GFP^{G120A}-BamHI-R	AAAAGGATCCTTACACTGACAATTTCATGGCGAAGG
SQSTM1-EcoRI-F	AAAAGAATTCTATGGCGTCGCTCACCGTGAAGGCC
SQSTM1-SalI-R	AAAAGTCGACTGCAACGGCGGGGGATGCTTTGAATAC
UBA-XhoI-F	AAAACTCGAGCTGGTGGATCGCTCCCGCCAGAGGCTGACCCG
UBA-EcoRI-R	AAAAGAATTCTCACAACGGCGGGGGATGCTTTGAATAC
SQSTM1-NheI-F	AAAAGCTAGCATGGCGTCGCTCACCGTGAAGGCC
SQSTM1-385-AgeI-R	AAAAACCGGTAGCGATCCACCATGTGGGTACAAGGCAGCTTCCTTCAGCC