Supplementary Information

Supplementary Methods

Autophagy induction and autophagosome maturation assay

Autophagy was induced by starvation. For amino acid and serum starvation, the cells were cultured in Earle's balanced salt solution (EBSS; Sigma) for 2–4 h.

Autophagosome maturation was analyzed by two different methods. One is based on the co-localization of GFP-LC3 with LAMP1 and in another method we measured the RFP-positive ratio in cells transiently expressing tandem fluorescent LC3 (tfLC3) as described before (Kimura *et al*., 2007). A549 cells stably expressing GFP-LC3 were incubated in nutrient-rich medium and examined by immunofluorescence microscopy using an anti-LAMP1 antibody. The areas of GFP-LC3 and LAMP1 co-localization were counted and are shown as the percentage of LAMP1-positive GFP-LC3.

Assay for Rab7 GAP activity

Rab7 GAP activity was assayed as previously described (Fukui *et al*., 1997) with slight modifications. Briefly, lipid-modified Rab7 was incubated for 20 min at 30°C in a reaction mixture (10 μl) containing 25 mM Tris/HCl at pH 8.0, 10 mM EDTA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and 2 mM [γ-^{32}P]GTP. The reaction was terminated by adding 2.5 μl of 80 mM $MgCl_2$. To this mixture, purified recombinant hRubicon (RH domain), hRubicon (FL), PLEKHM1 (RH domain), or PLEKHM1 (FL) in a buffer containing 40 mM Tris/HCl at pH 8.0, 12 mM $MgCl_2$, 1 mM DTT, and 2 mM cold GTP was added in a total volume of 50 μl and the reaction mixture was further incubated for 2 min at 30°C. The samples were applied to a nitrocellulose filter, and the radioactivity retained on the filter was determined by Cerenkov counting.

Preparation and purification of recombinant Rab7 using a baculovirus/Sf9 cell system

The entire coding region of mouse Rab7 cDNA was subcloned into pFASTBAC HTb (Invitrogen). The plasmid was subjected to recombination with the baculovirus genome in DH10BAC (Invitrogen), and the resulting recombinant viral genome was transfected

into Sf9 cells in order to generate recombinant baculovirus. An Sf9 monolayer was infected with the recombinant virus and cultured for 48 h. To prepare the lipid-modified form of Rab7, the cells were then treated with 5 mM mevalonic acid and incubated for 24 h. Lipid-modified His6-tagged Rab7 protein was purified from the membrane fraction of cells by TALON column chromatography (Clontech).

Supplementary References

Geourjon, C. and Deleage, G. SOPMA: Significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Comput. Appl. Biosci.* **11**, 681-684 (1995).

Gille, C. and Frommel, C. STRAP: editor for STRuctural Alignments of Proteins. *Bioinformatics* **17**, 377-378 (2001).

Fukui, K., et al. Isolation and Characterization of a GTPase Activating Protein Specific for the Rab3 Subfamily of Small G Proteins. *J. Biol. Chem.* **272**, 4655–4658 (1997).

Kimura, S., Noda, T. and Yoshimori, T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* **3**, 452-460 (2007).

Thompson, J. D., Higgins, D. G. and Gibson, T. J. Clustal-W - Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Res.* **22**, 4673-4680 (1994).

Figure Legends

Figure S1. PLEKHM1 and Rubicon specifically interact with Rab7.
(A) Association of PLEKHM1 and Rubicon with Rabs. The interactions between full-length PLEKHM1 or Rubicon and each Rab mutant were analyzed by a yeast two-hybrid analysis. In all cases, two independent colonies were tested. (B) Rab7 interactions in HEK293A cells. Lysates from HEK293A cells expressing GFP-Rab7 mutants and FLAG-PLEKHM1/Rubicon (wild type or CGHL) or the empty vector were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with an anti-GFP antibody.

Figure S2. Rubicon family proteins and the RH domain.
(A) Rubicon homologous proteins and their structure. The domain structures were revealed by Motif Scan (SBI). Rubicon homologous (RH) domains in each protein are indicated by magenta. (B) Multiple sequence alignment and secondary structure prediction of Rubicon and its closest homologues in the RH domain. The alignment was automatically generated by the program ClustalW (Thompson *et al*., 1994) and visualized using STRAP (Gille and Frommel, 2001). The consensus secondary α-helix prediction structures obtained using the SOPMA (Geourjon and Deleag *et al*., 1995) are shown as green H. NCBI accession codes: *Homo sapiens*, NP_055502.1 (Rubicon); *Homo sapiens*, NP_055613.1 (PLEKHM1, KIAA0356); *Homo sapiens*, NP_997397.1 (DEF8 isoform1); *Homo sapiens*, NP_079389.2 (C13orf18) and *Homo sapiens*, NP_001073944.1 (PLEKHM3). The cysteines in the RH domain are represented by yellow. Four asterisks indicate mutation sites for CGHL mutants.

Figure S3. The effects of PLEKHM1 over-expression.
(A) The effects of PLEKHM1 over-expression on endosomal pH. A549 cells over-expressing the control or PLEKHM1 were treated with Lysotracker-Red, an agent that causes weakly basic amines to selectively accumulate in cellular compartments with a low internal pH, for 1 h. The cells were analyzed by immunofluorescence microscopy using an anti-EEA1 antibody and anti-LAMP1 antibody. Bars indicate 20 μm. (B) The effects of PLEKHM1 over-expression on the fusion between autophagosomes and lysosomes. A549 cells stably expressing GFP-LC3 were infected

with adenovirus harboring the control, PLEKHM1 or Rubicon and stained with an anti-LAMP1 antibody. The projected z-stack images obtained by confocal microscopy are shown. Arrows indicate the luminal signal of GFP-LC3 in LAMP1-positive endosomes. Bars represent 20 μm. (C) The effects of over-expression on the size of LAMP1-positive endosome. Cross-sections of LAMP1-positive endosomes were measured by FV1000 software (Olympus) after staining with an anti-LAMP1 antibody. Data are indicated as mean +/- s.d. (n>30).

Figure S4. The effects of knockdown on the efficiency of EGF transport to LAMP1-positive endosomes.
Knocked down cells were treated with Alexa 488 EGF and stained with an anti-LAMP1 antibody at each time point. Arrows indicate EGF-positive dots merged with LAMP1 signals. The percentages of co-localization are shown in Figure 3E. Bars indicate 20 μm.

Figure S5. The effects of PLEKHM1 knockdown on autophagic pathway.
(A and B) PLEKHM1 knockdown did not disturb autophagosome-lysosome fusion. (A) A549 cells depleted PLEKHM1 were transfected with tfLC3 and subjected to immunostaining with anti-LAMP1 antibody. Bars indicate 20 μm. (B) The percentages of co-localization are shown as red-positive dots/total red dots (mean ± s.d., n>20). (C-D) the effects of PLEKHM1 knockdown on autophagy flux. (C) A549 cells depleted PLEKHM1 were incubatied in growth medium, EBSS or EBSS containing protease inhibitors (50 ng/μl E64d and 50 ng/μl pepstatin A) for 2 hours and subjected to immunoblotting with anti-LC3 antibody. (D) LC3-II level were quantified and showed as percentages based on (C). N, S and PI show Nutrient condition, Starvation condition and Protease Inhibitors treatment condition, respectively.

Figure S6. The effects of expressing Rab mutants on Rubicon localization.
HeLa cells co-expressing mStrawberry-Rubicon and each Rab mutant were examined by confocal fluorescent microscopy. Arrows indicate mStrawberry-Rubicon, which did not co-localize with the Rab proteins in cells expressing the SN mutants. Bars indicate 20 μm.

Figure S7. The effects of CGHL mutants over-expression.
(A) Over-expression of PLEKHM1 (CGHL) mutant did not disturb EGFR degradation. A549 cells over-expressed PLEKHM1 were subjected to EGFR degradation assay. Immunodetectable levels of EGFR (relative to α-tubulin) were shown as mean ± s.e.m.. (B and C) Over-expression of CGHL mutant did not affect the formation of GFP-LC3 dots. (B) Rubicon (CGHL) mutant were over-expressed in A549 cells stably expressing GFP-LC3. The cells were treated with EBSS for 2 hours for starvation and subjected to confocal microscopy. Bars indicate 20 μm. (C) GFP-LC3 positive dots were counted. Data are indicated as mean +/- s.d. (n>30). N and S show Nutrient condition and Starvation condition, respectively. (D and E) The effects of over-expression of CGHL mutant on the autophagosome-lysosome fusion. (D) Over-expressed cells were incubated with EBSS containing protease inhibitors (50 ng/μl E64d and 50 ng/μl pepstatin A) for 2 hours. Arrows indicate colocalization signals. (E) The percentages of co-localization are shown as GFP-LC3 /LAMP1 (mean ± s.d., n>20).

Figure S8. Characterization of the Rab7 GAP activity of Rubicon and PLEKHM1.
(A-E) The hydrolysis of [γ-^{32}P]GTP bound to lipid-modified Rab7 was assayed in the presence of various concentrations of recombinant proteins for (A) Rubicon (FL), (B) Rubicon (RH domain), (C) PLEKHM1 (FL), (D) PLEKHM1 (RH domain). (E) Bacterial lysates containing GST (vector) were used as a negative control. (F) The histogram shows the percentage of [γ-^{32}P]GTP bound to lipid-modified Rab7 in the presence of 5 pmol of the indicated recombinant proteins. The hydrolysis rates of [γ-^{32}P]GTP bound to Rab7 were normalized to the control that did not contain recombinant proteins or bacterial cell lysates (blank).

Figure S9. PLEKHM1 and Rubicon are in different complexes.
Cell lysates were subjected to immunoprecipitation and immunoblot analysis using the indicated antibodies. IgG bands and unknown bands were detected by immunoblot analysis with a rabbit anti-Beclin 1 antibody and rabbit anti-PLEKHM1 antibody, respectively.

Figure S10. The effects of over-expressing the BA domain on autophagy.

(A) The effects of over-expressing the BA domain (a. a. 393–521) on the number of punctate structures with GFP-LC3. A549 cells stably expressing GFP-LC3 were infected with adenovirus to over-express the control or BA domain and incubated in growth medium or EBSS for 2 h. GFP-LC3-positive punctate structures were counted in more than 30 cells and are shown as the mean ± s.d. Bars indicate 20 μm. (B) The effects of over-expressing the BA domain on autophagosome maturation. A549 cells stably expressing GFP-LC3 were infected with adenovirus to over-express the control or BA domain and incubated in growth medium containing protease inhibitors (50 μg/ml E64d and 50 μg/ml pepstatin A) for 4 h. Areas of GFP-LC3 and LAMP1 co-localization were counted in more than 30 cells and are shown as the mean ± s.d. Bars indicate 20 μm.

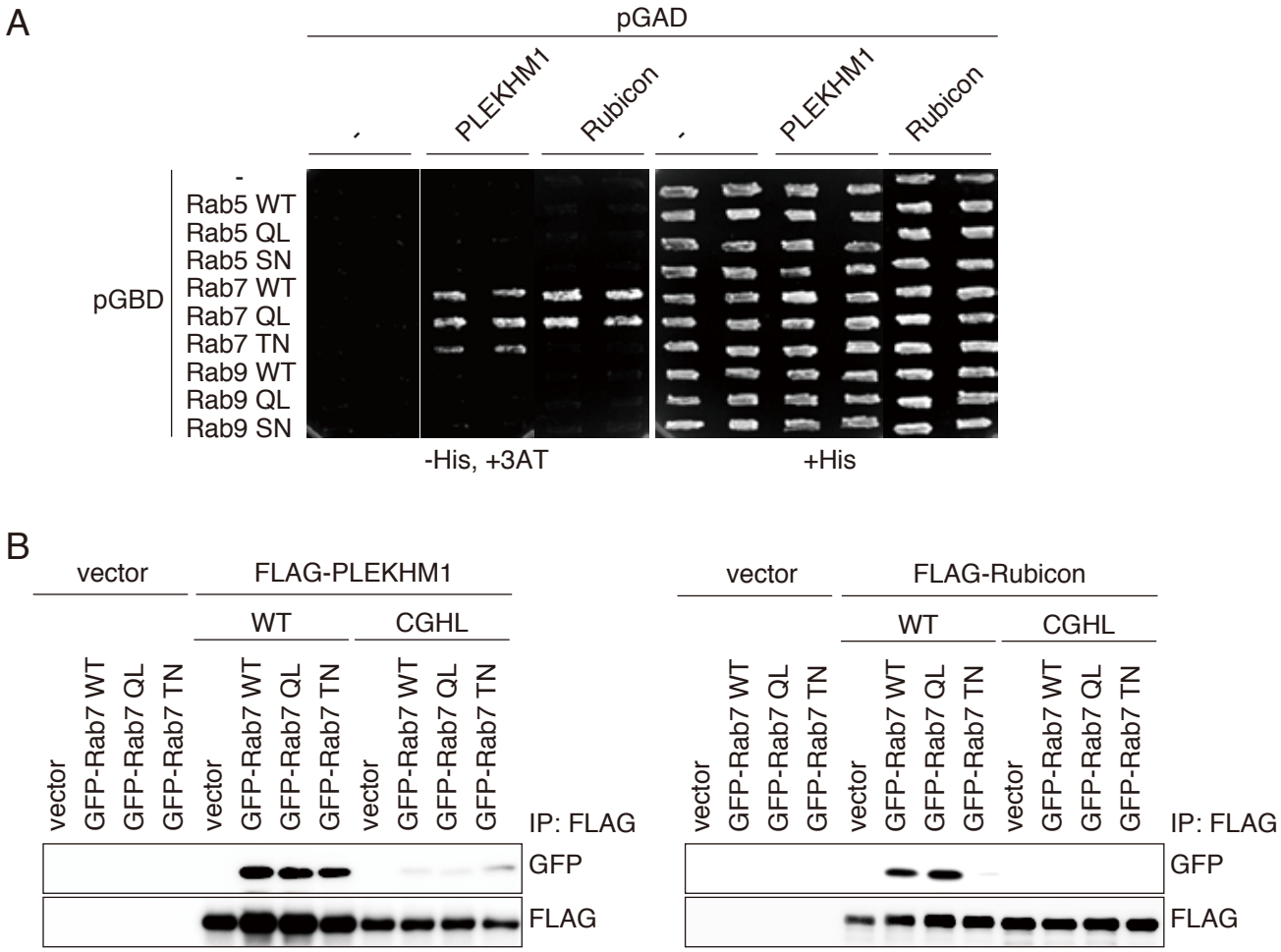
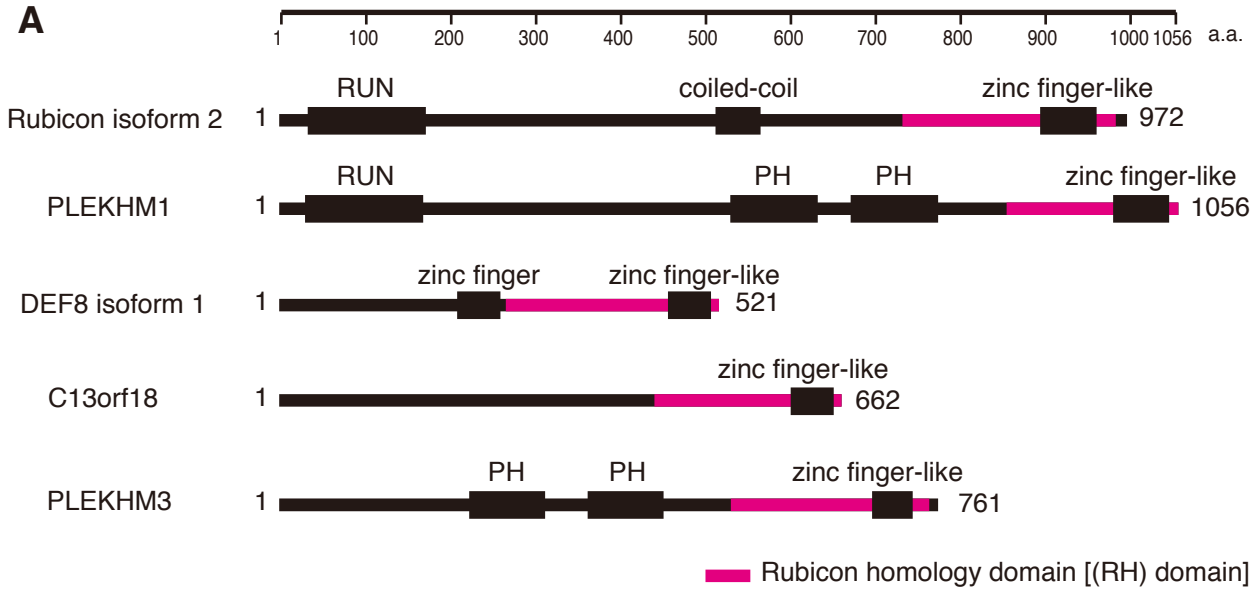

A
pGAD
-
PLEKHM1
Rubicon
-
PLEKHM1
Rubicon
pGBD
-
Rab5 WT
Rab5 QL
Rab5 SN
Rab7 WT
Rab7 QL
Rab7 TN
Rab9 WT
Rab9 QL
Rab9 SN
-His, +3AT
+His
B
vector
FLAG-PLEKHM1
WT
CGHL
vector
GFP-Rab7 WT
GFP-Rab7 QL
GFP-Rab7 TN
IP: FLAG
GFP
FLAG
vector
FLAG-Rubicon
WT
CGHL
IP: FLAG
GFP
FLAG

Figure S1.



B

```
hRubicon isoform2  721 ..............................CAGCGIRTDP
hPLEKHM1           831 ..............................CAGCSRQIGF
hDEF8 isoform1     278 ..............................CAECRAPISL
hC13orf18          434 ..............................CAGCGTPVEP
hPLEKHM3           514 ..............................CAGCQRSIGL

hRubicon isoform2  731 D.YIKRLRYCEYLGKYFCQCCHENAQMAIPSRVLRKWDFS
hPLEKHM1           841 S..FVRPKLCAFSGLYYCDICHQDDASVIPARIIHNWDLT
hDEF8 isoform1     288 RGVPSEARQCDYTGQYYCSHCHWNDLAVIPARVVHNWDFE
hC13orf18          444 K.FVKRLRYCEYLGKYFCDCCHSYAESCIPARILMMWDFK
hPLEKHM3           524 S..NGKAKVCNYSGWYYCSSCHVDDSFLIPARIVHNWDTS

                       HHHHHHHHHHHH          HHHHHHHHHHHHHHHHH
hRubicon isoform2  770 KYYVSNFSKDLLIKIWNDPLFNVQDINSALYRKVKLLNQV
hPLEKHM1           879 KRPICRQALKFLTQIRAQPLINLQMVNASLYEHVERMHLI
hDEF8 isoform1     328 PRKVSRCSMRYLALMVSRPVLRLREINPLLFSYVEELVEI
hC13orf18          483 KYYVSNFSKQLLDSIWHQPIFNLLSIGQSLYAKAKELDRV
hPLEKHM3           562 KYKVSKQAKEFLEYVYEEPLIDIQQENAMLYHHAEPLAAV

                       HHHHHHHHHHHHHHHHHHHHHHHHHHH
hRubicon isoform2  810 RLLRVQLCHMKNMFKTCRLAKELLDSFDTVPG.HLTEDLH
hPLEKHM1           919 GRRREQLKLLGDYLGLCRSGALKELSKRLNHRNYLLESPH
hDEF8 isoform1     368 RKLRQDILLMKPYFITCREAMEARLLLQLQDRQHFVENDE
hC13orf18          523 KEIQEQLFHIKKLLKTCRFANSALKEFEQVPG.HLTDELH
hPLEKHM3           602 LRLRQRLKSLRAYLFSCRAAVAEDLRRRIFPREYLLQQIH

                       HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
hRubicon isoform2  849 LYSLNDLTATRKGELGPRLAELTRAGATHVER.CMLCQAK
hPLEKHM1           959 RFSVADLQQIADGVYEGFLKALIEFASQHVYH.CDLCTQR
hDEF8 isoform1     408 MYSVQDLLDVHAGRLGCSLTEIHTLFAKHIKLDCERCQAK
hC13orf18          562 LFSLEDLVRIKKGLLAPLLKDILKASLAHVAG.CELCQGK
hPLEKHM3           642 LYSLADLQQVIEGKLAPFLGKVIKFATSHVYS.CSLCSQK

                                                 *  *    *  *
hRubicon isoform2  888 GFICEFCQNEDDIIFPFELHKCRTCEECKACYHKACFKS.
hPLEKHM1           998 GFICQICQH.HDIIFPFEFDTTVRCAECKTVFHQSCQAVV
hDEF8 isoform1     448 GFVCELCRE.GDVLFPFDSHTS.VCADCSAVFHRDCYYDN
hC13orf18          601 GFICEFCQN.TTVIFPFQTATCRRCSACRACFHKQCFQS.
hPLEKHM3           681 GFICEICNN.GEILYPFEDISTSRCESCGAVFHSECKEK.

                              HHHHHHHHHHHHHHHHHHHHH
hRubicon isoform2  927 .GSCPRCERLQARREALARQSLESYLSDYEEEPAEALALE AAVLEAT
hPLEKHM1          1037 KKGCPRCARRRKYQEQNIFA
hDEF8 isoform1     486 STTCPKCARLSLRKQSLFQEPGPDVEA
hC13orf18          639 .SECPRCARITARRKLLESVASAAT
hPLEKHM3           719 SVPCPRCVRRELQKKQKSFWQRLNMDESLEEACTMFELSY QNT
```

Figure S2.

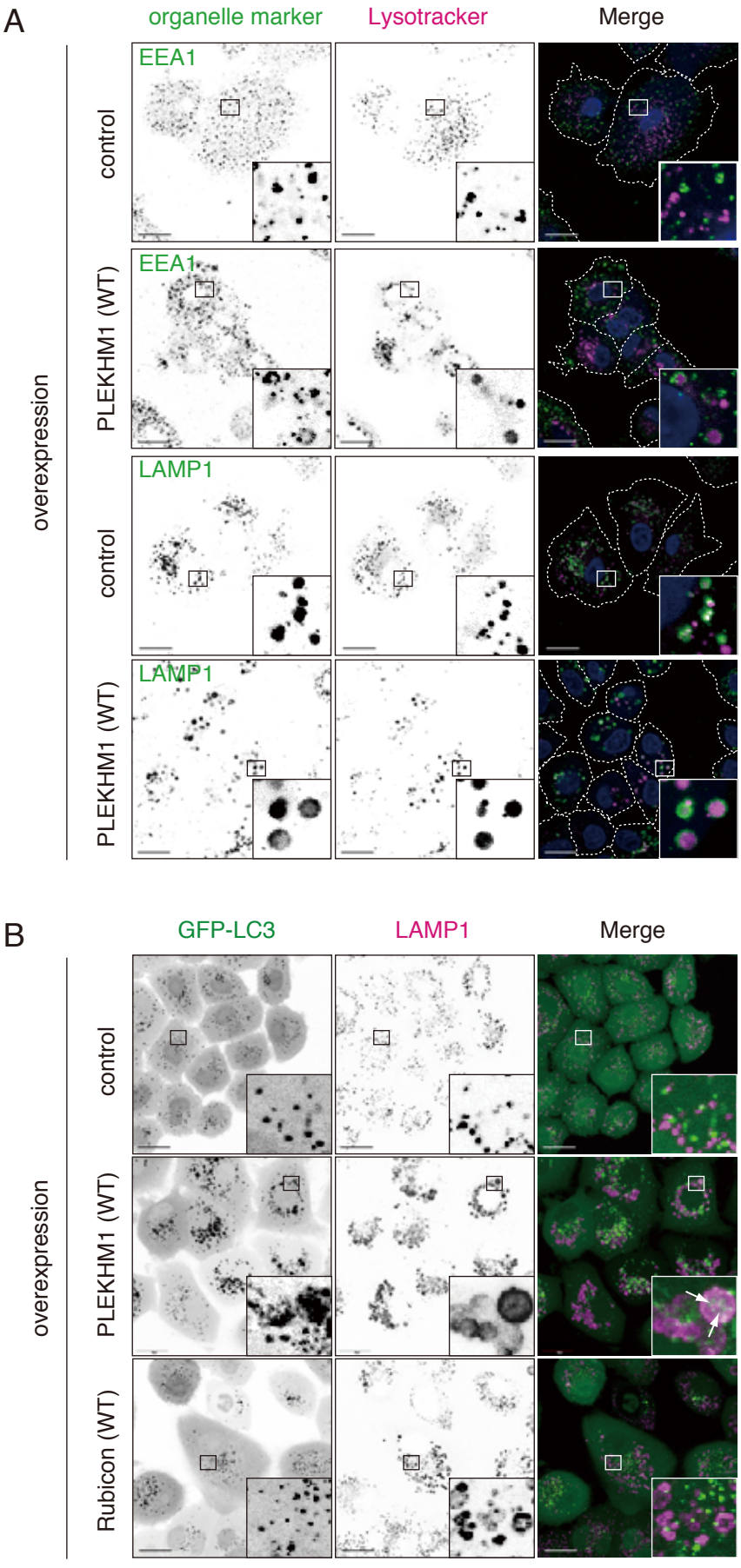

A
organelle marker
Lysotracker
Merge
overexpression
control
EEA1
PLEKHM1 (WT)
EEA1
control
LAMP1
PLEKHM1 (WT)
LAMP1
B
GFP-LC3
LAMP1
Merge
overexpression
control
PLEKHM1 (WT)
Rubicon (WT)

Figure S3.

C

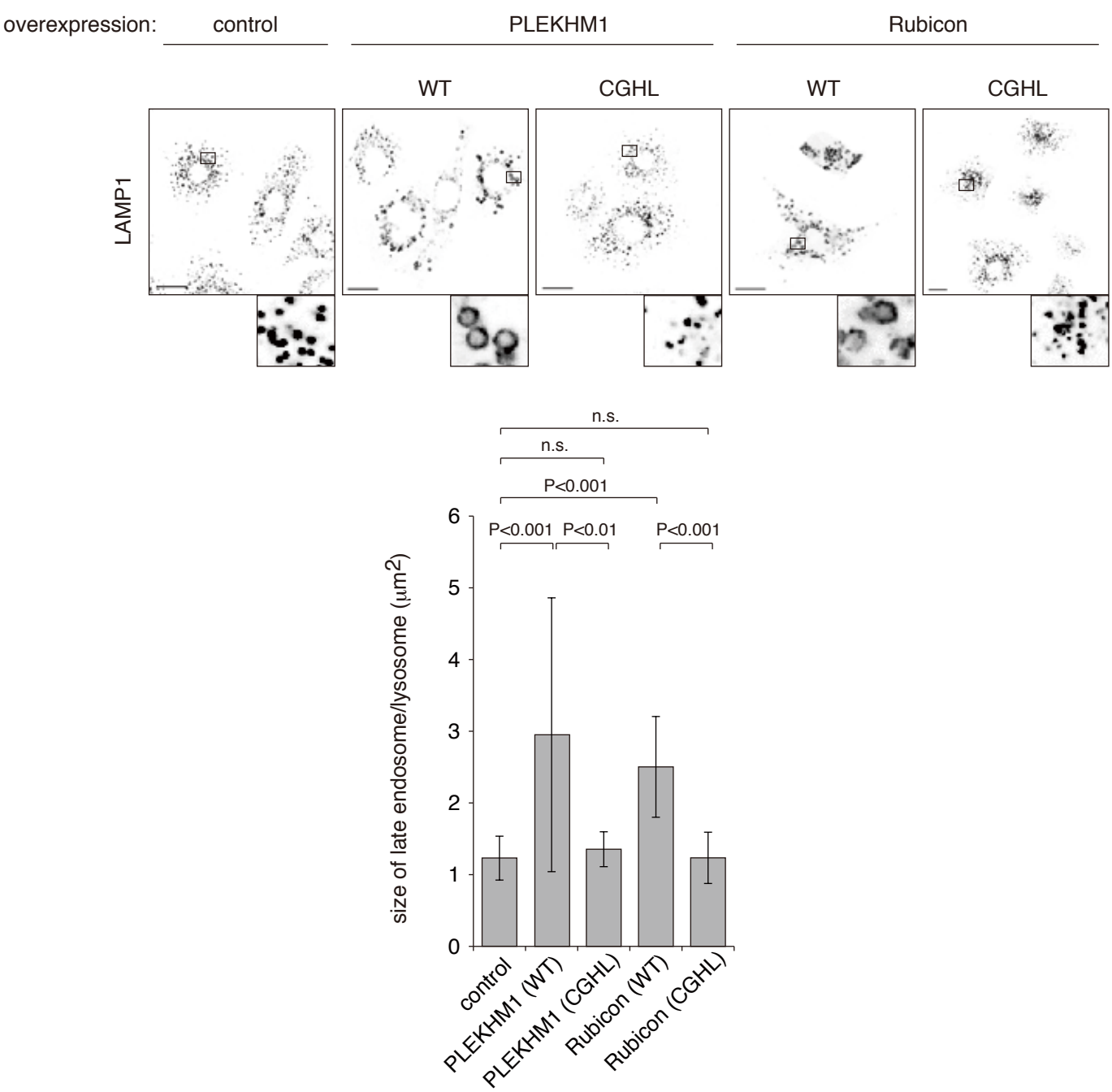


Figure S3._continued

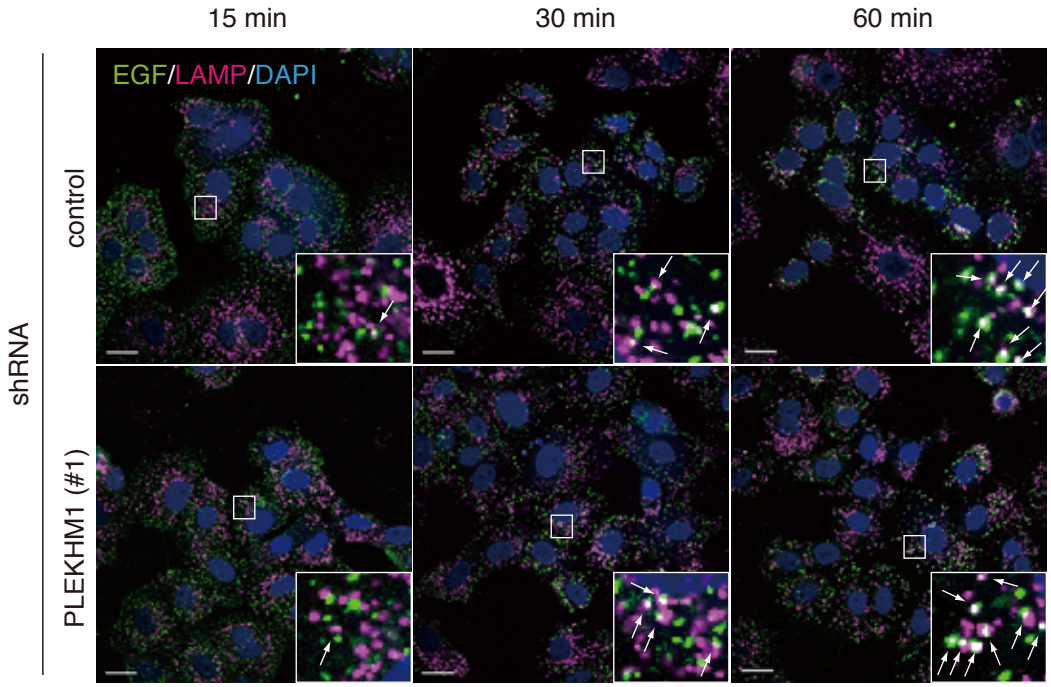


Figure S4.

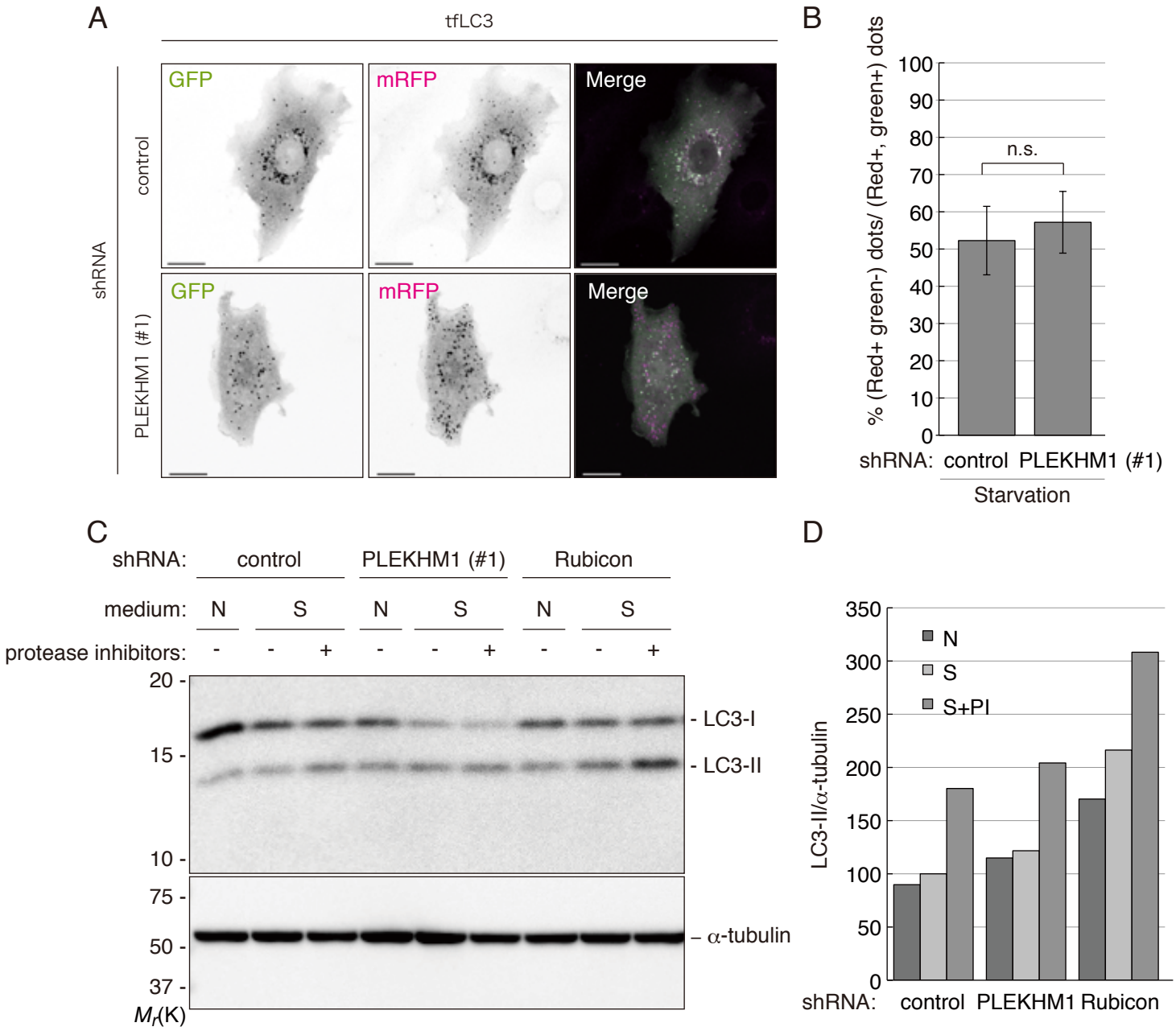

A
tfLC3
shRNA
control
PLEKHM1 (#1)
GFP
mRFP
Merge
B
% (Red+ green-) dots/ (Red+, green+) dots
n.s.
shRNA: control PLEKHM1 (#1)
Starvation
C
shRNA: control PLEKHM1 (#1) Rubicon
medium: N S N S N S
protease inhibitors: - - + - - + - - +
- LC3-I
- LC3-II
– α-tubulin
M_r(K)
D
LC3-II/α-tubulin
N
S
S+PI
shRNA: control PLEKHM1 Rubicon

Figure S5.

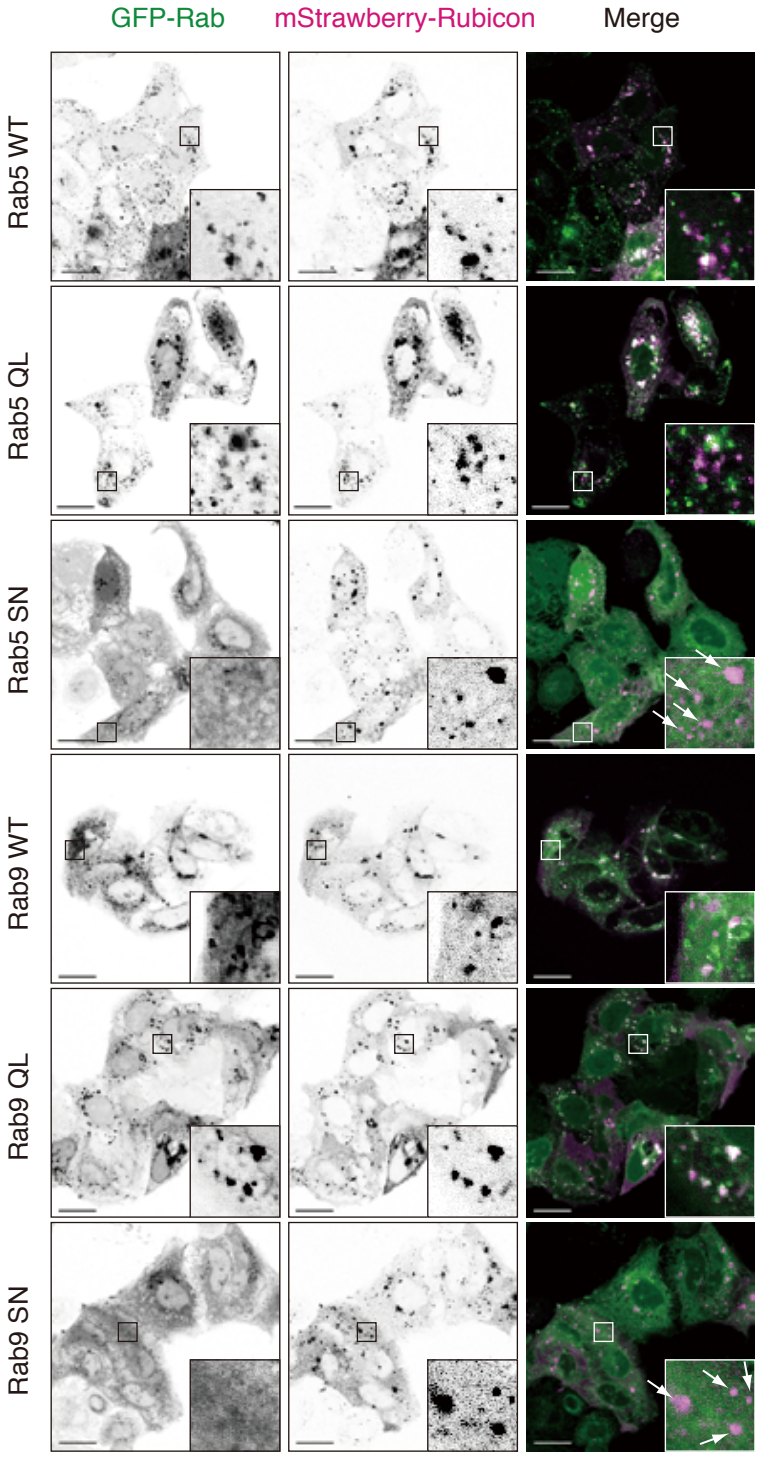

GFP-Rab
mStrawberry-Rubicon
Merge
Rab5 WT
Rab5 QL
Rab5 SN
Rab9 WT
Rab9 QL
Rab9 SN

Figure S6.

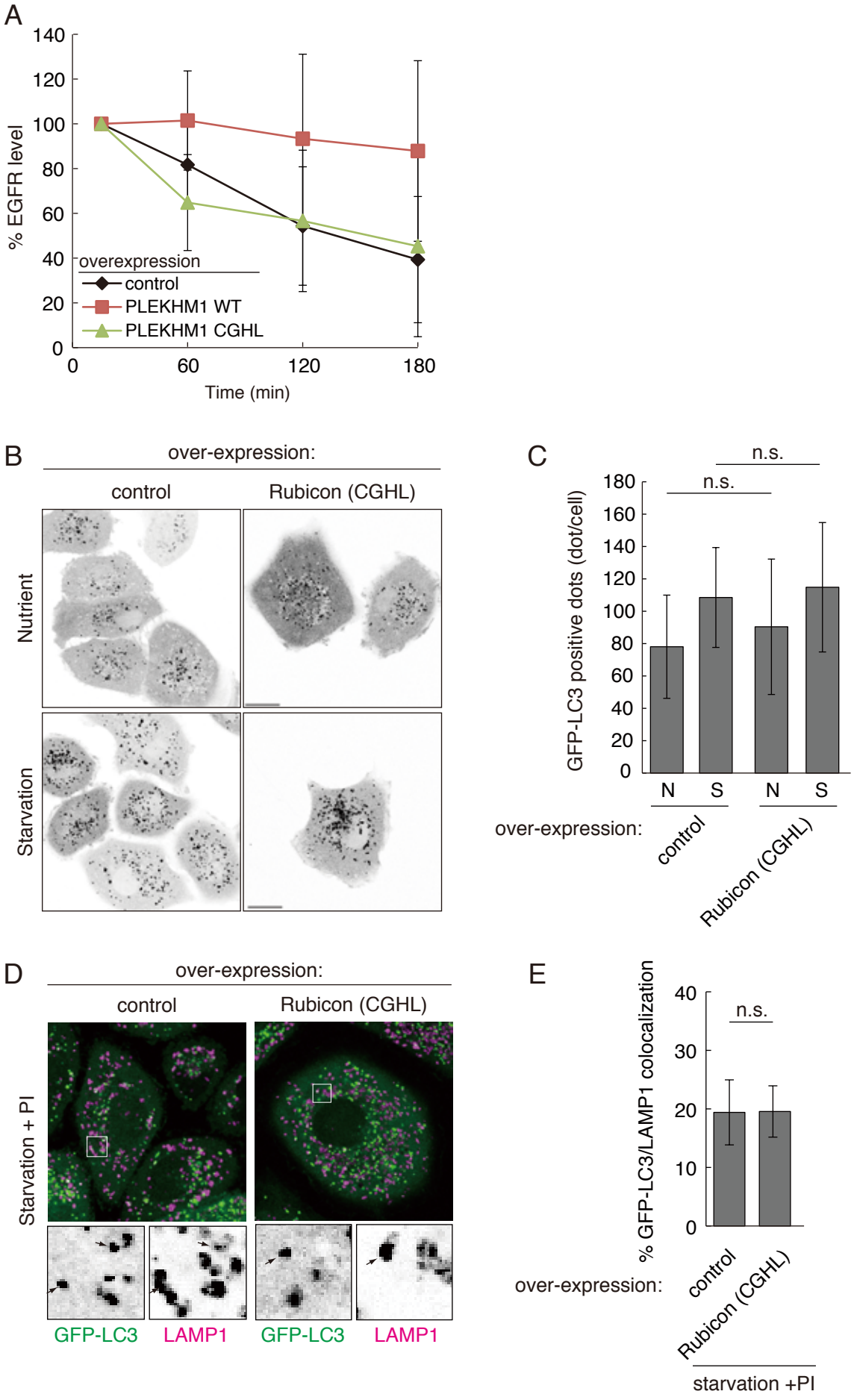

A
% EGFR level
Time (min)
overexpression
control
PLEKHM1 WT
PLEKHM1 CGHL
B
over-expression:
control
Rubicon (CGHL)
Nutrient
Starvation
C
GFP-LC3 positive dots (dot/cell)
n.s.
N
S
over-expression:
control
Rubicon (CGHL)
D
over-expression:
control
Rubicon (CGHL)
Starvation + PI
GFP-LC3
LAMP1
E
% GFP-LC3/LAMP1 colocalization
n.s.
over-expression:
control
Rubicon (CGHL)
starvation +PI

Figure S7.

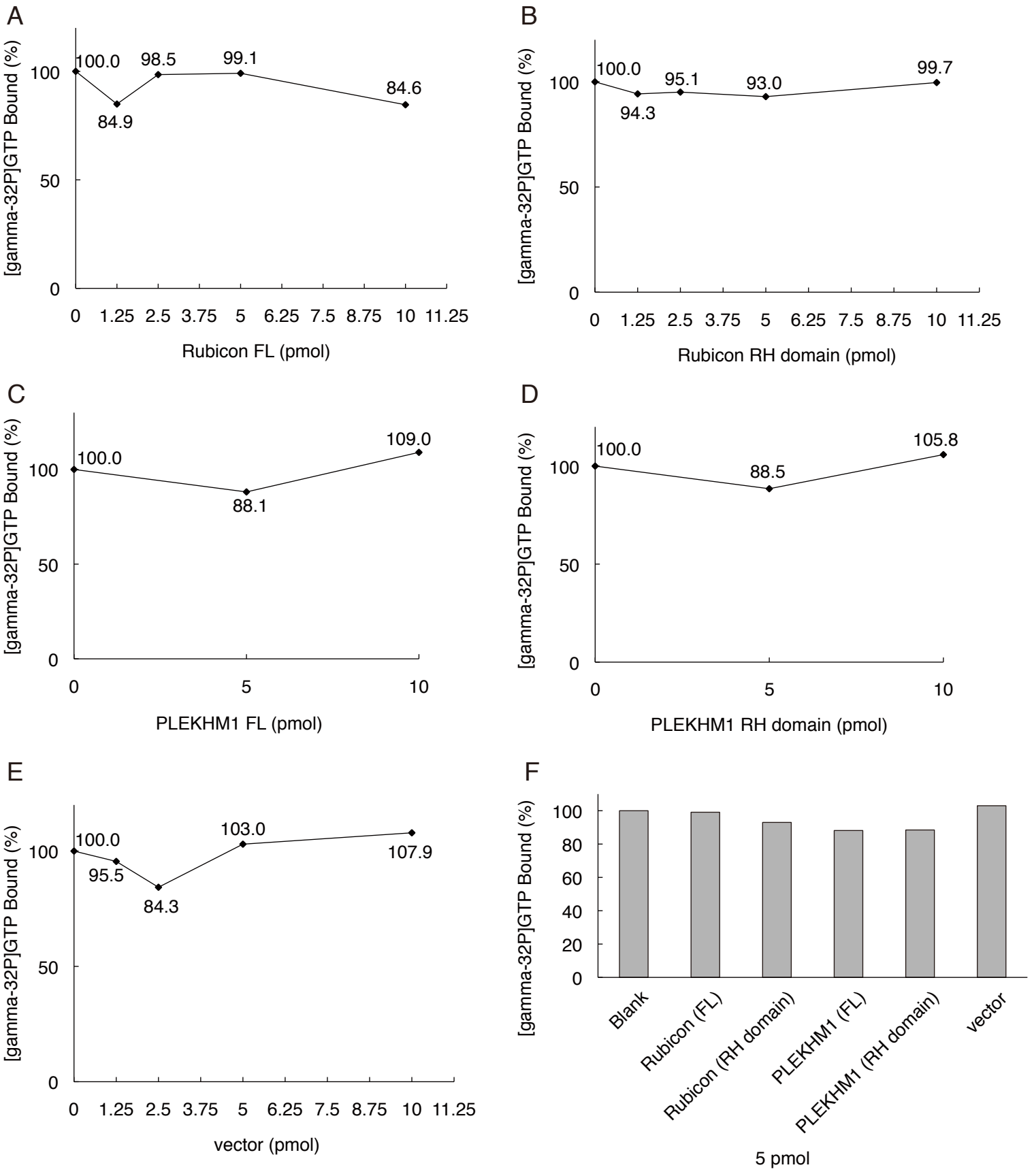

A
[gamma-32P]GTP Bound (%)
100.0
84.9
98.5
99.1
84.6
0
50
100
0 1.25 2.5 3.75 5 6.25 7.5 8.75 10 11.25
Rubicon FL (pmol)
B
[gamma-32P]GTP Bound (%)
100.0
94.3
95.1
93.0
99.7
0
50
100
0 1.25 2.5 3.75 5 6.25 7.5 8.75 10 11.25
Rubicon RH domain (pmol)
C
[gamma-32P]GTP Bound (%)
100.0
88.1
109.0
0
50
100
0 5 10
PLEKHM1 FL (pmol)
D
[gamma-32P]GTP Bound (%)
100.0
88.5
105.8
0
50
100
0 5 10
PLEKHM1 RH domain (pmol)
E
[gamma-32P]GTP Bound (%)
100.0
95.5
84.3
103.0
107.9
0
50
100
0 1.25 2.5 3.75 5 6.25 7.5 8.75 10 11.25
vector (pmol)
F
[gamma-32P]GTP Bound (%)
0
20
40
60
80
100
Blank
Rubicon (FL)
Rubicon (RH domain)
PLEKHM1 (FL)
PLEKHM1 (RH domain)
vector
5 pmol

Figure S8.

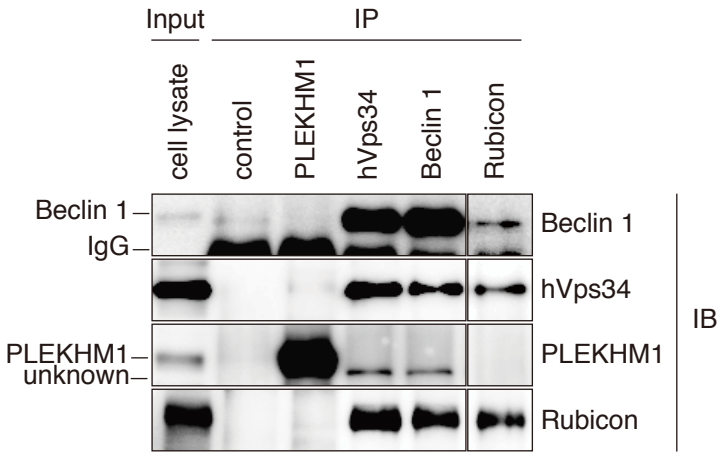

Input
IP
cell lysate
control
PLEKHM1
hVps34
Beclin 1
Rubicon
Beclin 1
IgG
PLEKHM1
unknown
Beclin 1
hVps34
PLEKHM1
Rubicon
IB

Figure S9.

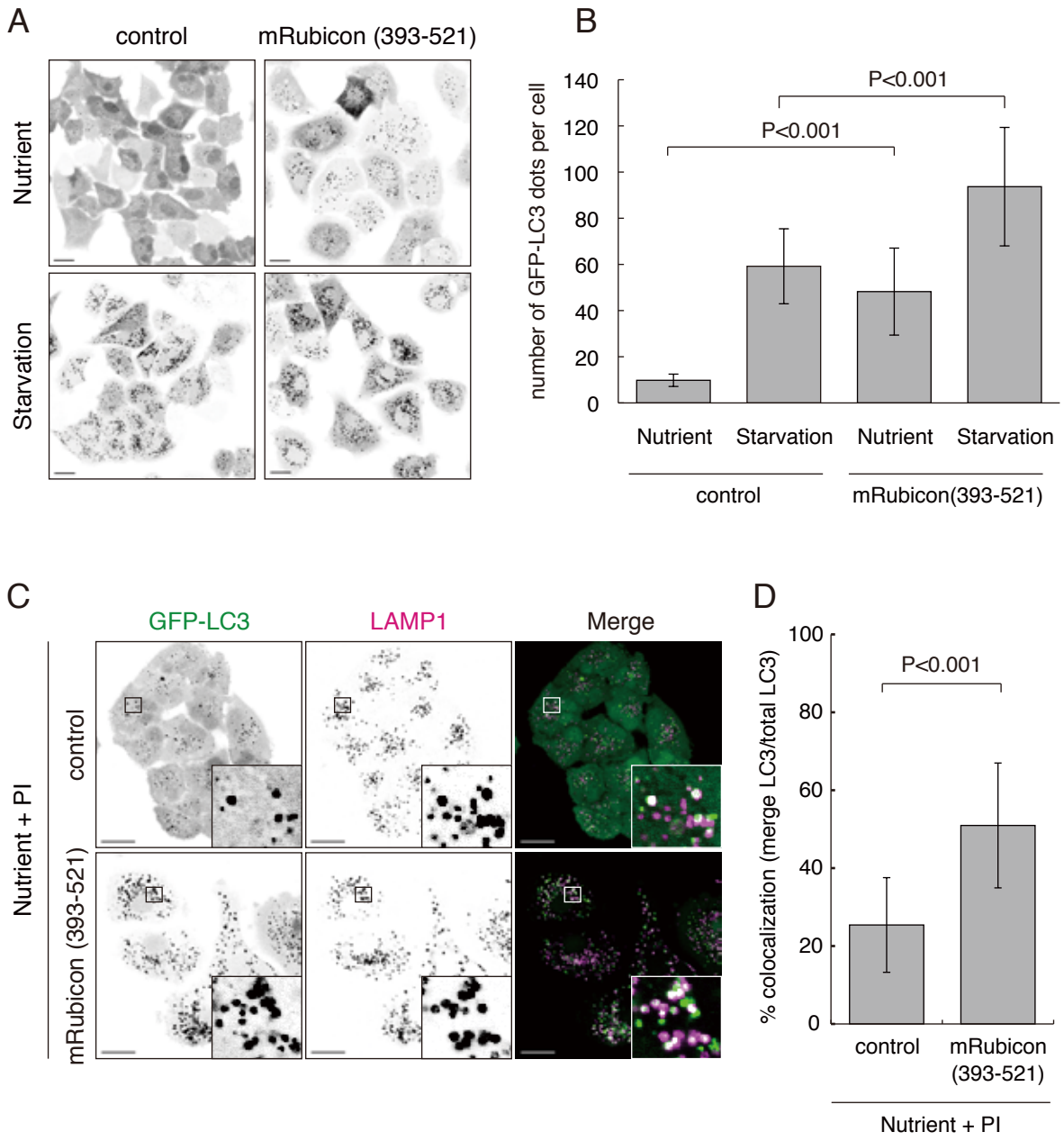

A
control
mRubicon (393-521)
Nutrient
Starvation
B
number of GFP-LC3 dots per cell
140
120
100
80
60
40
20
0
P<0.001
P<0.001
Nutrient
Starvation
Nutrient
Starvation
control
mRubicon(393-521)
C
GFP-LC3
LAMP1
Merge
Nutrient + PI
control
mRubicon (393-521)
D
% colocalization (merge LC3/total LC3)
100
80
60
40
20
0
P<0.001
control
mRubicon
(393-521)
Nutrient + PI

Figure S10.