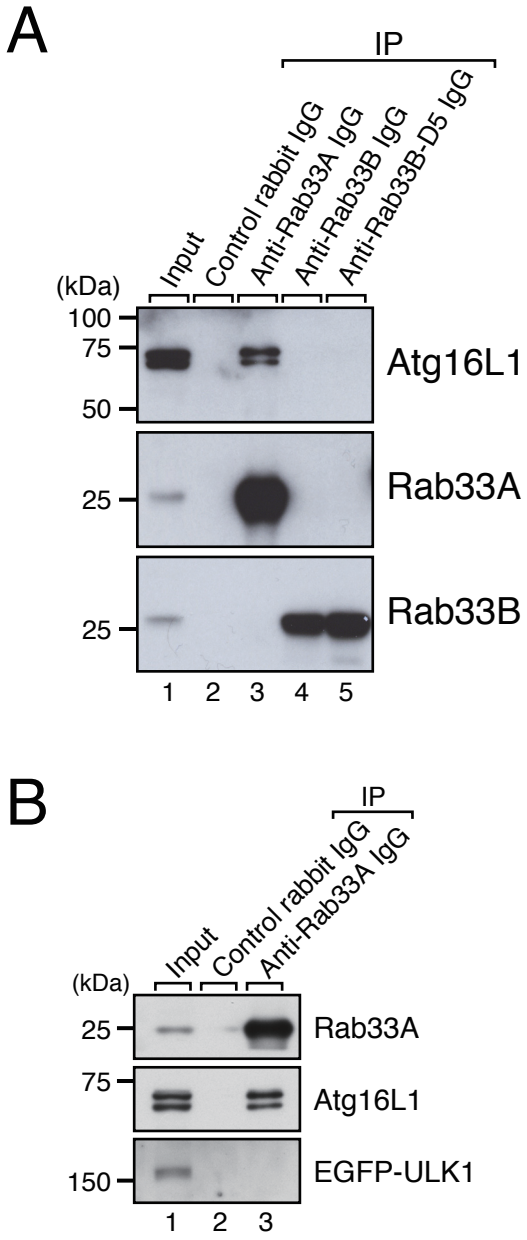
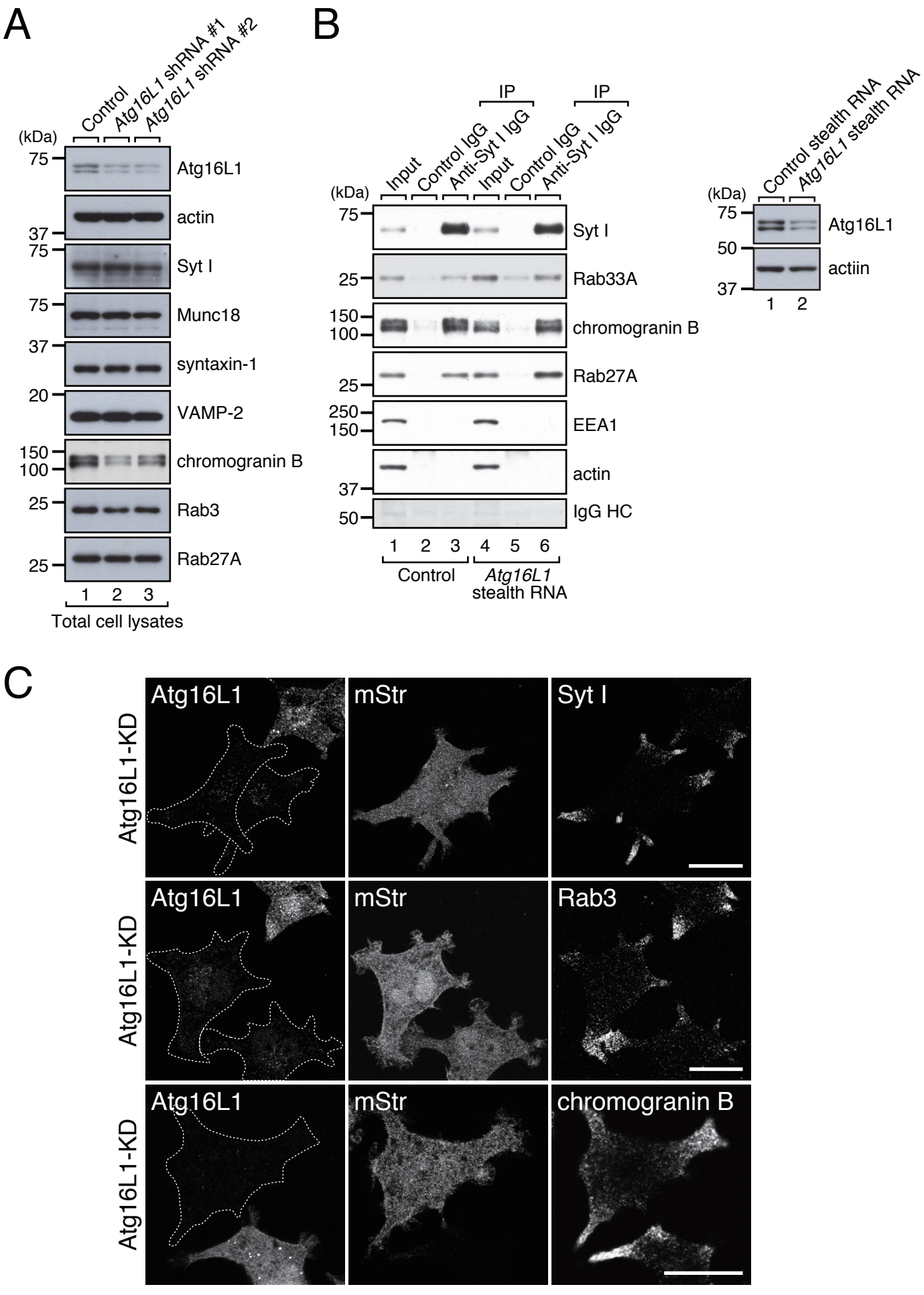
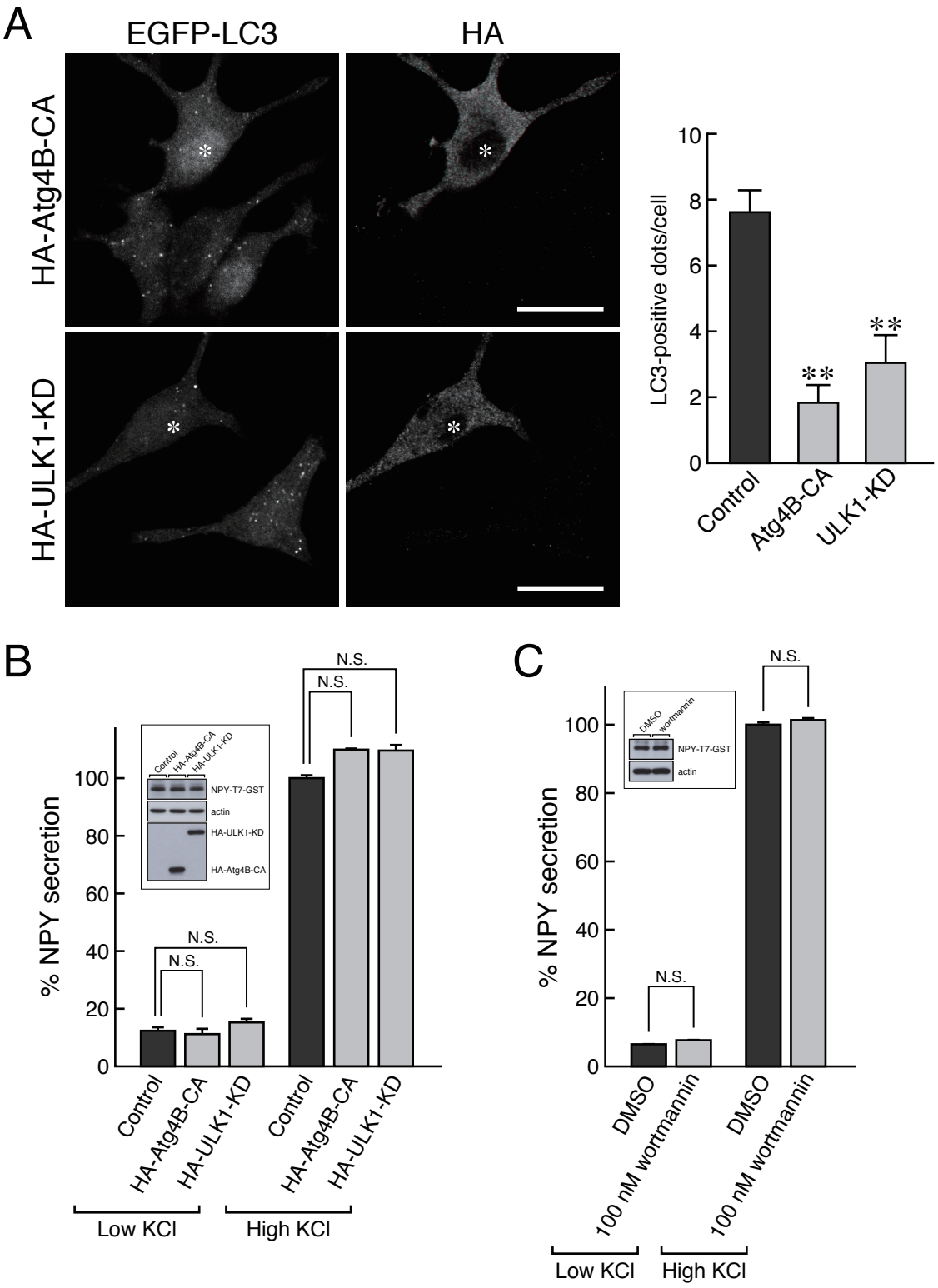


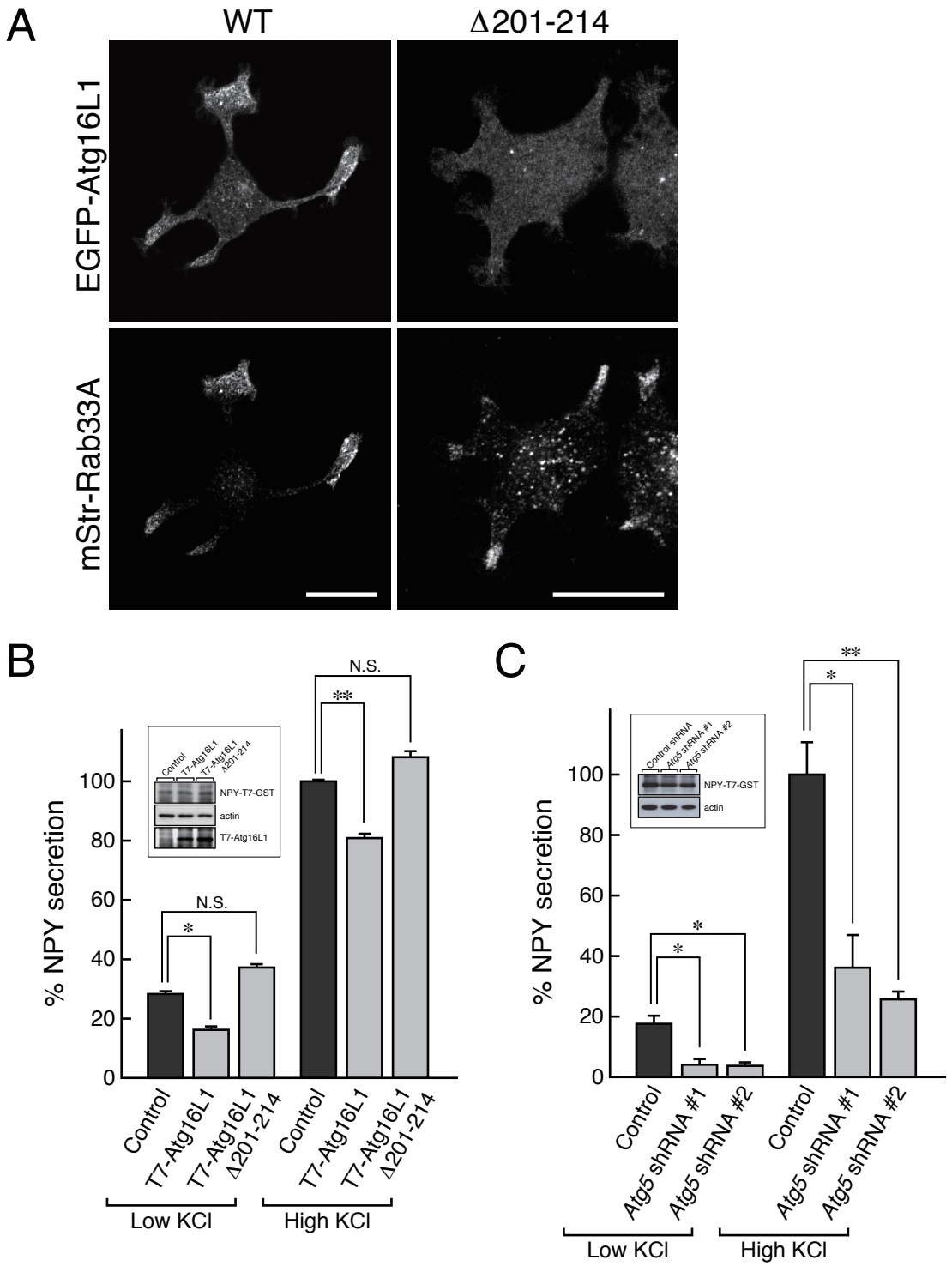
SUPPLEMENTAL FIGURE S1: Rab33A preferentially interacts with Atg16L1 in PC12 cells. (A) Atg16L1 interacts preferentially with Rab33A in PC12 cells rather than with Rab33B. Co-immunoprecipitation (IP) assays were performed as described in the Materials and Methods section. Protein A-Sepharose beads coupled with anti-Rab33A-specific antibody (lane 3) or anti-Rab33B-specific antibody (lanes 4 and 5) were incubated with PC12 cell lysates. The total cell lysates (input; lane 1) and the proteins bound to the beads (lanes 2-5) were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-Atg16L1 antibody (top panel), anti-Rab33A antibody (middle panel), and anti-Rab33B antibody (bottom panel). (B) Rab33A does not interact with ULK1 in PC12 cells. Co-immunoprecipitation (IP) assays in EGFP-ULK1-expressing PC12 cells were similarly performed as described above. The size of the molecular mass markers (in kDa) is shown at the left.


A
Control
Atg16L1 shRNA #1
Atg16L1 shRNA #2
(kDa)
75
Atg16L1
actin
37
75
Syt I
75
Munc18
37
syntaxin-1
20
VAMP-2
150
100
chromogranin B
25
Rab3
25
Rab27A
1 2 3
Total cell lysates
B
IP
IP
Input
Control IgG
Anti-Syt I IgG
Input
Control IgG
Anti-Syt I IgG
(kDa)
75
Syt I
25
Rab33A
150
100
chromogranin B
25
Rab27A
250
150
EEA1
actin
37
50
IgG HC
1 2 3 4 5 6
Control
Atg16L1
stealth RNA
Control stealth RNA
Atg16L1 stealth RNA
(kDa)
75
Atg16L1
50
actiin
37
1 2
C
Atg16L1-KD
Atg16L1
mStr
Syt I
Atg16L1-KD
Atg16L1
mStr
Rab3
Atg16L1-KD
Atg16L1
mStr
chromogranin B

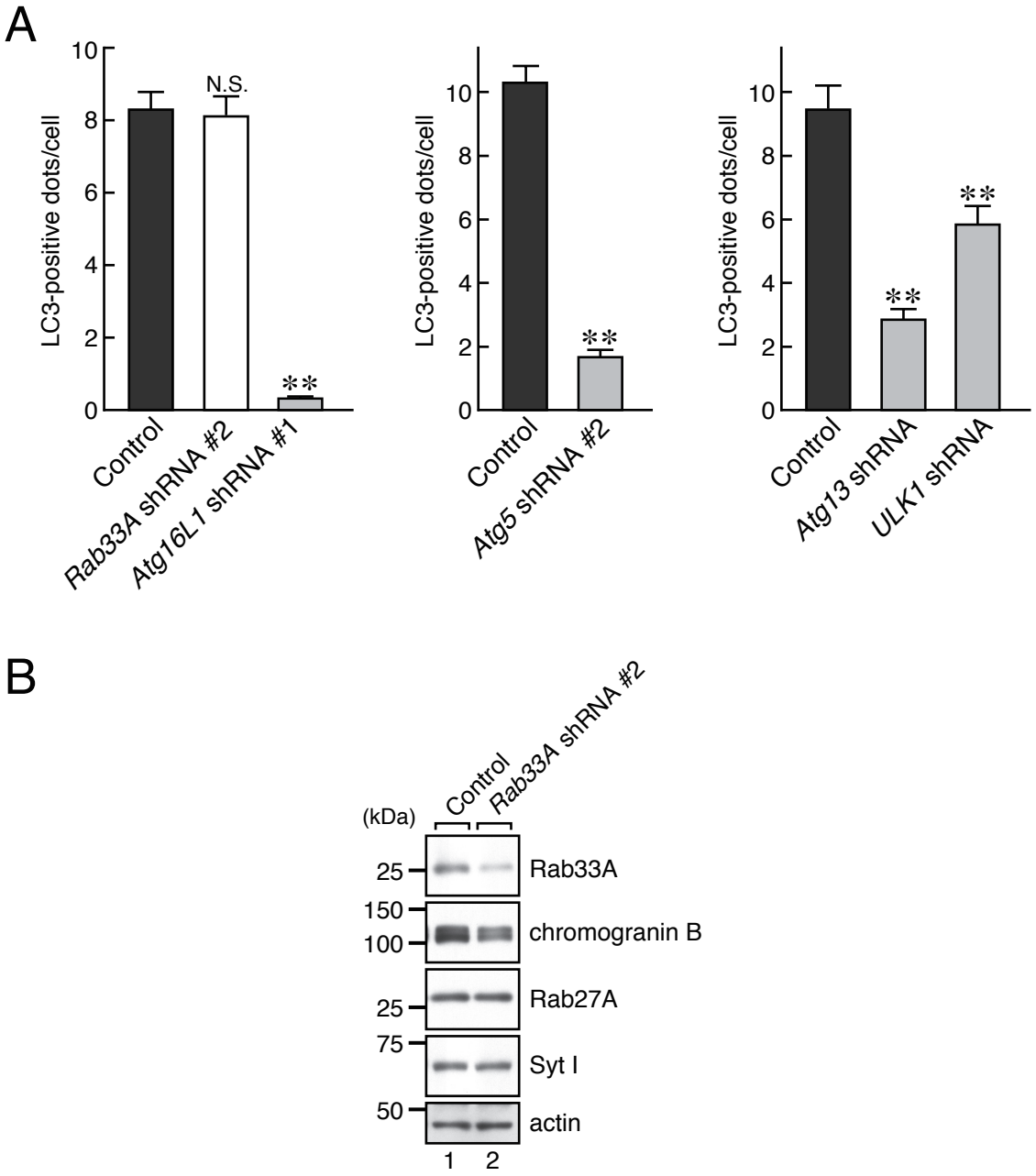
SUPPLEMENTAL FIGURE S2: Knockdown of Atg16L1 in PC12 cells had little or no effect on the level of expression (or distribution) of proteins involved in hormone secretion or of dense-core vesicle-associated proteins. (A) Total lysates of PC12 cells that had been transfected with *Atg16L1* shRNAs were prepared as described in the legend of Figure 1C and subjected to 10% SDS-PAGE followed by immunoblotting with anti-Atg16L1 antibody, anti-actin antibody, anti-Syt I antibody, anti-Munc18 antibody, anti-syntaxin-1 antibody, anti-VAMP-2 antibody, anti-chromogranin B antibody, anti-Rab3 antibody, and anti-Rab27A antibody. Note that only the level of expression of chromogranin B, a dense-core vesicle cargo protein, was clearly decreased by Atg16L1 knockdown. (B) Dense-core vesicles were immuno-isolated from PC12 cells that had been transfected with control stealth RNA or *Atg16L1* stealth RNA, by using anti-Syt I antibody as described in the Materials and Methods section. The dense-core vesicle fraction was subjected to 10% SDS-PAGE followed by immunoblotting with the antibodies indicated (left blots). Note that although the total level of chromogranin B was decreased as shown in (A), no clear differences in the amounts of dense-core vesicle-associated proteins (i.e., Syt I, Rab27A, and Rab33A) in the dense-core vesicle fraction were observed between the control PC12 cells and Atg16L1-knockdown PC12 cells. The knockdown efficiency of *Atg16L1* stealth RNA in PC12 cells is shown at the right. The size of the molecular mass markers (in kDa) is shown at the left. (C) PC12 cells were co-transfected with *Atg16L1* shRNA #1 and pmStr-C1 vector as a transfection marker and then immuno-stained with anti-Atg16L1 antibody and anti-Syt I antibody (anti-Rab3 antibody or anti-chromogranin B antibody). Note that the distribution of the three dense-core vesicle markers in the neurites of PC12 cells was not affected by the knockdown of Atg16L1. Atg16L1-KD cells are outlined in broken lines. Scale bars, 20 μm.


A
EGFP-LC3
HA
HA-Atg4B-CA
HA-ULK1-KD
LC3-positive dots/cell
10
8
6
4
2
0
**
**
Control
Atg4B-CA
ULK1-KD
B
% NPY secretion
100
80
60
40
20
0
N.S.
N.S.
N.S.
N.S.
Control
HA-Atg4B-CA
HA-ULK1-KD
NPY-T7-GST
actin
HA-ULK1-KD
HA-Atg4B-CA
Low KCl
High KCl
C
% NPY secretion
100
80
60
40
20
0
N.S.
N.S.
DMSO
100 nM wortmannin
NPY-T7-GST
actin
Low KCl
High KCl

SUPPLEMENTAL FIGURE S3: Effect of Atg4B-CA/ULK1-KD overexpression or wortmannin on hormone secretion from PC12 cells. (A) Inhibition of canonical autophagy by overexpression of Atg4B-CA or ULK1-KD in PC12 cells. (Left images) The HA-Atg4B-CA mutant or HA-ULK1-KD mutant was transiently expressed in PC12 cells stably expressing EGFP-LC3. PC12 cells expressing HA-Atg4B-CA (upper panels, asterisk) or HA-ULK1-KD (lower panels, asterisk) contained fewer EGFP-LC3-positive dots than the adjacent untransfected cells. (Right graph) The number of EGFP-LC3 dots in the cells (n >70) was counted as described previously (Itoh *et al*., 2008; Itoh *et al*., 2011). **, $p < 0.01$ (Student's unpaired *t* test). (B) Overexpression of Atg4B-CA or ULK1-KD in PC12 cells had no effect on hormone secretion. The HA-Atg4B-CA mutant or HA-ULK1-KD mutant was transiently co-expressed with NPY-T7-GST in PC12 cells to inhibit autophagy. The NPY-T7-GST secretion assays were performed as described in the Materials and Methods section. Note that inhibition of autophagy had no effect on either low KCl-dependent or high KCl-dependent NPY secretion (shaded bars). Bars indicate the means and S.E. of three determinations. N.S., not significant in comparison with the control cells. Total cell lysates (or immunoprecipitates of anti-HA tag antibody) of PC12 cells that had been co-transfected with pShooter-NPY-T7-GST and pEF-HA-Atg4B-CA (pEF-HA-ULK1-KD or control pEF-BOS vector) were subjected to 10% SDS-PAGE followed by immunoblotting with anti-T7 tag antibody, anti-actin antibody, and anti-HA tag antibody (inset). (C) Inhibition of canonical autophagy by wortmannin had no effect on hormone secretion from PC12 cells. PC12 cells transfected with pShooter-NPY-T7-GST were treated with 100 nM wortmannin (or DMSO) for 30 min to inhibit autophagy, and the NPY-T7-GST secretion assays were then performed as described in the Materials and Methods section.. Note that inhibition of autophagy had no effect on either low KCl- or high KCl-dependent NPY secretion (shaded bars). N.S., not significant in comparison with the control cells. Under our experimental conditions, inhibition of autophagy had no effect on the total amount of hormone (NPY-T7-GST) (inset). The results shown are representative of at least two independent experiments.



SUPPLEMENTAL FIGURE S4: Effect of Atg16L1 overexpression and Atg5 knockdown on hormone secretion from PC12 cells. (A) A Rab33A-binding-deficient mutant, Atg16L1-Δ201-214, is unable to target dense-core vesicles in PC12 cells. Note that wild-type Atg16L1 was clearly targeted to the Rab33A-positive neurites, but that no enrichment of the mutant protein in the neurites was observed in any of the Atg16L1-Δ201-214-expressing cells tested (n >30). Scale bars, 20 μm. (B) Overexpression of Atg16L1, but not of Atg16L1-Δ201-214, in PC12 cells inhibited hormone secretion. T7-Atg16L1 or T7-Atg16L1-Δ201-214 was transiently co-expressed with NPY-T7-GST in PC12 cells. The NPY-T7-GST secretion assays were performed as described in the Materials and Methods section. *, p <0.05 and **, p <0.01 (Student's unpaired t test). N.S., not significant in comparison with the control cells. Total cell lysates of PC12 cells that had been co-transfected with pShooter-NPY-T7-GST and pEF-T7-Atg16L1 (pEF-T7-Atg16L1-Δ 201-214 or control pEF-BOS vector) were subjected to 10% SDS-PAGE followed by immunoblotting with anti-T7 tag antibody and anti-actin antibody (inset). (C) Effect of Atg5 knockdown on hormone secretion from PC12 cells. NPY-T7-GST secretion assays were performed as described above. *, p <0.05 and **, p <0.01 (Student's unpaired t test). Note that both low KCl-dependent and high KCl-dependent hormone secretion were significantly lower in the Atg5-knockdown cells, the same as in the Atg16L1-knockdown cells (Figure 5A). Total cell lysates of PC12 cells that had been co-transfected with pShooter-NPY-T7-GST and pSilencer-Atg5 (or control empty pSilencer vector) were subjected to 10% SDS-PAGE followed by immunoblotting with anti-T7 tag antibody and anti-actin antibody (inset). Under our experimental conditions, Atg5-knockdown cells had a propensity to decrease the total amount of NPY-T7-GST, and this effect may be partly attributable to the reduced viability of *Atg5* shRNA-transfected cells.



SUPPLEMENTAL FIGURE S5: Characterization of shRNAs used in this study. (A) Effect of shRNAs against Rab33A, Atg16L1, Atg5, Atg13, or ULK1 on starvation-induced autophagy. PC12 cells stably expressing EGFP-LC3 were transfected with each of the shRNAs indicated. At 72 hours after transfection, the cells were starved in HBSS for 1.5 hours, and then the number of EGFP-LC3 dots in the cells (n >50) was counted as described previously (Itoh *et al*., 2008; Itoh *et al*., 2011). Note that knockdown of endogenous Atg16L1, Atg5, Atg13, or ULK1, but not of Rab33A, in PC12 cells significantly inhibited starvation-induced autophagy as monitored by LC3-dot formation. **, p <0.01 in comparison with the control cells (Student's unpaired t test). (B) Effect of knockdown of Rab33A on the amount of dense-core vesicle proteins. Total lysates of PC12 cells that had been transfected with *Rab33A* shRNA #2 were prepared as described in the legend of Figure 1C and subjected to 10% SDS-PAGE followed by immunoblotting with the antibodies indicated. Note that knockdown of Rab33A in PC12 cells decreased the level of expression of chromogranin B, the same as Atg16L1 knockdown did (Supplemental Figure S2A). The size of the molecular mass markers (in kDa) is shown at the left.