E07-10-1074 Pool

Figure S1. Rab33B-specific antibody and siRNA.

(A) Specificity of anti-Rab33B antibody. COS-7 cell lysates containing FLAG-Rab33A or FLAG-Rab33B were analyzed by 10% SDS-PAGE and immunoblotting with affinity-purified anti-Rab33B rabbit polyclonal antibody (upper panel) or HRP-conjugated anti-FLAG tag mouse monoclonal antibody (lower panel). Our anti-Rab33B antibody detected Rab33B, but not its close homologue Rab33A. (B) Specificity of siRNA against Rab33B. COS-7 cells were co-transfected with pEGFP-C1-Rab33B and pSilencer control vector (lane 1), pEGFP-C1-Rab33B and pSilencer-Rab33B (lane 2), pEGFP-C1-Rab33A and pSilencer control vector (lane 3), or pEGFP-C1-Rab33A and pSilencer-Rab33B (lane 4). Each cell lysate was analyzed by 10% SDS-PAGE and immunoblotting with anti-GFP antibody (upper panel) or anti-actin antibody (lower panel). Note that Rab33B-siRNA down-regulated the expression of GFP-Rab33B efficiently and specifically.

Figure S2. Rab33B interacts with Atg5-12/16L complex regardless of nutrient conditions.

NIH3T3 cells stably expressing FLAG-Rab33B (lanes 3, 4, 7, and 8) or nothing (lanes 1, 2, 5, and 6) were cultured in DMEM and the medium was switched to HBSS for the final 2 hours (lanes 2, 4, 6, and 8, Medium "H") or not switched (lanes 1, 3, 5, and 7, Medium "D"), and the cell lysates were incubated with anti-FLAG M2 agarose beads. Total cell lysates (lanes 1-4) and proteins bound to the agarose beads (lanes 5-8) were

analyzed by SDS-PAGE followed by immunoblotting with anti-Atg16L antibody (top panel), anti-Atg5 antibody (middle panel), and anti-FLAG tag antibody (bottom panel). Not only Atg16L, a direct interactor of Rab33B, but also Atg12-conjugated Atg5 was co-immunoprecipitated with FLAG-Rab33B, and these interactions were unaffected by nutrient conditions (compare lanes 7 and 8).

Figure S3. Golgi localization of Rab33B.

(A) HeLaS3 cells were fixed with 2% paraformaldehyde and stained with anti-Rab33B mouse monoclonal antibody and anti-giantin rabbit polyclonal antibody. Rab33B clearly co-localized with giantin (a cis-Golgi marker). Scale bar, 20 μm. (B-E) NIH3T3 cells transiently expressing GFP-Rab33B (green) under nutrient-rich conditions were fixed and stained with anti-GM130 (B, red), anti-γ-adaptin (C, red), anti-EEA1 (D, red), or anti-Lamp-1 antibody (E, red). Note that GFP-Rab33B was co-localized specifically with GM130, a cis-Golgi marker protein, and was not co-localized with γ-adaptin, a trans-Golgi network marker, EEA1, an early endosome marker, or Lamp-1, a lysosome marker. Scale bar, 20 μm.

Figure S4. Localization of other Atg16L-binding Rabs in NIH3T3 cells.

NIH3T3 cells transiently expressing (A) GFP-Rab18, (B) GFP-Rab18-QL, (C) GFP-Rab33A, (D) GFP-Rab33A-QL, (E) GFP-Rab35, or (F) GFP-Rab35-QL (green) under nutrient-rich conditions were fixed and stained with anti-Atg16L (red). Rab33A and Rab33A-QL recruited endogenous Atg16L to Rab33A-positive dots in cytosol like

Rab33B. Rab18 and Rab35 hardly recruited Atg16L. Scale bar, 10 μm.

Figure S5. Rab33B and Rab33B-QL recruit Atg12 to the Golgi and cytoplasmic dot structures.

NIH3T3 cells transiently expressing GFP-Rab33B (A; green) or GFP-Rab33B-QL (B; green) under nutrient-rich conditions were fixed and stained with anti-Atg12 antibody (red) and anti-GM130 antibody (blue). Both GFP-Rab33B and GFP-Rab33B-QL were localized in the Golgi (indicated by GM130) and cytoplasmic dot structures. Atg12 was co-localized with GFP-Rab33B in the Golgi (A; large arrows), but not with GFP-Rab33B-QL (B; large arrows). Note that Atg12 was not localized in the Golgi in untransfected control cells (A; small arrows). Scale bar, 20 μm.

Figure S6. Binding activity and autophagy-inhibition activity of Atg16L are highly correlated.

(A) Schematic representation of Atg16L and the truncated mutants are shown. (B) Lysates from PC12 cells transiently expressing nothing (lane 1), FLAG-Atg16L-M (lane 2), FLAG-Atg16L-M1 (lane 3), FLAG-Atg16L-M2 (lane 4), or FLAG-Atg16L-M3 (lane 5) and cultured in HBSS for 2 hours were analyzed by SDS-PAGE followed by immunoblotting with anti-FLAG tag antibody (top panel), anti-LC3 antibody (middle panel), and anti-α-tubulin antibody (bottom panel). FLAG-Atg16L-M and -M1 inhibited LC3-lipidation. (C) Anti-FLAG M2 agarose beads were incubated with lysates from PC12 cells transiently expressing nothing (lanes 1 and 7), only GFP-

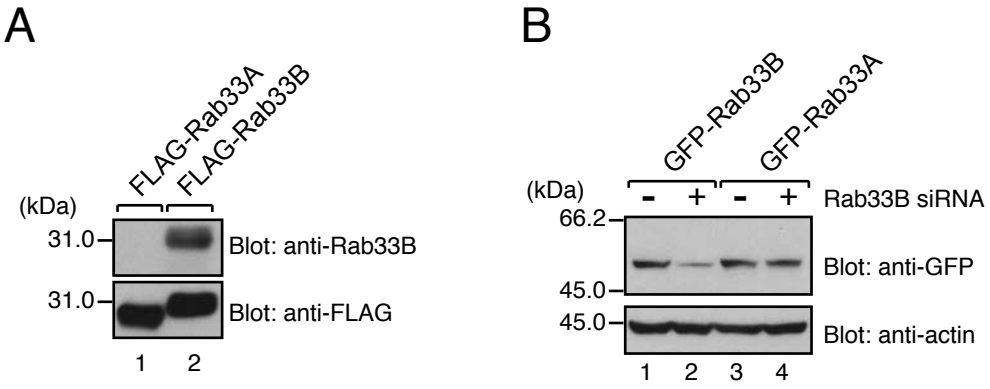
Rab33B (lanes 2 and 8), GFP-Rab33B and FLAG-Atg16L-M (lanes 3 and 9), GFP-Rab33B and FLAG-Atg16L-M1 (lanes 4 and 10), GFP-Rab33B and FLAG-Atg16L-M2 (lanes 5 and 11), or GFP-Rab33B and FLAG-Atg16L-M3 (lanes 6 and 12). Cell lysates (Input; lanes 1-6) and proteins bound to the beads (IP; lanes 7-12) were analyzed by SDS-PAGE followed by immunoblotting with anti-FLAG tag antibody (upper panel) and anti-GFP antibody (lower panel). GFP-Rab33B was co-immunoprecipitated with FLAG-Atg16L-M and -M1. (D) Lysates from PC12 cells transiently expressing nothing (lanes 1 and 2), FLAG-Atg16L-ΔN (lane 3), FLAG-Atg16L-ΔN1 (lane 4), FLAG-Atg16L-ΔN2 (lane 5), or FLAG-Atg16L-ΔN3 (lane 6) and cultured in DMEM (lane 1) or HBSS (lanes 2-6) for 2 hours were analyzed by SDS-PAGE followed by immunoblotting with anti-FLAG tag antibody (top panel), anti-LC3 antibody (middle panel), and anti-α-tubulin antibody (bottom panel). FLAG-Atg16L-ΔN and -ΔN1 inhibited LC3-lipidation. (E) Anti-FLAG M2 agarose beads were incubated with lysates from PC12 cells transiently expressing nothing (lanes 1 and 8), only GFP-Rab33B (lanes 2 and 9), GFP-Rab33B and FLAG-Atg16L-ΔN (lanes 3 and 10), GFP-Rab33B and FLAG-Atg16L-ΔN1 (lanes 4 and 11), GFP-Rab33B and FLAG-Atg16L-ΔN2 (lanes 5 and 12), GFP-Rab33B and FLAG-Atg16L-ΔN3 (lanes 6 and 13), or GFP-Rab33B and FLAG-Atg16L-C (lanes 7 and 14). Cell lysates (Input; lanes 1-7) and proteins bound to the beads (IP; lanes 8-14) were analyzed by SDS-PAGE followed by immunoblotting with anti-FLAG tag antibody (upper panel) and anti-GFP antibody (lower panel). GFP-Rab33B was co-immunoprecipitated with FLAG-Atg16L-ΔN and -ΔN1.

Figure S7. Expression profiles of Rab33A/B in NIH3T3 cells.

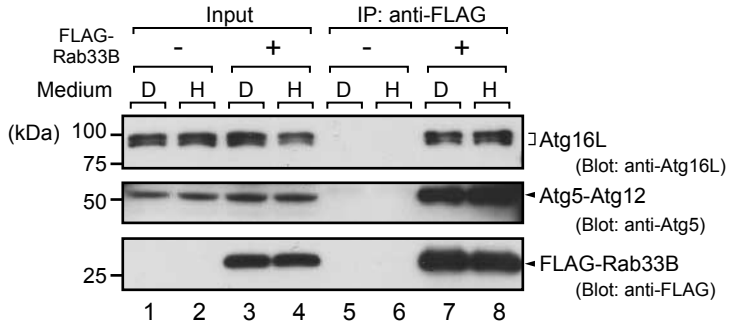
Endogenous expression of Rab33A and Rab33B in NIH3T3 cells. Beads coupled with FLAG-Atg16L were incubated with NIH3T3 cell lysates. The lysates (lane 1) and the proteins (lane 2) bound to the beads were analyzed by SDS-PAGE followed by immunoblotting with anti-Rab33B antibody (top panel), anti-Rab33A antibody (middle panel), and HRP-conjugated anti-FLAG tag antibody (bottom panel). Rab33B was concentrated by FLAG-Atg16L (compare lanes 1 and 2 in the top panel). The specificity of anti-Rab33A antibody was confirmed previously (Tsuboi and Fukuda, 2006). Rab33A seems not to be expressed in NIH3T3 cells. The asterisk presumably corresponds to the non-specific band.

Figure S8. Down-regulation of Rab33B by siRNA treatment.

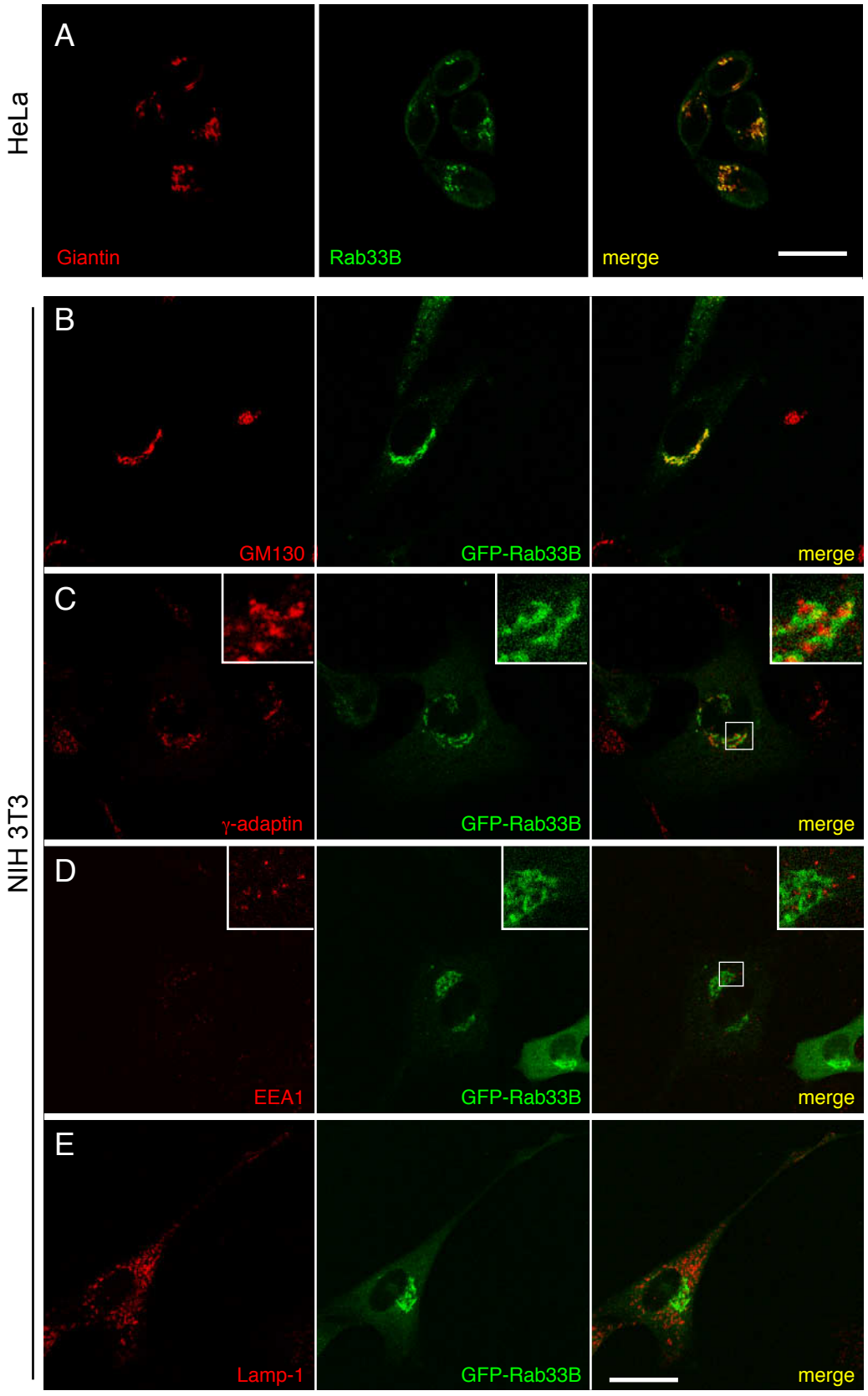
(A) The lysates from NIH3T3 cells treated with control (lanes 1-3) or Rab33B (lanes 4-6) siRNA and cultured in HBSS for 1 hour were analyzed by SDS-PAGE followed by immunoblotting with anti-actin antibody (top panel), anti-Rab33B antibody (middle panel) and anti-LC3 antibody (bottom panel). Results from three independent dishes under the same condition are shown. Ratio between LC3-I and LC3-II was not affected by treatment of Rab33B siRNA. (B) NIH3T3 cells cultured in HBSS for 1 hour were fixed and stained with anti-LC3 antibody. The number of LC3-positive dots in the cells was counted. Bars represent the means ± S.E. of representative data. No significant differences between control and Rab33B siRNA was observed.



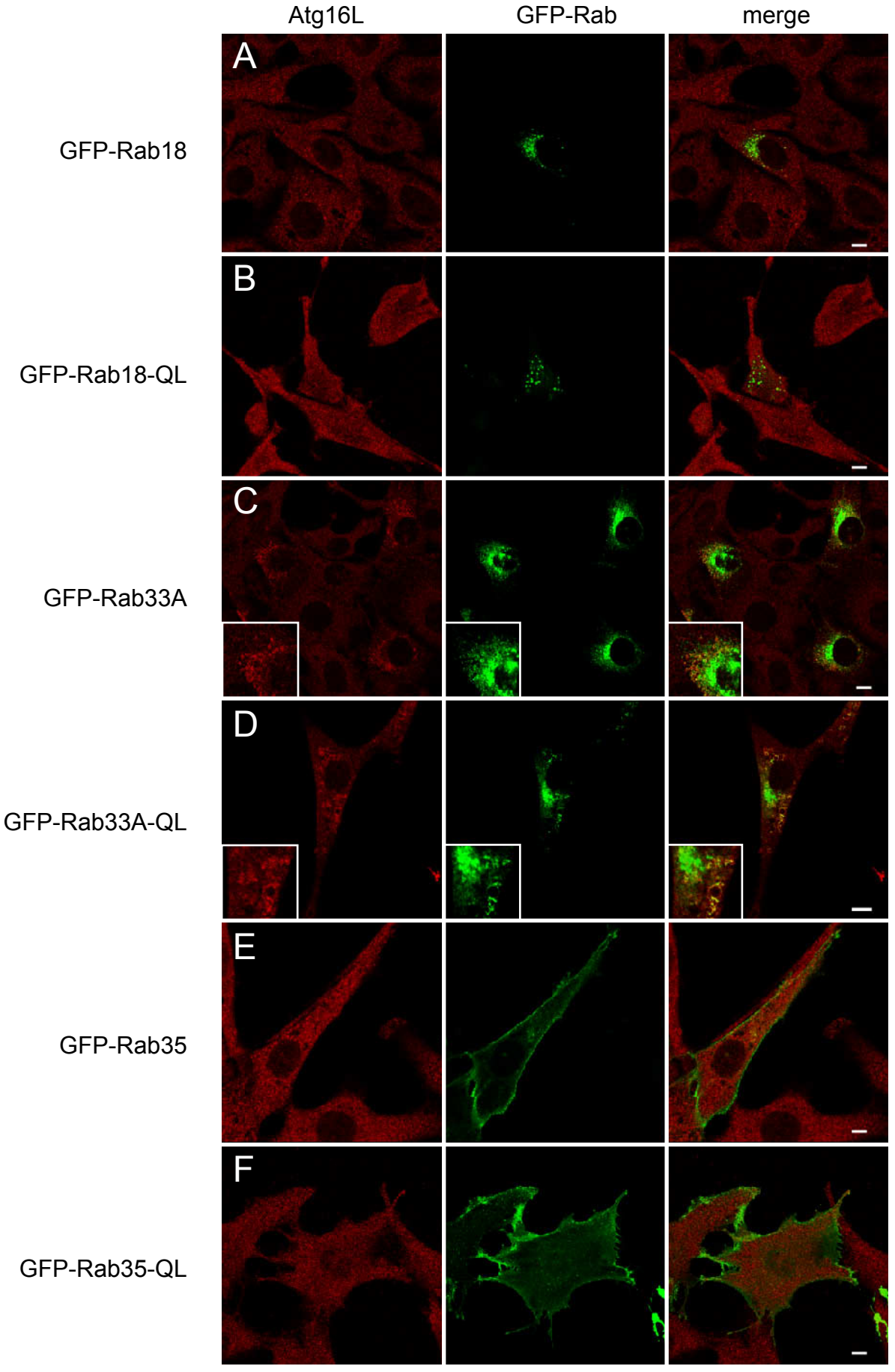
Itoh et al. Supplemental information Figure S1, Top ⇑



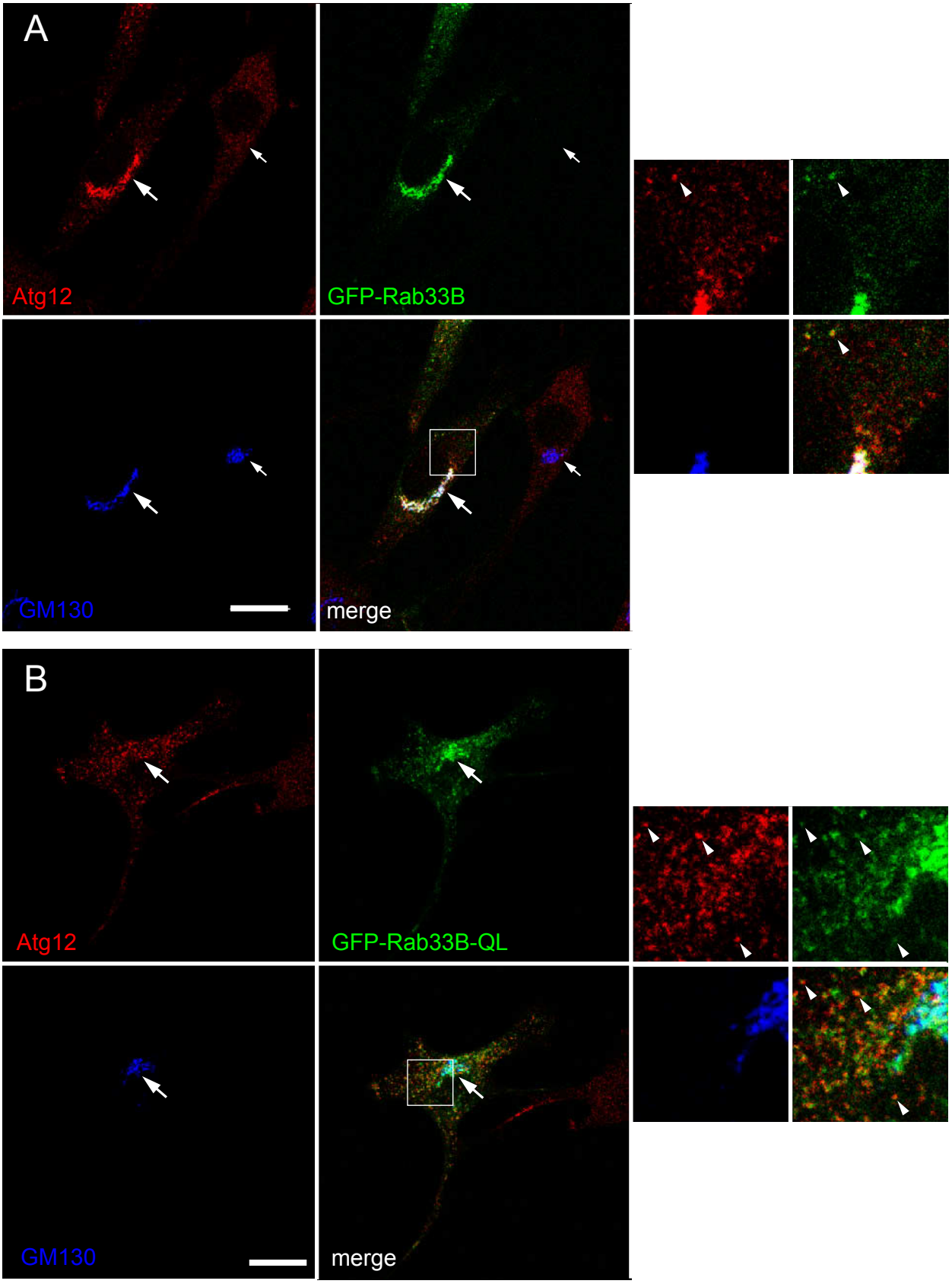
Itoh et al. Supplemental information Figure S2, Top ⇑



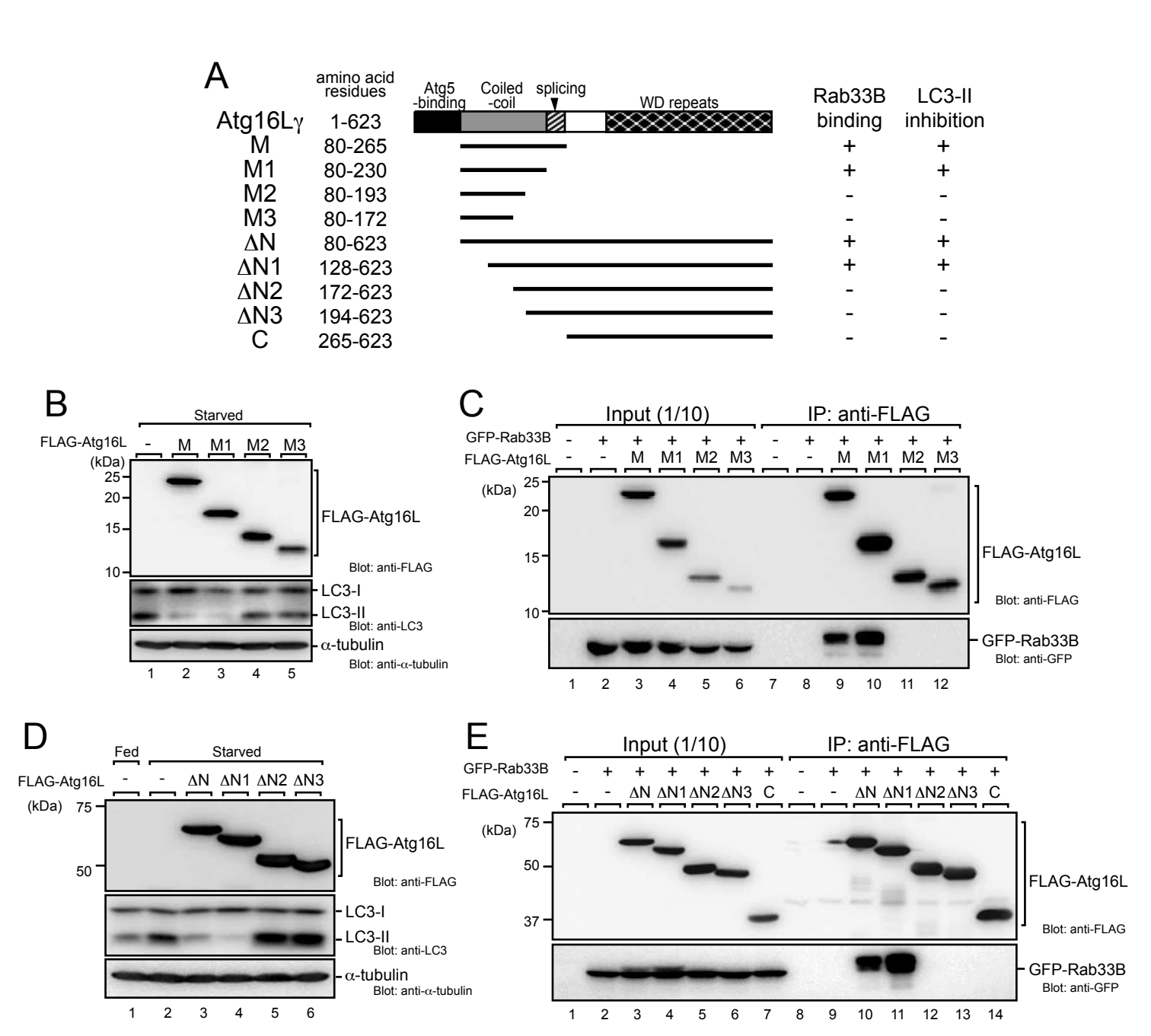
Itoh et al. Supplemental information Figure S3, Top ⇑



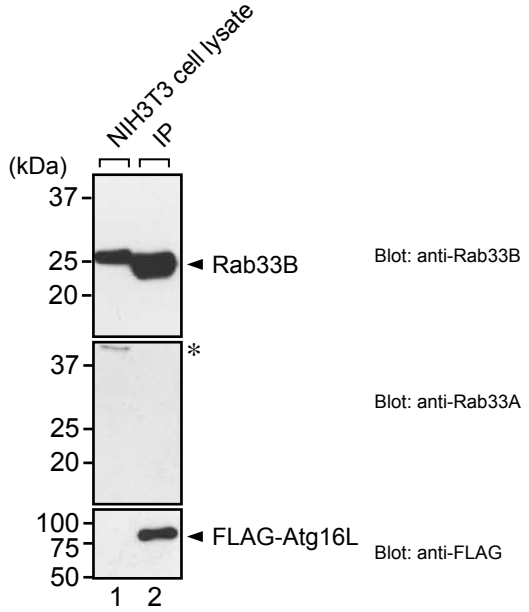
Itoh et al. Supplemental information Figure S4, Top ⇑



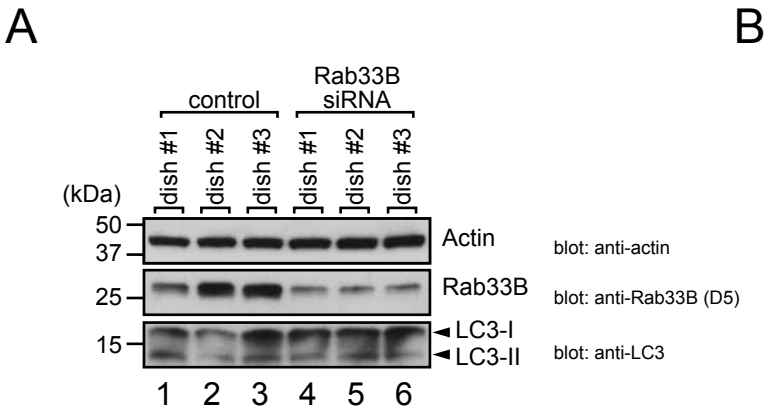
Itoh et al. Supplemental information Figure S5, Top ⇑

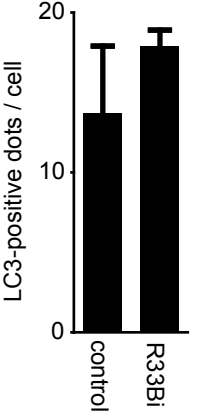


Itoh et al. Supplemental information Figure S6, Top ⇑



Itoh et al. Supplemental information Figure S7, Top ⇑





Itoh et al. Supplemental information Figure S8, Top ⇑