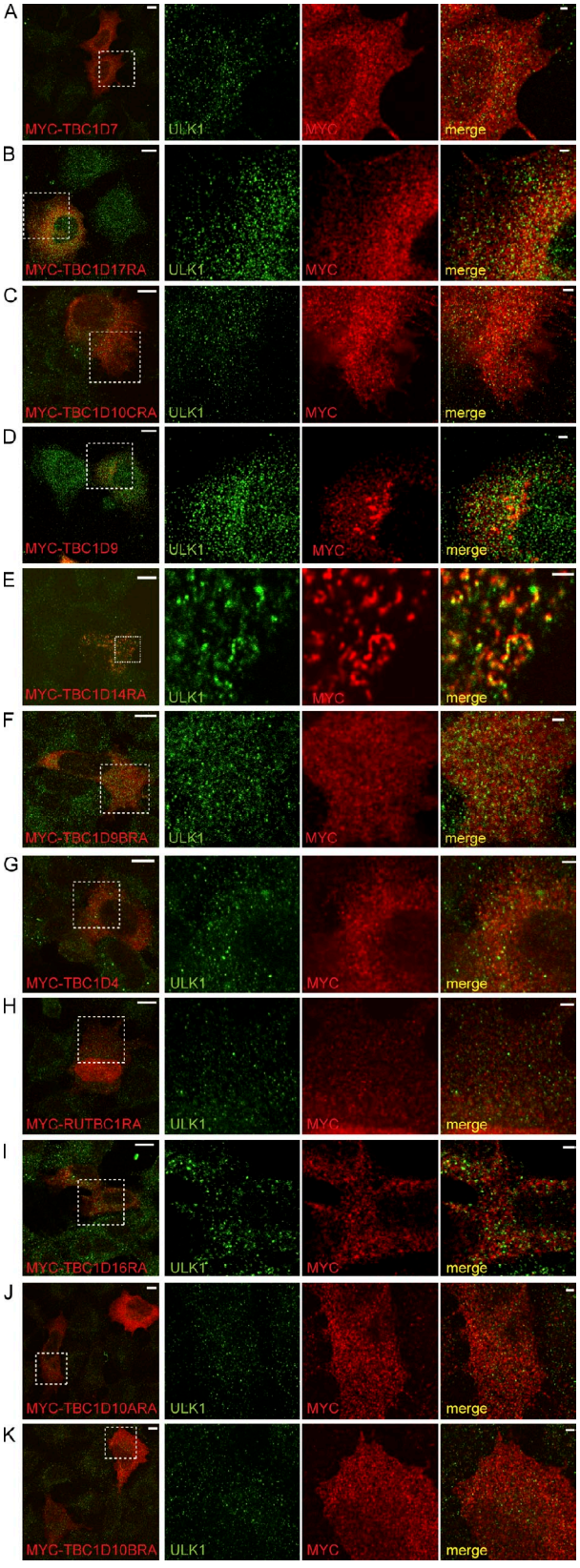
Longatti et al., http://www.jcb.org/cgi/content/full/jcb.201111079/DC1

Figure S1. **Screening candidates from the RabGAP screen for colocalization with ULK1.** (A–K) HEK293A cells were transfected with the indicated TBC protein, WT, or RA mutant as indicated. After 24-h expression, cells were stained with antimyc and anti-ULK1 antibodies. Insets shown in merge images on left are shown in the right images. Images show myc-TBC1D7 (A), myc-TBC1D17R381A (B), myc-TBC1D10CR141A (C), myc-TBC1D9 (D), myc-TBC1D14R472A (E), myc-TBC1D9BR559A (F), myc-TBC1D4 (G), RUTBC1R848A (H), TBC1D16R494A (I), TBC1D10AR160A (J), and TBC1D10BR134A (K). Bars: (main images) 10 µm; (merge) 2 µm.



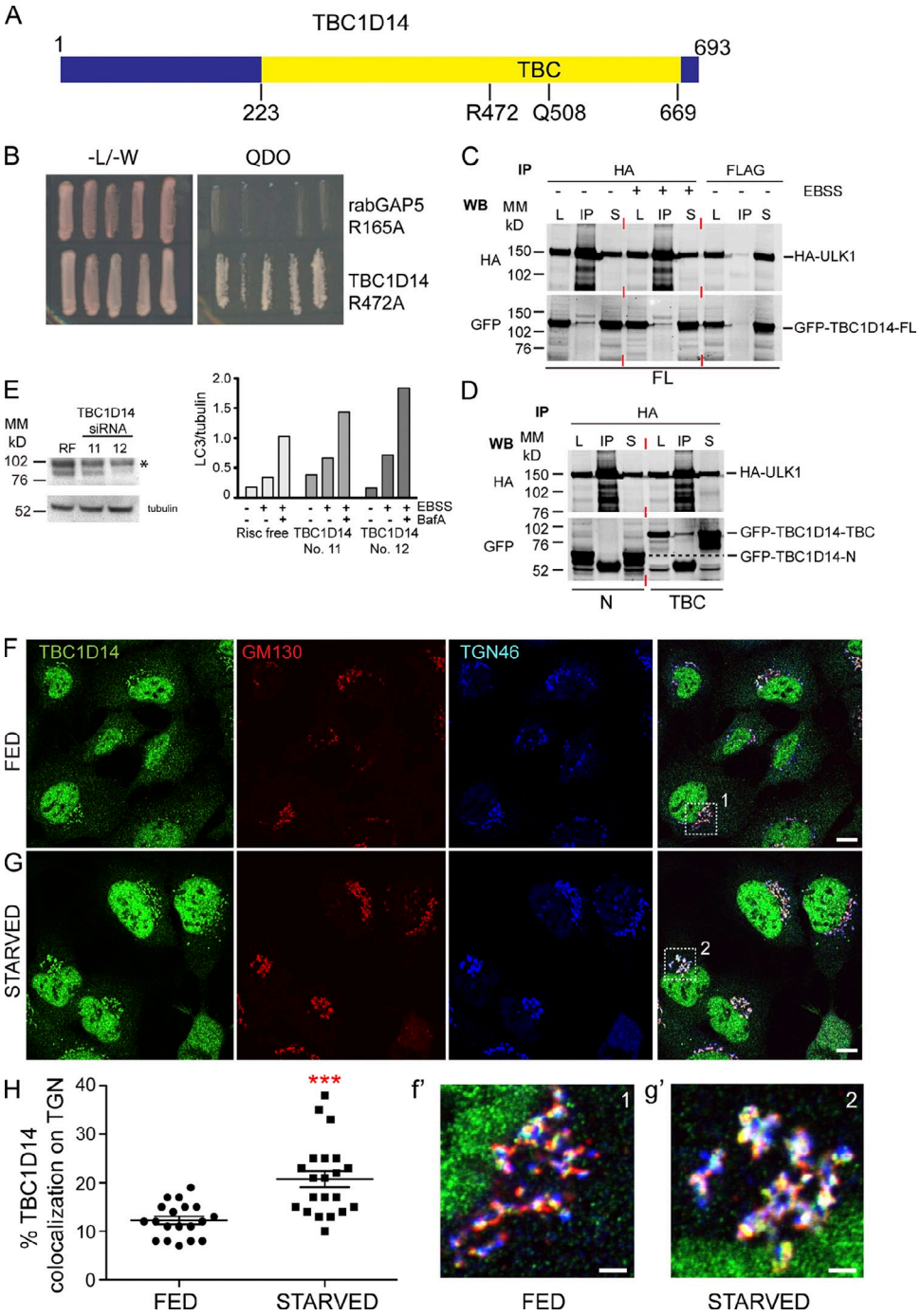


Figure S2. **TBC1D14 binds ULK1 directly.** (A) Domain structure of TBC1D14, showing an N terminus with no known motifs, the Tre-2/Bub2/Cdc16 (TBC) domain, and the location of the conserved arginine and glutamine residues. (B) Yeast two-hybrid assay performed as described in Fig. 4 A. ULK1 (bait) binds directly to TBC1D14 R472A but not to an unrelated GAP RabGAP5 R165A (prey). −L/−W, plates lacking leucine and tryptophan; QDO, medium lacking leucine, tryptophan, histidine, and adenine. (C and D) HEK293A cells were transfected with HA-ULK1 and the indicated GFP-TBC1D14 construct for 24 h, and cells were fed or starved in EBSS for 2 h. Cell lysates were then immunoprecipitated with anti-HA or anti-FLAG as a control for specific immunoprecipitation. L, 3% lysate; IP, immunoprecipitated pellet; S, supernatant or unbound fraction. Western blots were stained with antibodies against HA and GFP using the Odyssey system. (C) Full-length (FL) GFP-TBC1D14 coimmunoprecipitated with HA-ULK1 in both fed and starved conditions. No HA- or GFP-tagged protein was immunoprecipitated when the irrelevant antibody, anti-FLAG, was used. (D) The GFP-tagged C-terminal TBC domain (aa 224–669), but not the GFP-tagged N terminus (N; aa 1–223), of TBC1D14 binds to HA-ULK1. Red lines separate groups of load, immunoprecipitation, and supernatant. (E) siRNA depletion of TBC1D14 with oligonucleotides 11 and 12 for 24 h. (left) TBC1D14 was detected using an antibody to TBC1D14 to confirm knockdown. The asterisk indicates a nonspecific band. siRNA-treated cells were incubated for 2 h in full medium, EBSS, or EBSS with Bafilomycin A (BafA). RF, RISCfree. (right) LC3-II levels were determined by Western blotting and normalized to tubulin. The data shown are from a single experiment performed in duplicate. TBC1D14 relocates to the TGN upon starvation. (F–H) Endogenous TBC1D14 localizes to the Golgi and TGN and a peripheral punctate pool. HEK293A cells were fed (F and f′) or starved (G and g′) for 2 h in EBSS, fixed, stained with antibodies against TBC1D14, GM130, and TGN46, and imaged using confocal microscopy. Merged inset 1 is enlarged in f′, and inset 2 is enlarged in g′. (H) The Golgi/TGN pool and the extent of colocalization with TGN46 and GM130 increase upon starvation. Colocalization was measured using Imaris software. Fed (n = 19) versus starved (n = 21) cells: ***, $P < 0.0001$ by two-way unpaired t test. Note that the nuclear TBC1D14 antibody staining is nonspecific, as it does not decrease after siRNA depletion of TBC1D14. Error bars show SEM. Bars: (F and G) 10 µm; (f′ and g′) 2 µm. MM, molecular mass; WB, Western blot.

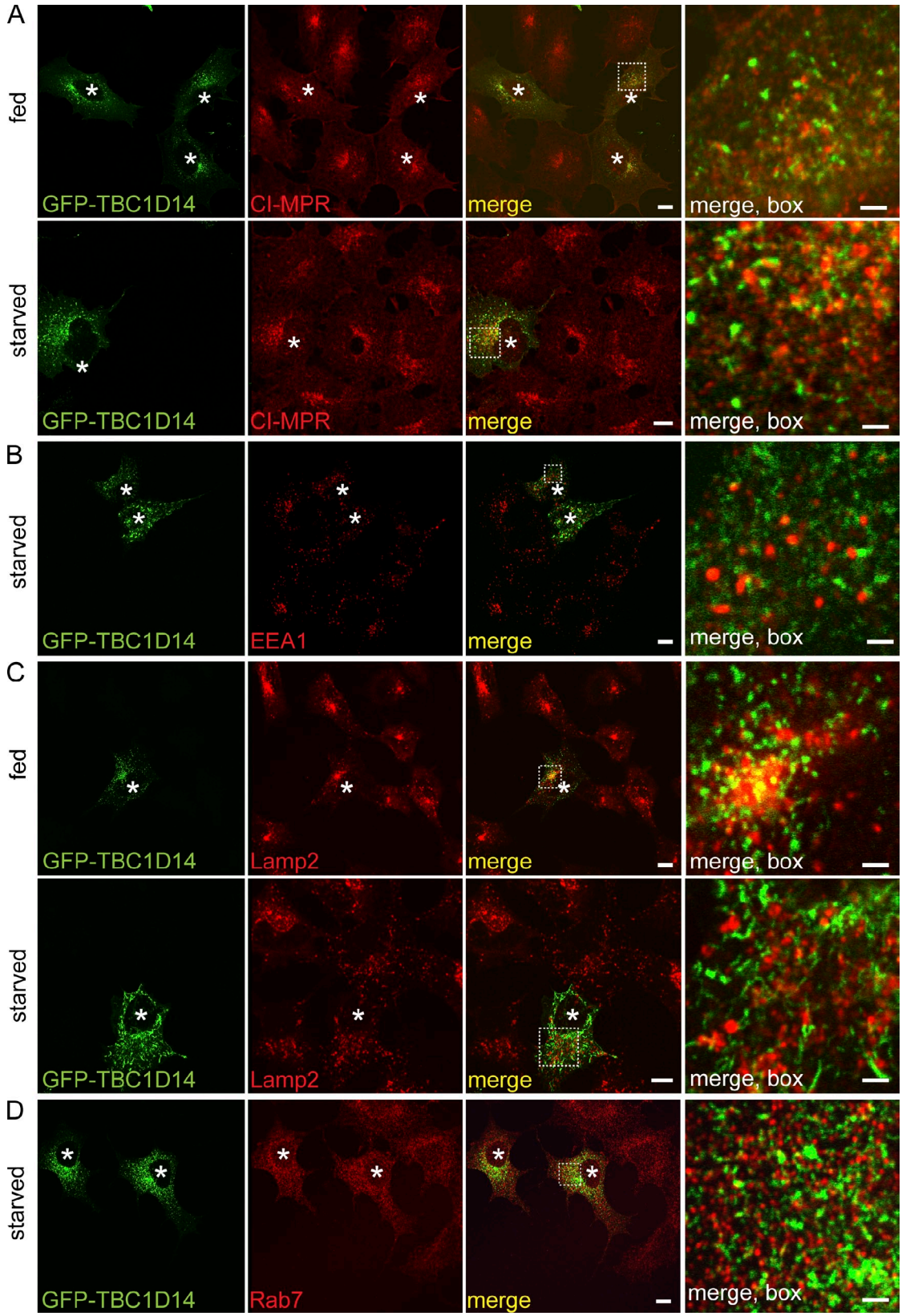


Figure S3. **TBC1D14 overexpression does not disturb other trafficking pathways.** (A–D) HEK293A cells were transfected with GFP- or myc-TBC1D14 and, 24 h later, starved for 2 h in EBSS where indicated. Where only starved images are shown, no difference in localization was observed in fed cells (not depicted). Cells were then stained with antibodies against (red) the cation-independent mannose-6-phosphate receptor (CI-MPR; A), EEA1 (early endosomal autoantigen 1; B), Lamp2 (Lysosomal-associated membrane protein 2; C), and Rab7 (D), which is found on late endosomes. Note that there is no colocalization with any of the markers shown, but early and sorting endosomal markers CI-MPR and EEA1 are often found juxtaposed to TBC1D14 tubules (insets on the right). Asterisks indicate transfected cells, with enlarged insets on the right indicated as boxes on the merged images. Bars: (main images) 10 µm; (merge images) 2 µm.

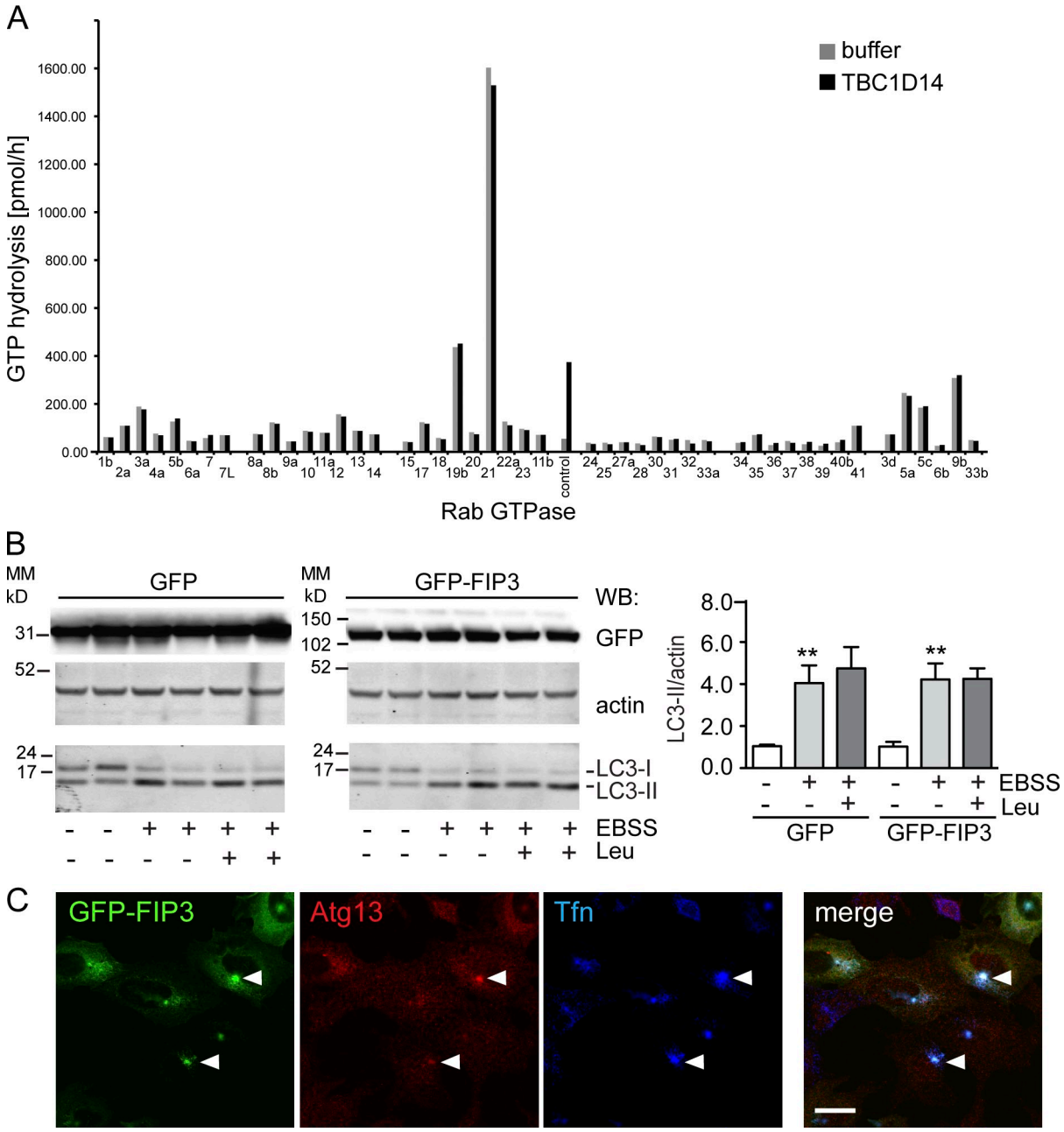


Figure S4. **In vitro GAP activity assay with TBC1D14 against the Rab proteins.** (A) Recombinant TBC1D14 protein was purified from Sf9 and High5 insect cells by the London Research Institute protein purification service. 5 µg of each Rab GTPase was loaded with γ-[^{32}P]GTP and cold GTP (1:5). 200 ng TBC1D14 or empty buffer was added to each Rab followed by 1-h incubation at 30°C. 2.5 µl of each GAP reaction was counted to allow calculation of the specific activity of each reaction. 5 µl of each GAP reaction was added to 800 µl of activated charcoal slurry and spun down, and 400 µl of the supernatant buffer containing released-free ^{32}P, as a result of GTP hydrolysis, was counted. GAP activity was calculated by dividing the amount of GTP hydrolyzed by the specific activity (Yoshimura et al., 2008). Control is TBC1D20 incubated with Rab1b, which is known to be its target (Zheng et al., 1998). All reactions were performed in duplicate and shown is the mean of the two. (B) Overexpression of FIP3 does not inhibit autophagy. HEK293A cell lysates were cotransfected with either GFP or GFP-FIP3, and 24 h after, transfection cells were starved with or without leupeptin (Leu). GFP, LC3, and actin levels were detected by Western blotting and quantified. Error bars indicate SEM (n = 3). **, $P < 0.005$; result of two-way unpaired t test. (C) Atg13 is found on tubulated RE. HEK293A cells were transfected as in B, and 24 h after transfection, Alexa Fluor 647–Tfn was added to the medium and internalized for 30 min. The cells were fixed and stained with anti-Atg13. The merge image shows GFP-FIP3, Atg13, and Alexa Fluor 647–Tfn. Arrowheads indicate colocalization of FIP3, Atg13, and Tfn. Bar, 20 µm. MM, molecular mass; WB, Western blot.

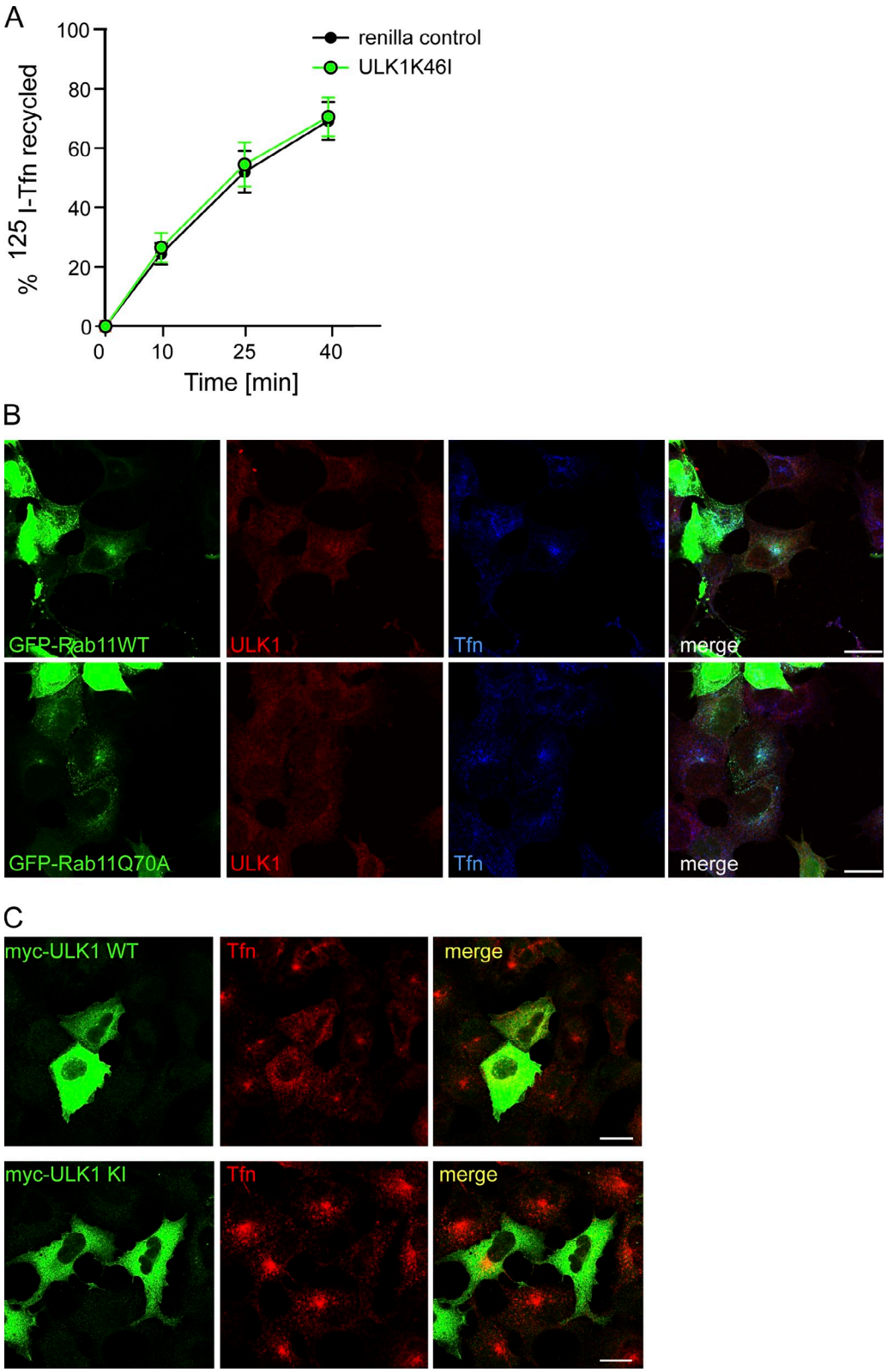


Figure S5. **Overexpression of ULK1K46I does not inhibit Tfn recycling.** (A) After 24-h expression of renilla vector (control) or myc-ULK1K46I, cells were serum starved for 2 h and allowed to internalize ^{125}I-Tfn for 20 min at 37°C. Cells were chased in serum-free medium for 10, 25, or 40 min. At each time point, the supernatant medium containing recycled ^{125}I-Tfn was removed and counted, the cells were lysed, and intracellular ^{125}I-Tfn was counted. All conditions were performed in duplicates, and the mean was used for calculations. Error bars indicate SEM (n = 3). (B) Overexpression of Rab11WT or Q70A does not affect Ulk1 localization. HEK293A cells were transfected with GFP-Rab11WT or GFP-Rab11Q70A plasmids, fixed 24 h after transfection, and stained for ULK1. (C) Overexpression of ULK1K46I does not affect distribution of Tfn. HEK293A cells were transfected with myc-ULK1WT or myc-ULK1K46I plasmids, and 24 h after transfection, Alexa Fluor 647–Tfn was added to the medium and internalized for 30 min. Note that overexpression of ULK1WT disrupts the distribution of Tfn. Bars, 20 µm. KI, kinase inactive.

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