Supporting Information

Shi et al. 10.1073/pnas.1205278109

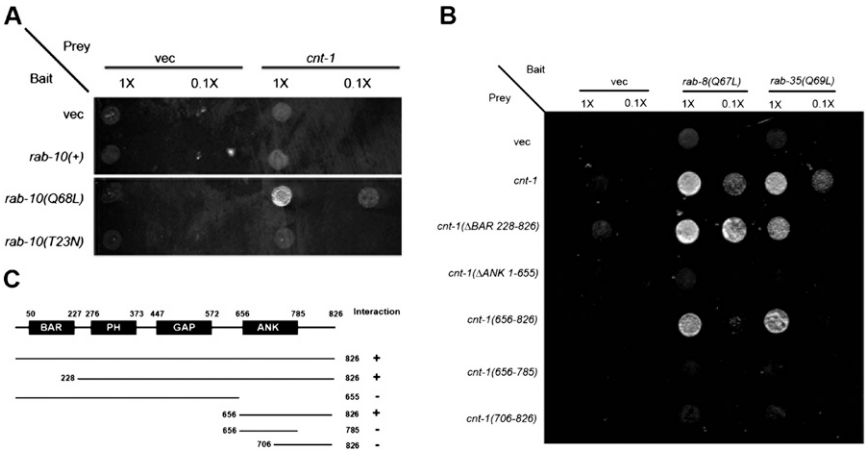

Fig. S1. (*A*) CNT-1 interacts preferentially with the GTP-bound active form of RAB-10. RAB-10, RAB-10(Q68L), and RAB-10(T23N) were expressed in a yeast reporter strain as a fusion with the DNA-binding domain of LexA (bait). CNT-1 truncated forms were expressed in the same yeast cells as fusions with the B42 transcriptional activation domain (prey). Interaction between bait and prey was assayed by complementation of leucine auxotrophy (LEU2 growth assay). Colonies were diluted in liquid and spotted on solid growth medium directly or after further 0.1× dilutions. (*B*) CNT-1 interacts physically with RAB-8(Q67L) and RAB-35(Q69L), and the interaction between CNT-1 and RAB-8(Q67L) and RAB-35(Q69L) requires the CNT-1 segment containing the C-terminal ANK repeat. RAB-8(Q67L) and RAB-35(Q69L) were expressed as bait, and CNT-1 truncated forms were expressed as prey. (*C*) Schematic representations of CNT-1 domains and the truncated fragments used in the yeast two-hybrid analysis. Protein domains [ankyrin repeat domain (ANK), bin-amphiphysin-rys domain (BAR),GTPase-activating protein domain (GAP), and pleckstrin homology domain (PH)] are shown as dark boxes above the protein sequences (shown as dark lines) used in the study. Amino acid numbers are indicated.

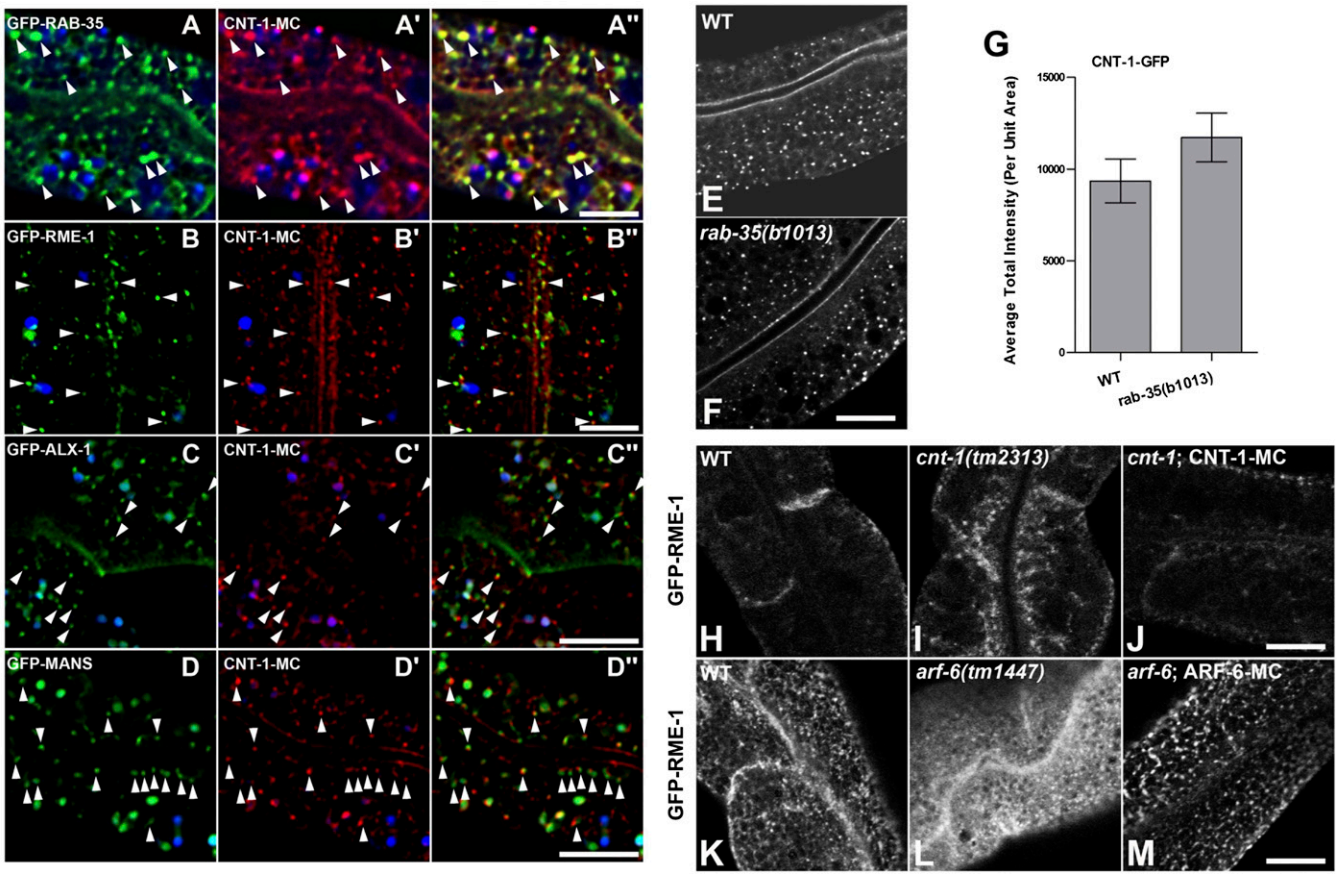

Fig. S2. (*A–D″*) Colocalization images are from deconvolved 3D image stacks acquired in intact living animals expressing GFP- and mCherry (MC)-tagged proteins specifically in intestinal epithelial cells. (*A–A″*) CNT-1-MC colocalizes with GFP-RAB-35. Arrowheads indicate endosomes labeled by both CNT-1-MC and GFP-RAB-35. (*B–B″*) CNT-1-MC partially colocalizes with recycling endosome marker GFP-RME-1. Arrowheads indicate endosomes labeled by both CNT-1-MC and GFP-RME-1. (*C–C″*) CNT-1-MC partially colocalizes with GFP-ALX-1. Arrowheads indicate CNT-1-MC–positive endosomes partially overlapping GFP-ALX-1–labeled structures. (*D–D″*) CNT-1-MC does not colocalize with Golgi marker GFP-Mannosidase (GFP-MANS). In each image autofluorescent lysosome-like organelles can be seen in all three channels with the strongest signal in blue. GFP appears only in the green channel, and mCherry appears only in the red channel. Signals observed in the green or red channels that do not overlap with signals in the blue channel are considered bone fide GFP or RFP signals, respectively. (Scale bars: 10 μm.) (*E* and *F*) Loss of RAB-35 had no effect on CNT-1-GFP localization. (Scale bar: 10 μm.) (*G*) Quantification of average total intensity of CNT-1-GFP per unit area. Error bars indicate SEM; $n = 18$; three different regions of intestine (defined by a 100 × 100 pixel box positioned at random) were sampled in six animals of each genotype. (*H–J*) GFP-RME-1 medial endosomal accumulation phenotype in *cnt-1(tm2313)* mutants can be rescued by expression of MC-tagged CNT-1 driven by the intestine-specific *vha-6* promoter. (Scale bar: 10 μm.) (*K–M*) In *arf-6(tm1447)* mutants the phenotype of decreased GFP-RME-1 labeling of basolateral tubules and puncta can be rescued by intestinal expression of MC-ARF-6. (Scale bar: 10 μm.)

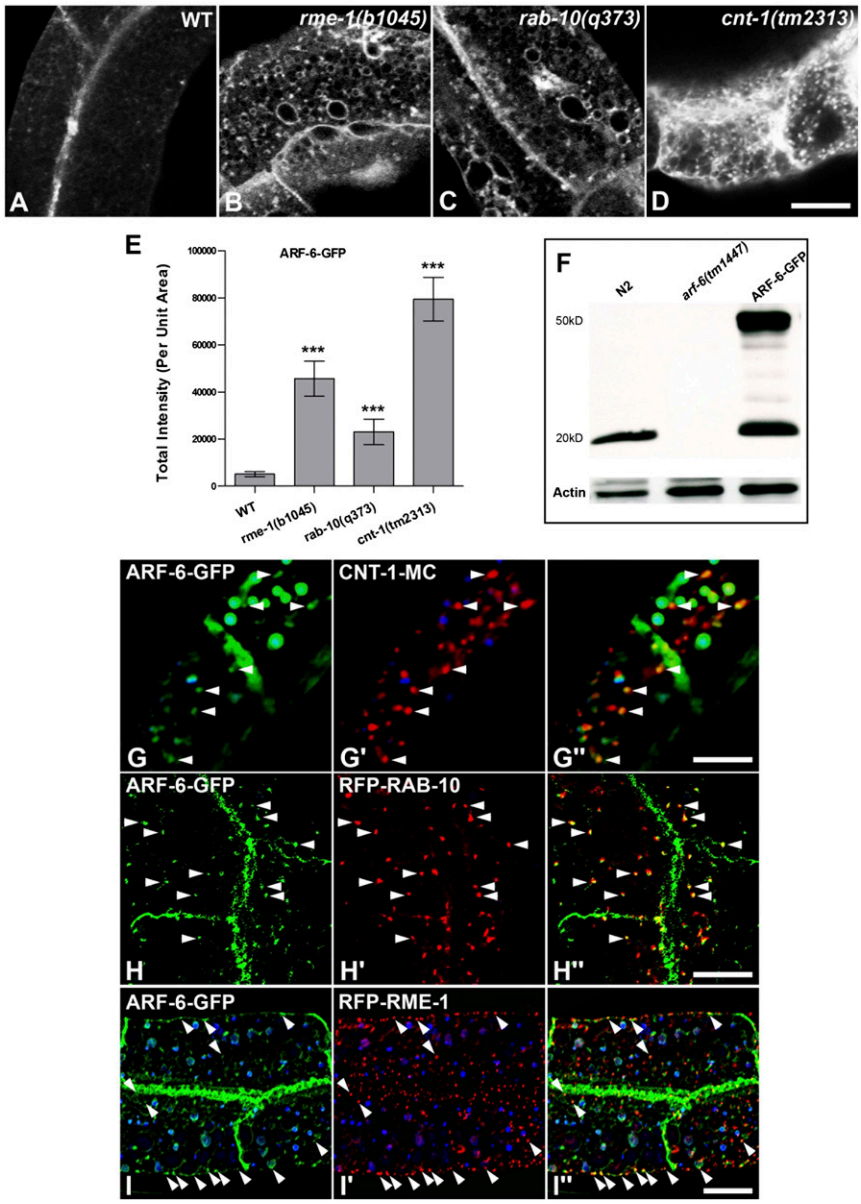

Fig. S3. (*A–E*) ARF-6-GFP labels recycling endosomes and accumulates on enlarged endosomes in recycling-defective mutants. (*A–D*) Representative confocal images are shown for ARF-6-GFP in wild-type animals (*A*) and in *rme-1(b1045)* (*B*), *rab-10(q373)* (C), and *cnt-1(tm2313)* (*D*) mutants. (*E*) Quantification of total intensity of ARf-6-GFP per unit area. (*F*) ARF-6 levels in wild-type animals and in *arf-6(tm1447)* and ARF-6-GFP–expressing transgenic animals. Protein ARF-6 was not detected in *arf-6(tm1447)*-mutant animals. (*G–I″*) Colocalization images are from deconvolved 3D image stacks acquired in intact living animals expressing fluorophore-tagged proteins specifically in intestinal epithelial cells. (*G–G″*) CNT-1-MC colocalizes with ARF-6-GFP. Arrowheads indicate endosomes labeled by both CNT-1-MC and ARF-6-GFP. (*H–H″*) ARF-6-GFP colocalizes with RFP-RAB-10. Arrowheads indicate ARF-6-GFP–positive endosomes with overlapping RFP-RAB-10–labeled puncta. (*I–I″*) Recycling endosomal marker RFP-RME-1 colocalizes with ARF-6-GFP mainly on puncta close to the basolateral membrane. Arrowheads indicate endosomes labeled by both RFP-RME-1 and ARF-6-GFP. (Scale bars: 10 μm.)

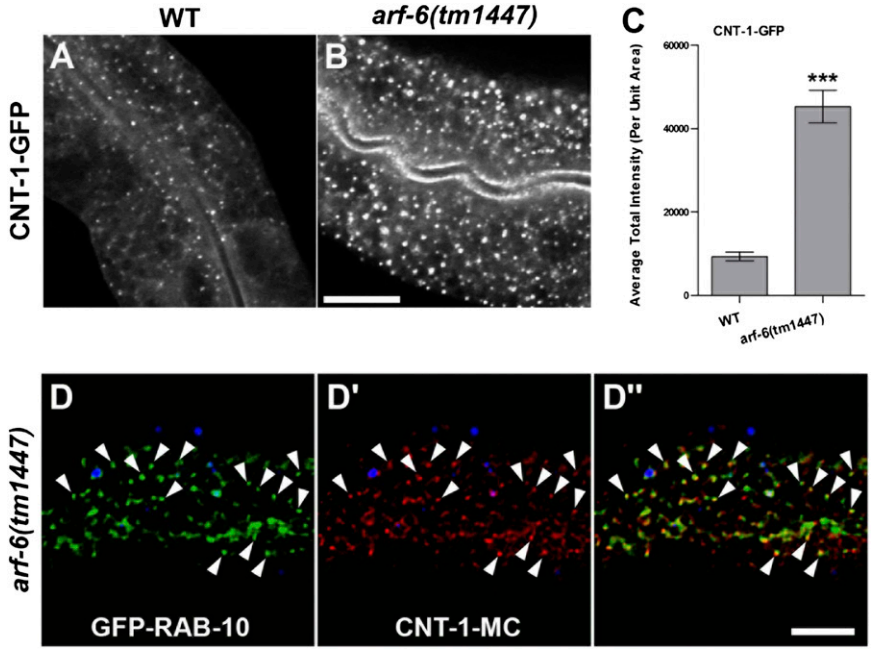

Fig. S4. (*A*–*C*) CNT-1 accumulates on endosomal structures in *arf-6* mutants. (*C*) Depletion of ARF-6 induces the accumulation of CNT-1-GFP–positive puncta, with an approximately fourfold increase in average total intensity per unit area. Error bars indicate SEM. $n = 18$; three different regions of intestine (defined by a 100 × 100 pixel box positioned at random) were sampled in six animals of each genotype. ***$P < 0.001$, one-tailed Student's t test. (Scale bar: 10 μm.) (*D*–*D*″) RAB-10 and CNT-1 coaccumulate on endosomes in *arf-6* mutants. Arrowheads indicate endosomes labeled by both CNT-1-MC and GFP-RAB-10. (Scale bar: 10 μm.)

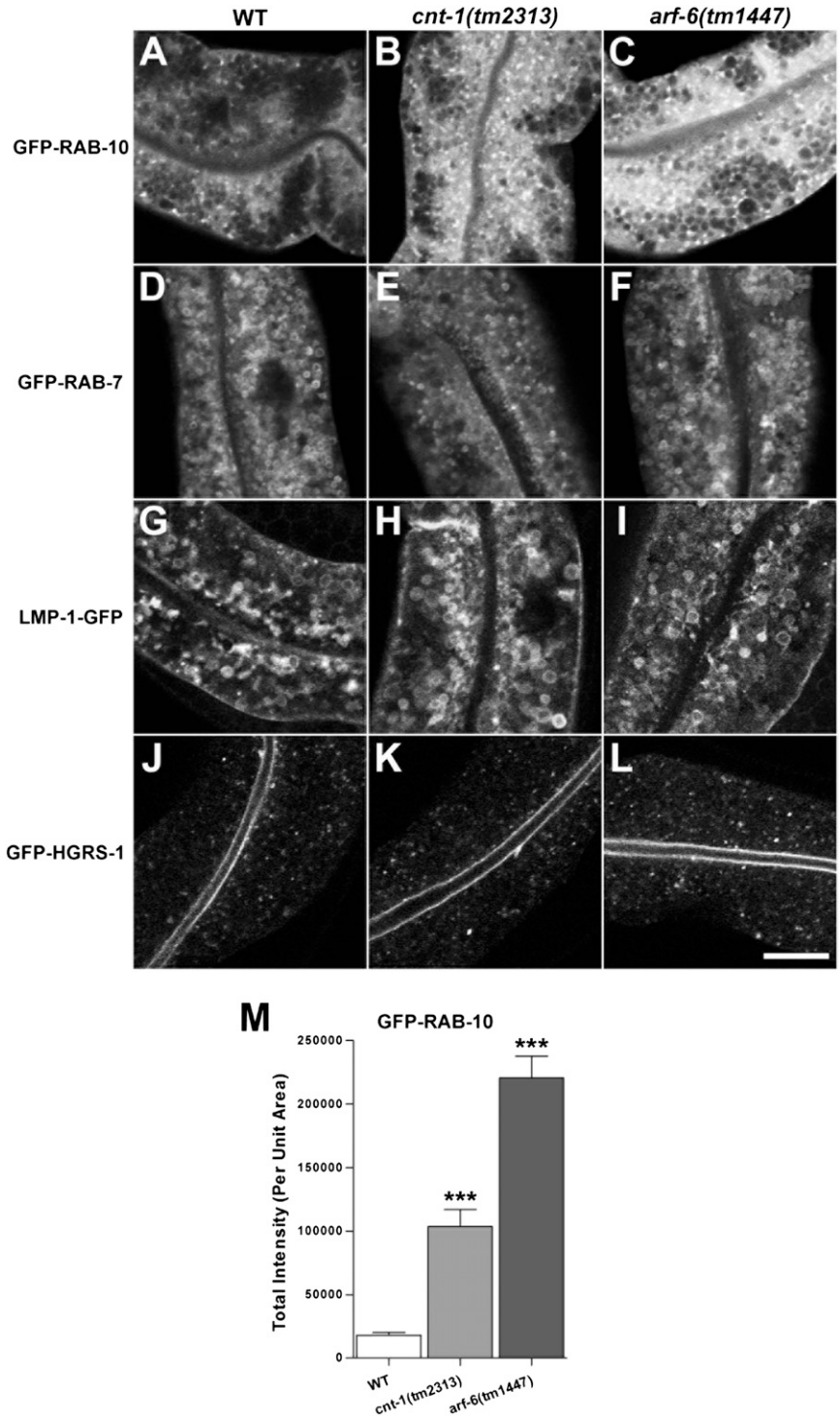

Fig. S5. (*A*–C) GFP-RAB-10–labeling of endosomes increases in *cnt-1*– and *arf-6*–mutant intestinal cells. (*D*–*F*) GFP-RAB-7–labeled late endosomes were not altered detectably in *cnt-1*– and *arf-6*–mutant intestinal cells. (*G*–*I*) LMP-1-GFP–labeled late endosomes were not affected in *cnt-1*– and *arf-6*–mutant intestinal cells. (*J*–*L*) The multivesicular endosome/multivesicular body marker HGRS-1/Hrs appears normal in *cnt-1* and *arf-6* mutants. (Scale bar: 10 μm.) (*M*) Quantification of total intensity per unit area of GFP-labeled puncta. Error bars indicate SEM. $n = 18$; three different regions of each intestine (defined by a 100 × 100 pixel box positioned at random) were sampled in six animals of each genotype. ***$P < 0.001$ one-tailed Student's t test.

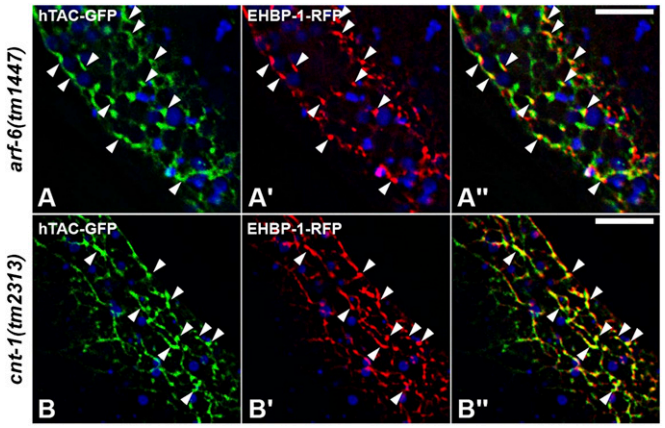

Fig. S6. (*A*–*A*′′) hTAC-GFP coaccumulates with EHBP-1-RFP on endosomes in *arf-6* mutants. Arrowheads indicate endosomes labeled by both hTAC-GFP and EHBP-1-RFP. (*B*–*B*′′) hTAC-GFP coaccumulates with EHBP-1-RFP on endosomes in *cnt-1* mutants. (Scale bar: 10 μm.)

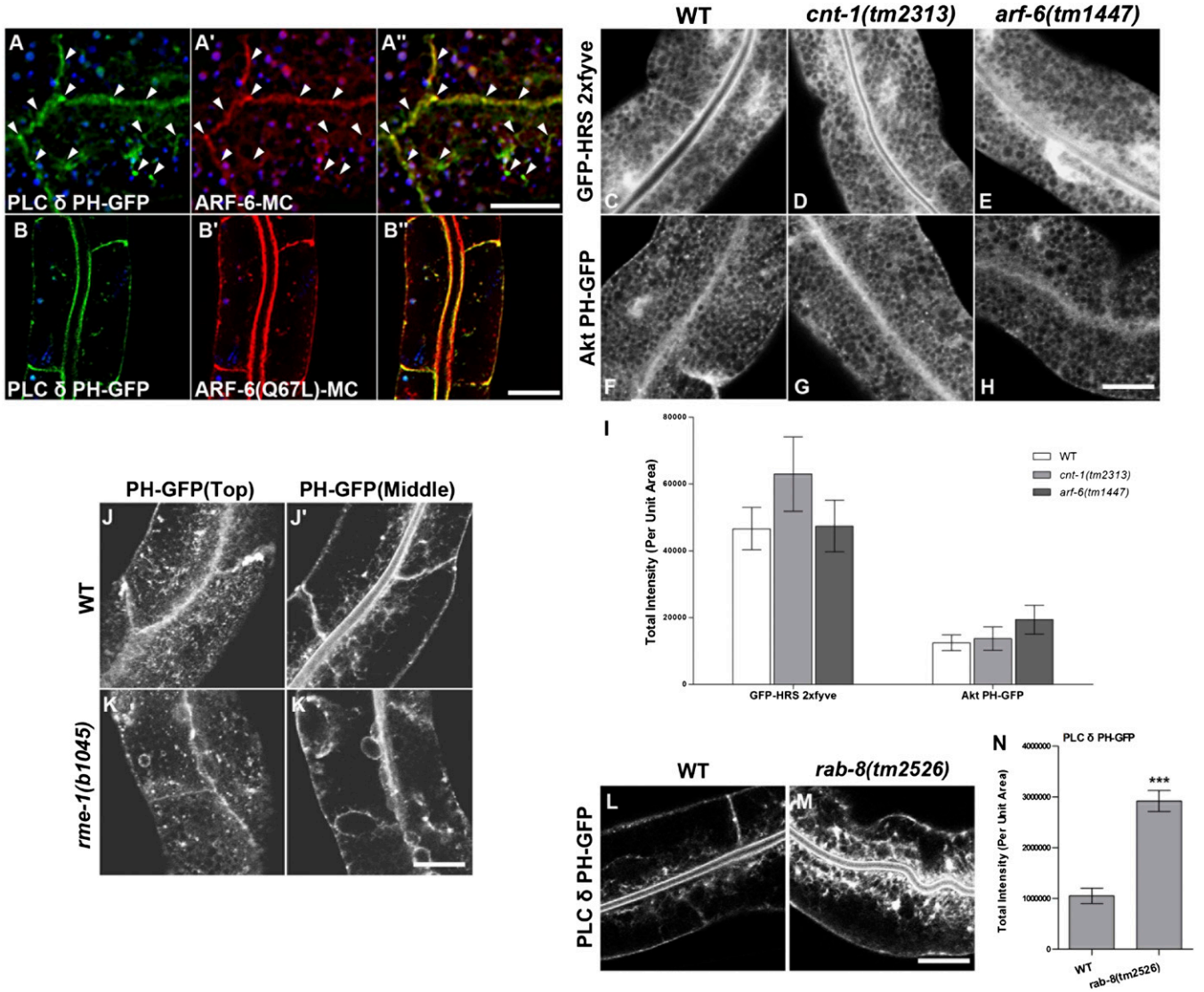

Fig. S7. (*A*–*A*′′′) ARF-6 colocalizes with PH-GFP on basolateral tubules and puncta. Arrowheads indicate tubules and puncta labeled by both ARF-6-MC and PH-GFP. (Scale bar: 10 μm.) (*B*–*B*′′) ARF-6(Q67L) colocalizes with PH-GFP on apical and lateral plasma membranes. (Scale bar: 10 μm.) (*C*–*I*) Levels of PI(3)P (labeled by GFP-2xFYVE) and PI(3,4,5)P3 [labeled by PH(Akt)-GFP] were unperturbed in *cnt-1* and *arf-6* mutants. (Scale bar: 10 μm.) Error bars in *I* indicate SEM. $n = 18$; three different regions of intestine (defined by a 100 × 100 pixel box positioned at random) were sampled in six animals of each genotype. (*J*–*K*′′) PH(PLCδ)-GFP labeling was not altered detectably in *rme-1* mutants. Some of the endosomes labeled by PH(PLCδ)-GFP were grossly enlarged in *rme-1* mutants. (Scale bar: 10 μm.) (*L*–*M*) Loss of RAB-8 causes significant cytosolic accumulation of PH-GFP. (Scale bar: 10 μm.) (*N*) Quantification of total intensity of PH-GFP per unit area. Error bars indicate SEM. $n = 18$; three different regions of each intestine (defined by a 100 × 100 pixel box positioned at random) were sampled in six animals of each genotype.

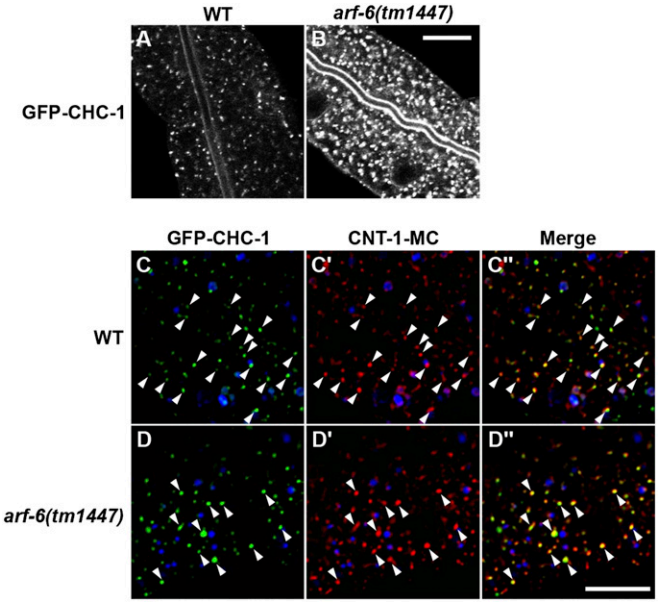

Fig. S8. Clathrin accumulates and overlaps with CNT-1 on enlarged endosomes in *arf-6* mutants. (*A* and *B*) Representative confocal images are shown for GFP-tagged clathrin heavy chain (GFP-CHC-1) in wild-type animals and *arf-6(tm1447)* mutants. (Scale bar: 10 μm.) (*C–D″*) Colocalization images of CHC-1 and CNT-1 in wild-type and *arf-6*–mutant backgrounds are from deconvolved 3D image stacks acquired in intact living animals expressing GFP- and mCherry-tagged proteins specifically in intestinal epithelial cells. (*C–C″*) CNT-1-MC colocalizes with GFP-CHC-1 in wild-type animals. Arrowheads indicate representative endosomes labeled by both CNT-1-MC and GFP-CHC-1. (*D–D″*) CNT-1-MC colocalizes with GFP-CHC-1 on enlarged endosomal structures in *arf-6* mutants. Arrowheads indicate enlarged puncta labeled by both CNT-1-MC and GFP-CHC-1. In each image autofluorescent lysosome-like organelles can be seen in all three channels with the strongest signal in blue. (Scale bar: 10 μm.)

Table S1. Transgenic and mutant strains used in this study

pwIs724[pvha6::CNT-1::GFP]
pwIs728[pvha6::CNT-1::mCherry]
pwIs601[pvha6::ARF-6::GFP]
pwIs206[pvha6::GFP::RAB-10] (1)
pwIs72[pvha6::GFP::RAB-5] (1)
pwIs87[pvha6::GFP::RME-1] (1)
pwIs524[pvha6::GFP::ALX-1] (2)
pwIs481[pvha6::MANS::GFP] (1)
pwIs112[pvha6::hTAC::GFP] (1)
pwIs90[pvha6::hTfR::GFP] (1)
pwIs722[pvha6::SDPN-1::GFP] (3)
pwIs170[pvha6::GFP::RAB-7] (1)
pwIs50[plmp-1::LMP-1::GFP] (4)
pwIs518[pvha6::GFP::HGRS-1] (2)
pwIs446[pvha6::PH::GFP]
pwIs140[pvha6::GFP::2xFYVE]
pwIs890[pvha6::Akt-PH::GFP]
dkIs8 [pvha6::GFP::CHC-1] (5)
pwIs68[pvha6::GFP::RAB-8]
pwIs625[pvha6::ARF-6::mCherry]
rme-1(b1045) (6)
rab-10(q373) (1)
alx-1 (gk275) (2)
*rab-10(ok1494)**
arf-6(tm1447)[†]
cnt-1(tm2313)[†]

**C. elegans* Gene Knockout Consortium.
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1. Chen CC, et al. (2006) RAB-10 is required for endocytic recycling in the Caenorhabditis elegans intestine. *Mol Biol Cell* 17:1286–1297.
2. Shi A, et al. (2007) A novel requirement for C. elegans Alix/ALX-1 in RME-1-mediated membrane transport. *Curr Biol* 17:1913–1924.
3. Pant S, et al. (2009) AMPH-1/Amphiphysin/Bin1 functions with RME-1/Ehd1 in endocytic recycling. *Nat Cell Biol* 11:1399–1410.
4. Treusch S, et al. (2004) Caenorhabditis elegans functional orthologue of human protein h-mucolipin-1 is required for lysosome biogenesis. *Proc Natl Acad Sci USA* 101:4483–4488.
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6. Grant B, et al. (2001) Evidence that RME-1, a conserved C. elegans EH-domain protein, functions in endocytic recycling. *Nat Cell Biol* 3:573–579.