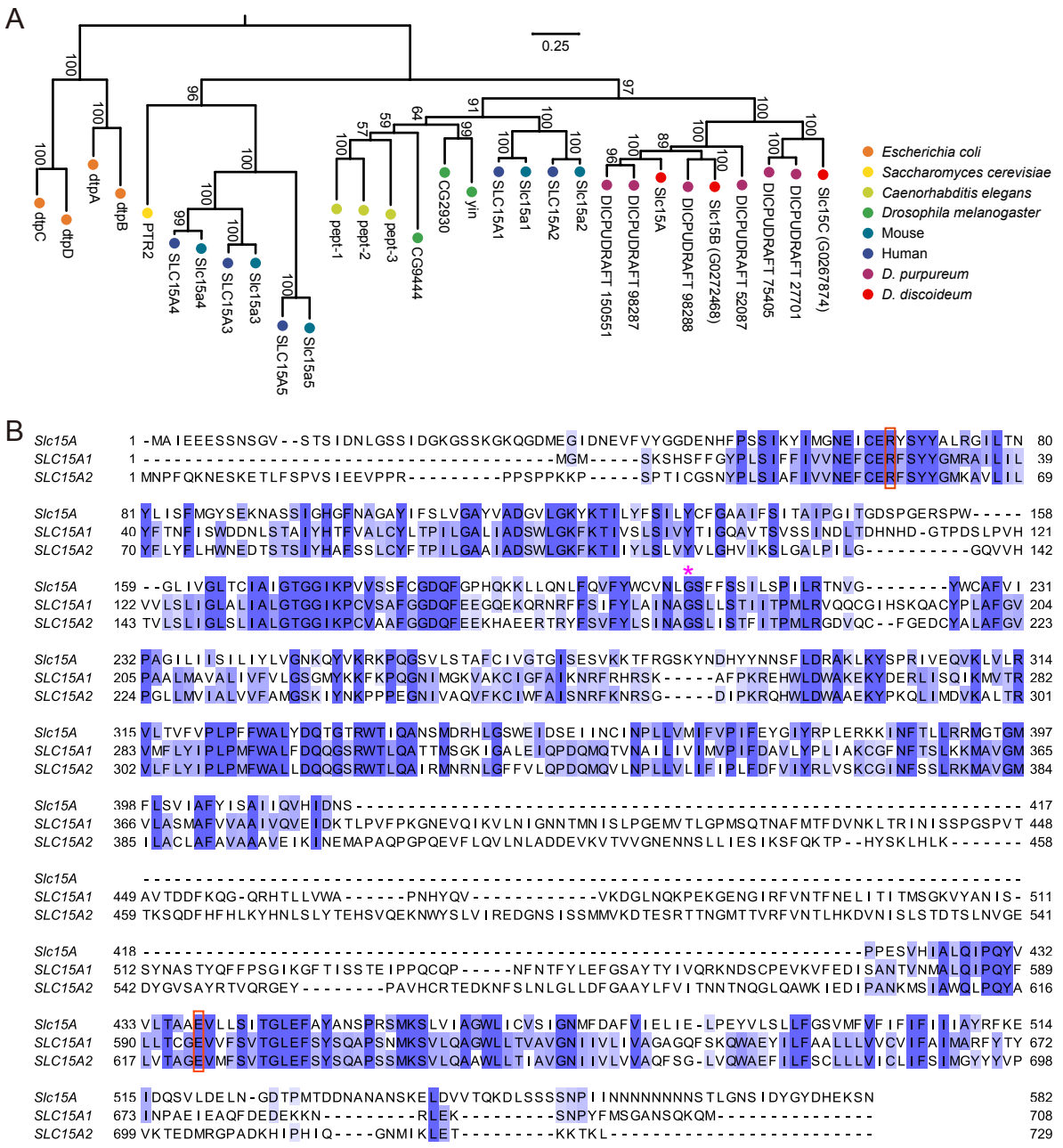


Fig. S1. Conservation of SLC15 family proteins. (A) Phylogenetic tree of the SLC15 family. Shown is a maximum likelihood tree built with LG+F+G4 model. Bootstrap values are from 1,000 iterations. In addition to Slc15A, *Dictyostelium* genome encodes two other proteins that are homologous to human SLC15 proteins. We named them Slc15B (DDB_G0272468) and Slc15C (DDB_G0267874). (B) Sequence alignment of Slc15A with human SLC15A1 and SLC15A2. Red boxes mark the two conserved residues, R68 and E438, in Slc15A. Mutation of the corresponding amino acid, R57H in SLC15A2 or E595R in SLC15A1, was shown to abolish the oligopeptide transport function of the protein. The magenta asterisk marks the position of REMI insertion.

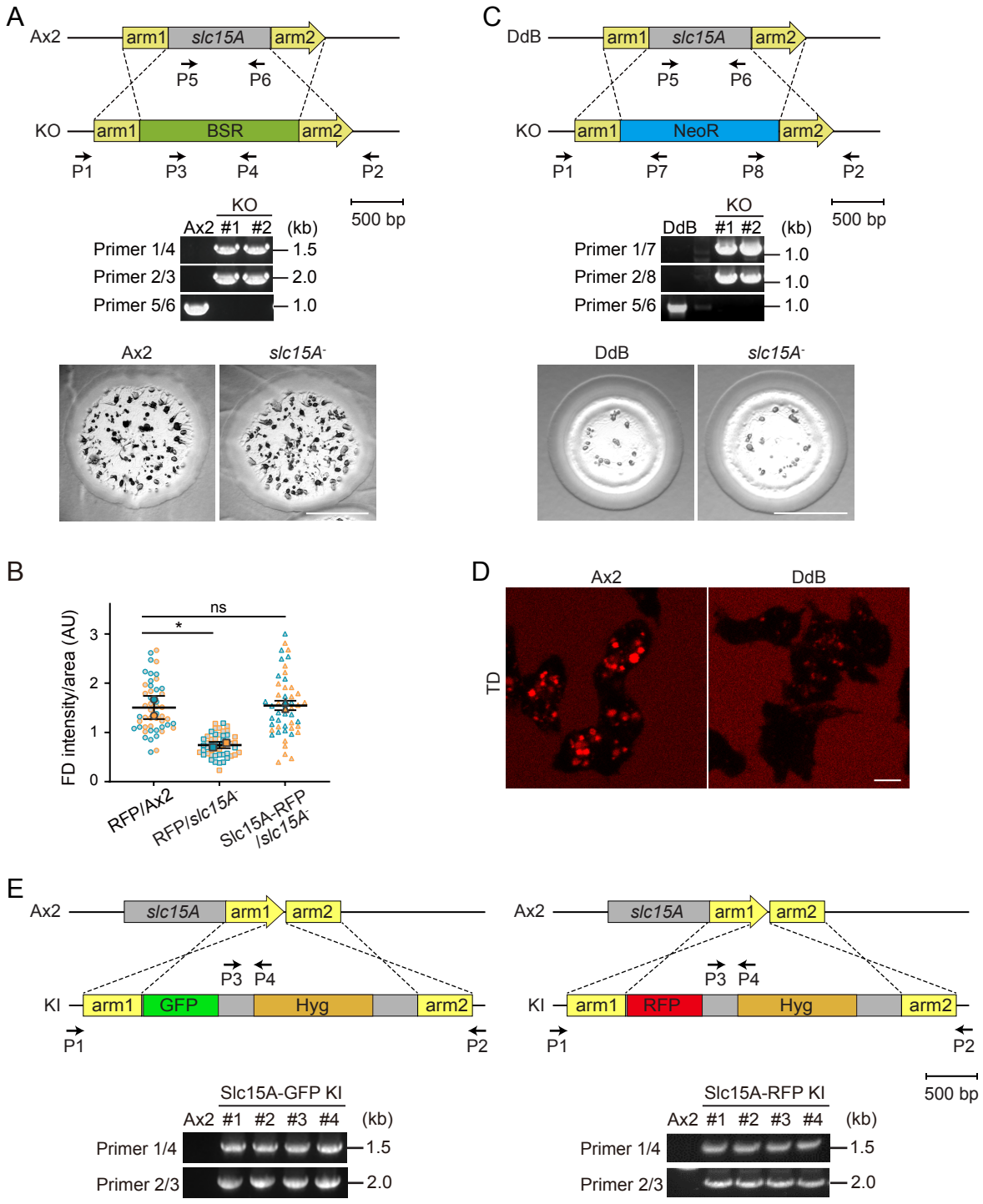


Fig. S2. Generation of *slc15A* deletion and knock-in cells. (A) Generation of *slc15A*$^-$ cell line in the Ax2 background. Top: A blasticidin resistant cassette (BSR) was used to replace part of the open reading frame of *slc15A*, and targeted clones were verified by PCR. Bottom: Ax2 and *slc15A*$^-$ cells were plated clonally with *K. aerogenes* on standard medium agar. Representative images were captured after 5 d. Scale bar, 5 mm. (B) Quantification of FD uptake in Ax2 and *slc15A*$^-$ cells expressing RFP or Slc15A-RFP. Data are from two independent experiments with at least 20 cells quantified per experiment (each experiment is shown in a different color). Mean ± SD. Statistical significance was determined by one-way ANOVA with Dunnett post-test. (C) Generation of *slc15A*$^-$ cell line in the DdB background. Top: A neomycin resistant cassette (NeoR) was used to replace part of the open reading frame of *slc15A*, and targeted clones were verified by PCR. Bottom: DdB and *slc15A*$^-$ cells were plated clonally with *K. aerogenes* on standard medium agar. Representative images were captured after 4 d. Scale bar, 5 mm. (D) Confocal images of TD uptake in Ax2 and DdB cells. Scale bar, 5 µm. (E) Design of knock-in constructs. Slc15A-GFP^{KI} and Slc14A-RFP^{KI} cells were generated by targeted in-frame integration of GFP or RPF at the C-terminus of Slc15A. Targeted clones were verified by PCR.

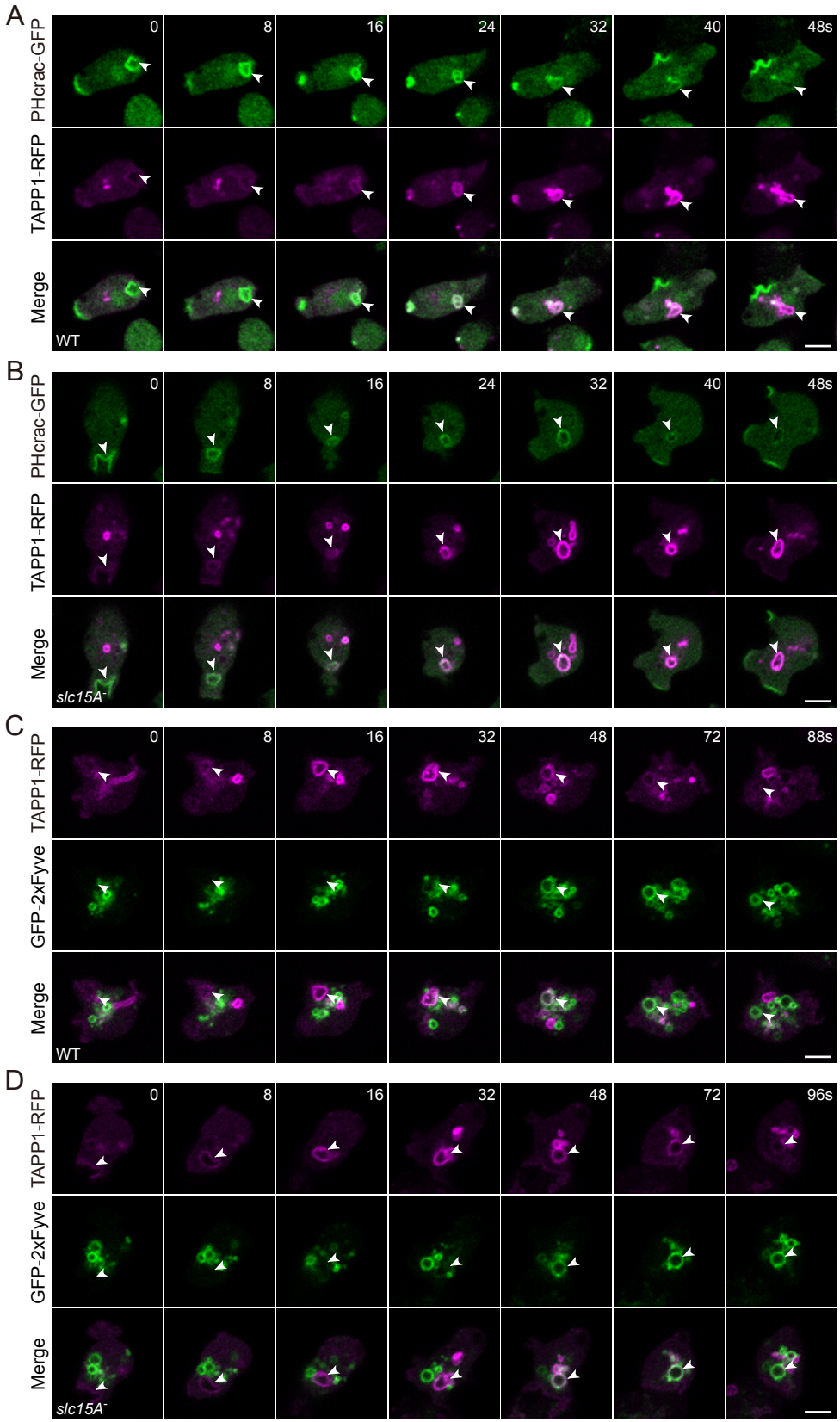


Fig. S3. PIP conversion during macropinocytosis in Ax2 and *slc15A*⁻ cells. (A-B) Time-lapse images showing the sequential accumulation of PHcrac-GFP and TAPP1-RFP in Ax2 and *slc15A*⁻ cells. (C-D) Time-lapse images showing the sequential accumulation of TAPP1-RFP and GFP-2xFYVE in Ax2 and *slc15A*⁻ cells. Scale bars, 5 µm.

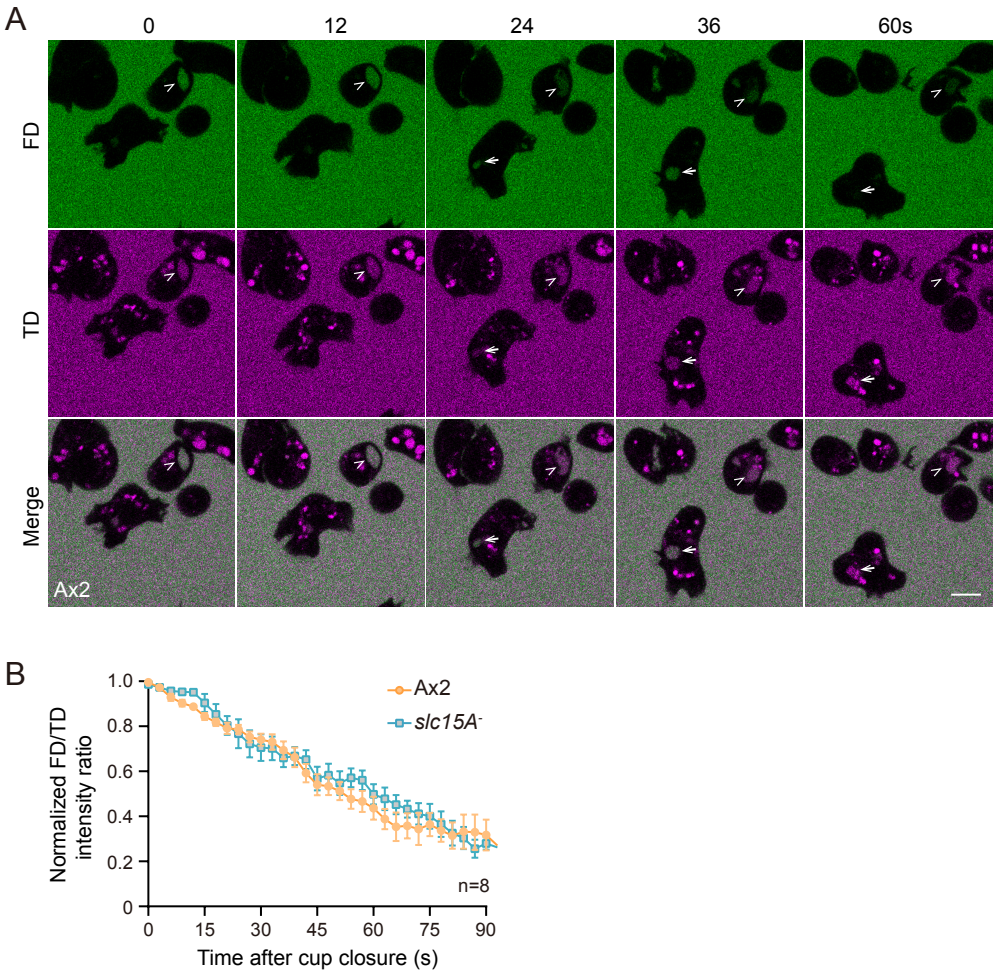


Fig. S4. Macropinosome acidification in Ax2 and *slc15A*⁻ cells. (A) Time-lapse images of Ax2 cells incubated with 1 mg/ml FD and 0.5 mg/ml TD. Arrows mark newly formed macropinosomes that quickly acquired an acidic environment indicated by the decrease of the fluorescence of FD. (B) Macropinosome acidification quantified by measuring the ratio of FD/TD as a function of time after cup closure in Ax2 and *slc15A*⁻ cells. Scale bar, 5 µm.

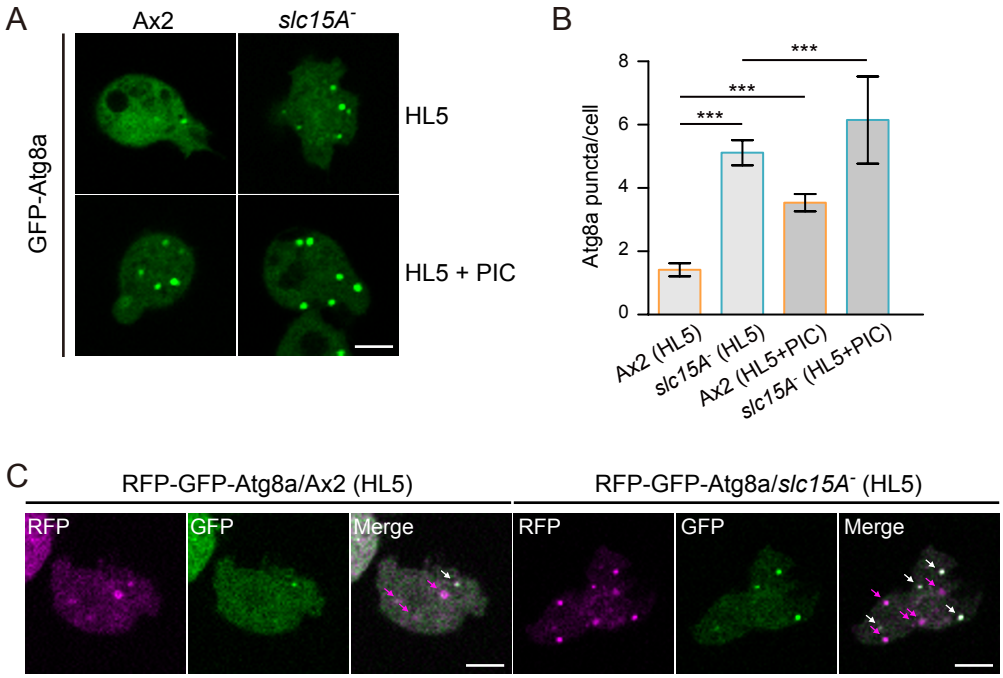


Fig. S5. Autophagy flux in Ax2 and *slc15A*⁻ cells. (A) Confocal images of GFP-Atg8a. Cells were cultured in HL5 in the absence or presence of a protease inhibitor cocktail (PIC). (B) Quantification of average Atg8a puncta per cell. At least 25 cells were quantified per condition. Mean ± SD. The addition of PIC further increased the number of GFP-Atg8a puncta in *slc15A*⁻ cells, indicating that autophagy flux was not blocked. (C) Confocal images of Atg8a fused to a tandem RFP-GFP tag. The presence of magenta-only puncta marked by the magenta arrows indicates that autophagy flux was not blocked in *slc15A*⁻ cells. White arrows point to Atg8a structures with both GFP and RFP signals. Scale bars, 5 µm.

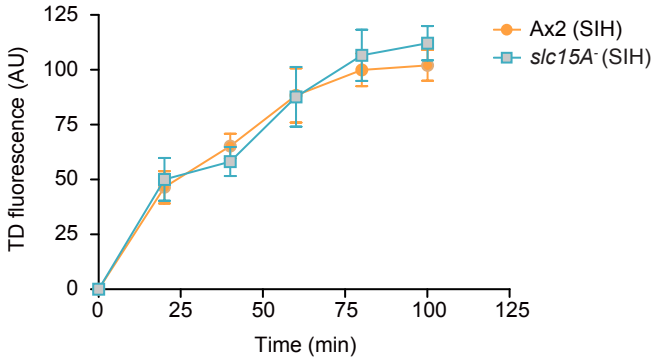


Fig. S6. Macropinocytosis in cells grown in SIH medium. Quantification of TD uptake in Ax2 and *slc15A*$^{-}$ cells by fluorimetric analysis. Data are from four independent experiments. Mean ± SD.

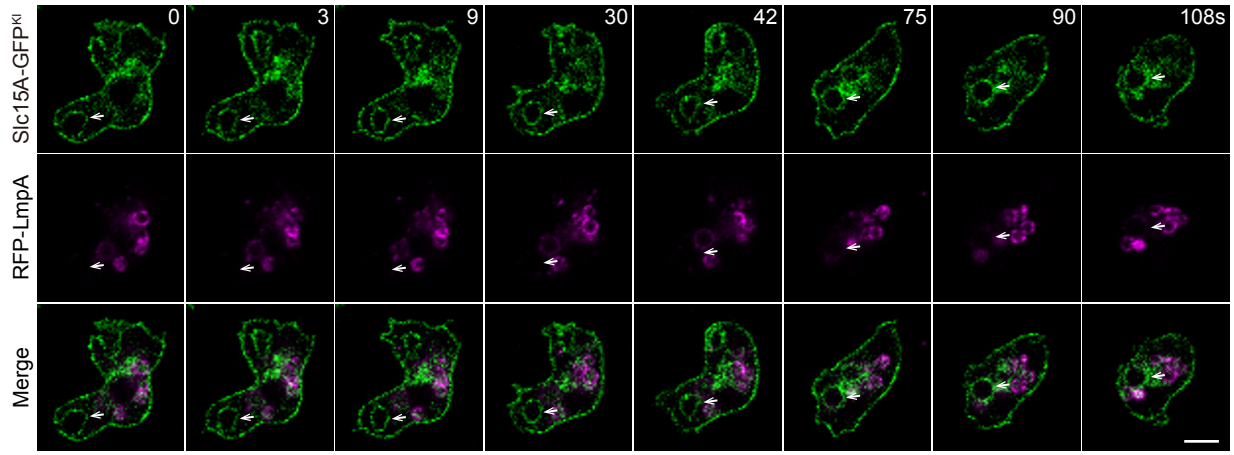


Fig. S7. Slc15A does not colocalize with the lysosomal/postlysosomal marker LmpA. Time-lapse images of Slc15A-GFP^{KI} and RFP-LmpA. The arrows point to a newly generated macropinosome marked by Slc15A. Scale bar, 5 µm.

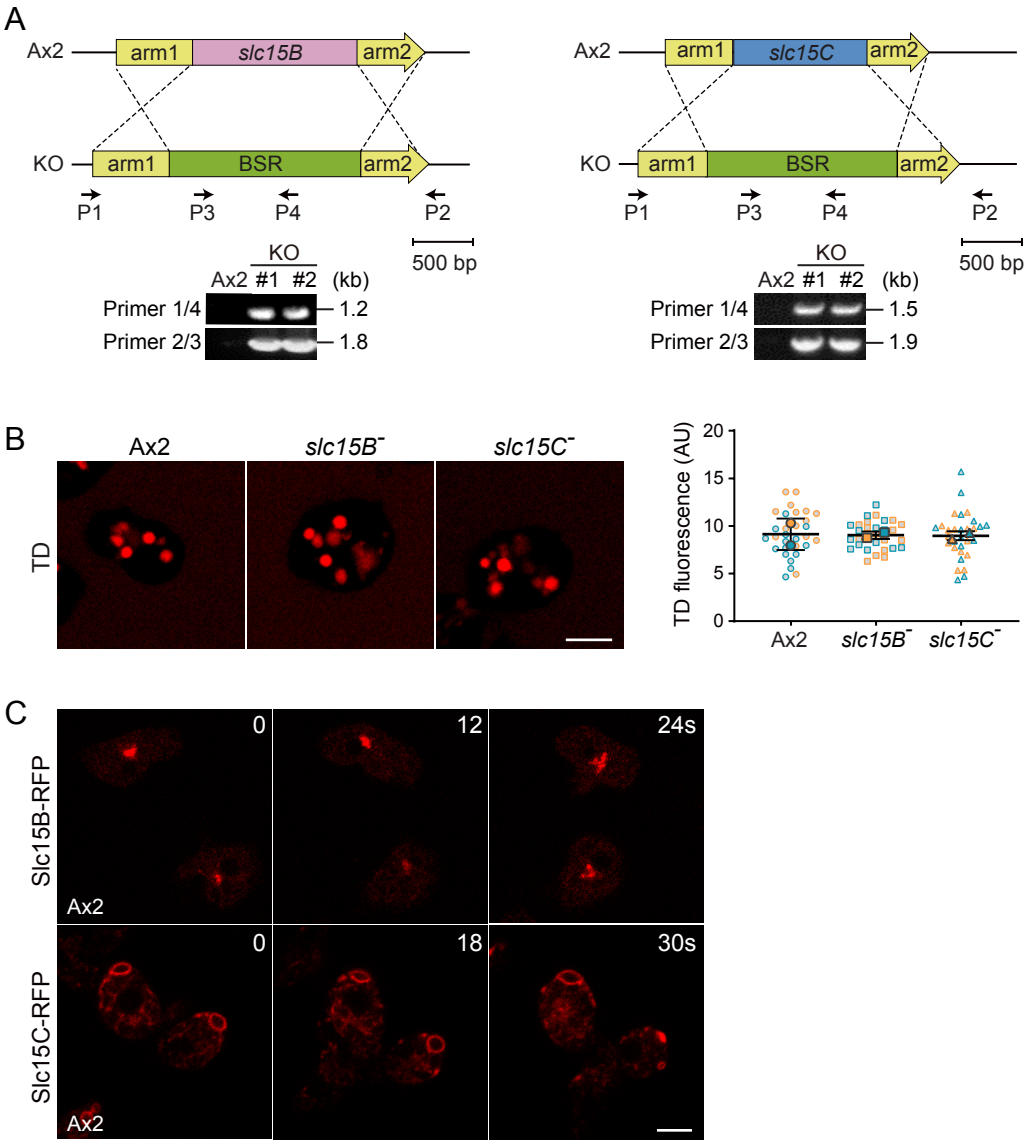
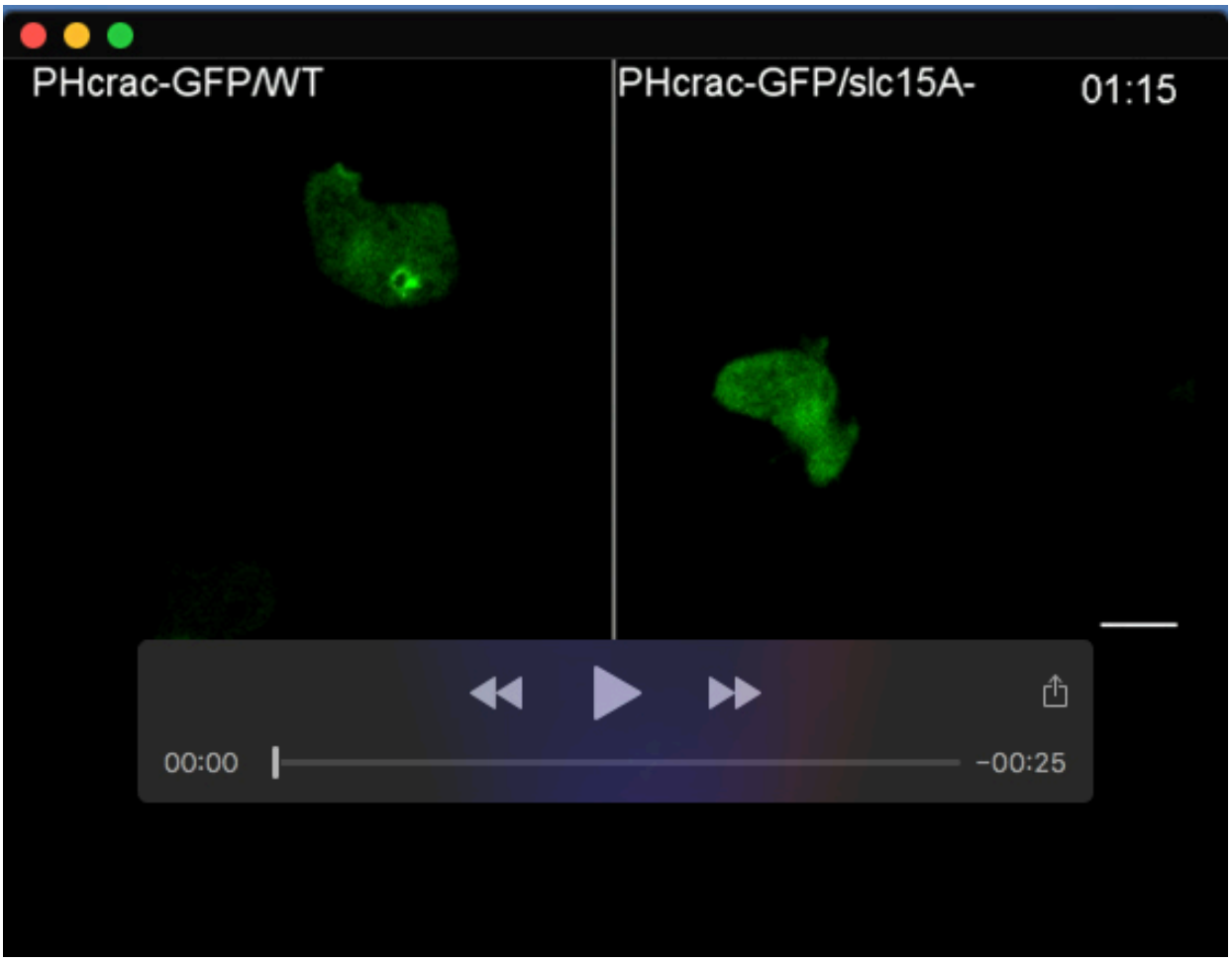


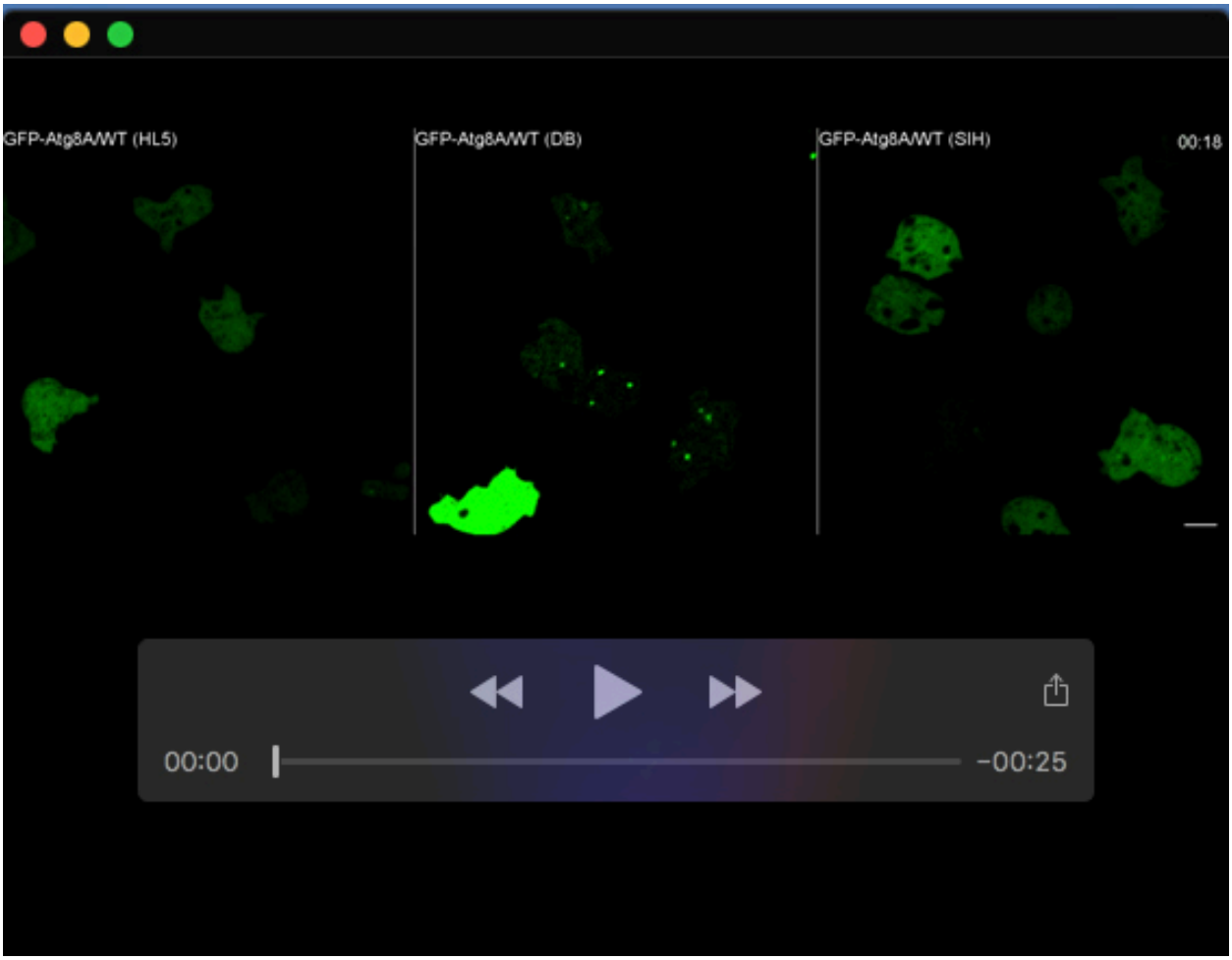
Fig. S8. Characterization of Slc15B and Slc15C. (A) Design of knockout constructs. The BSR was inserted via homologous recombination to replace part of the open reading frame of *slc15B* or *slc15C*. Targeted clones were verified by PCR. (B) Confocal images and quantification of TD uptake in Ax2, *slc15B*$^{-}$, and *slc15C*$^{-}$ cells. Data are from two independent experiments with at least 15 cells quantified per experiment (each experiment is shown in a different color). Mean ± SD. (C) Time-lapse images of Slc15B-RFP and Slc15C-RFP expressed in Ax2 cells. Slc15B localizes to the Golgi and Slc15C localizes to the contractile vacuole structure. Scale bars, 5 µm.

Table S1. Plasmids and primers used in this study. Each primer is designated as forward (F) or reverse (R).

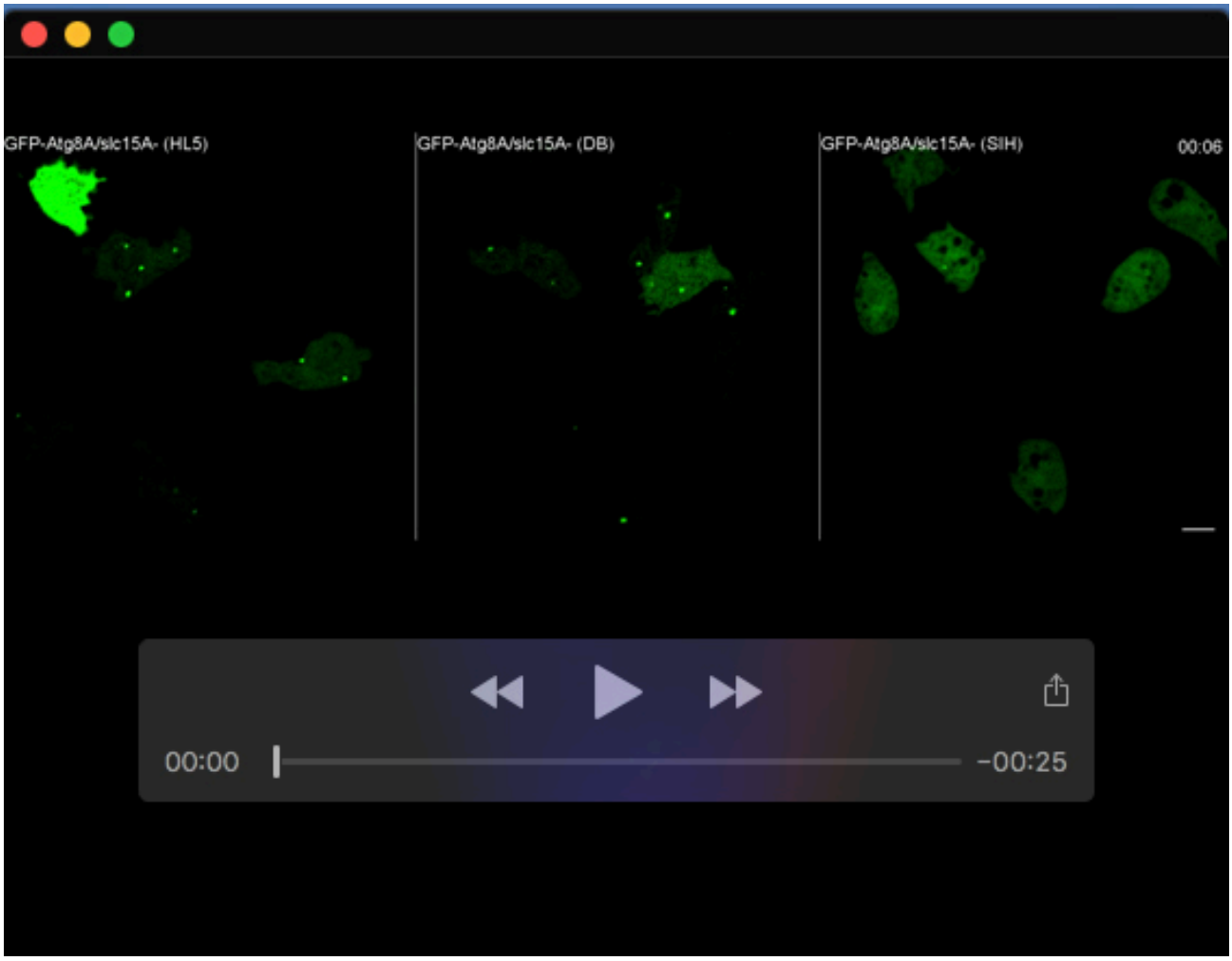
Usage	Plasmid backbone	Sequence, 5' - 3'
Expression in *Dictyostelium* cells		
Slc15A-GFP	pDM323	F: GCTCTAGAATGGCGATAGAAGAAGAATCATCAAATTCT R: ATAAGAATGCGGCCGCATTTGATTTTTCATGATCATAACCATAATCA
Slc15B-RFP	pDM324	F: GCTCTAGAATGGGTGGAATCGACGAAGATGG R: ATAAGAATGCGGCCGCTTTTGCATTTGATTTATTTGAATTTATTG
Slc15C-RFP	pDM324	F: GCTCTAGAATGAAAACAGACGATAAATATGCAAG R: ATAAGAATGCGGCCGCAATTAAATTATTTTCCAACTCTATAGATC
Slc15A$^{R68H/E438R}$-GFP	pDM323	R68H F: AATTTGTGAACATTATTCATACTATGCTTT R68H R: TGAATATAGTTCACAAATTTCATTACCCAT E438R F: GTCTTGACTGCCGCTCGTGTTTTATTATCA E438R R: ACACGAGCGGCAGTCAAGACAACATATTGTG
GFP-Rab5A	pDM317	F: CGGGAGCTCATGAATAATAATAATAAGATATTTC R: CGGACTAGTGTTACAACATTTGTTTTTCTTTCC
GFP-Rab7A	pDM317	F: CGGGAGCTCATGGCCACAAAGAAAAAGG R: CGGACTAGTACAACAACCTGATTTAGCTGG
TAPP1-RFP	pDM324	F: CGGGAGCTCATGCCTTATGTGGATCGTCAGAATC R: CTAGCTAGCCACGTCACTGACCGGAAGGCTCGC
GFP-Atg8a	pDM317	F: CTAAGATCTAGTGCTGGTGGTATGGTTCATGTATCAAGCTTTAAA R: CTAACTAGTTAAATCACTACCAAAAGATTTTCACC
RFP-GFP-Atg8a	pDM449	F: CGGGAGCTCATGGTTCATGTATCAAGCTTTAAAAAC R: CGGACTAGTTTATAAATCACTACCAAAAGTATTTTC GFP F: TGCTCTAGAATGGGTAAAGGAGAAGAACTTTTCAC GFP R: GGAAGATCTGGATCTGAGTCCGGACTTGTATAG
VatB-GFP	pDM323	F: CGGGAGCTCATGGTTGGATTTGAAGATCATATCG R: CGGACTAGTGTTAGTTGAATCTACAGTACCCTTTG
RFP-LmpA	pDM318	F: CGGGAGCTCATGGTAAAAAGAGGGTGTTGCCATAGAAAAATG R:CGGACTAGTTGGTTCTGTTAAACGACGACGTAATTCTTCTTCTAAACGACTTGG TTCATTATTAATAATTGCTTGATAAC
Generation of knockout cell		
slc15A knockout in Ax2	pBluescript-BSR	Insert 1 F: ACGGTCGACATGGCGATAGAAGAAGAATCATC Insert 1 R: ACGGAATTCAACACCATCAGCAACATAAGCAC Insert 2 F: ACGGGATCCGTGATTGCATTTTACATTTCAGC Insert 2 R: ACGACTAGTCAATACTATTACCCAAAGTTGAG
slc15A knockout in DdB	pDM1082	Insert 1 F: GGGGCCGGCATGGCGATAGAAGAAGAATC Insert 1 R: CCCAAGCTTAACACCATCAGCAACATAAGCA Insert 2 F: GGAAGATCTTGATTGCATTTTACATTTC Insert 2 R: CGGACTAGTTCAATACTATTACCCAAAGTTGAG
slc15B knockout	pBluescript-BSR	Insert 1 F: ACGCGTCGACATGGGTGGAATCGACGAAGATGGT Insert 1 R: CGGAATTCCTGCAATATATGCTCCAAATAATGTA Insert 2 F: GGACTAGTGAAGTTGATCAACAAAGATTAGATAATG Insert 2 R: ATAAGAATGCGGCCGCGCATTTGATTTATTTGAATTTATTGTGTAT
slc15C knockout	pBluescript-BSR	Insert 1 F: ACGCGTCGAC ATGAAAACAGACGATAAATATGCAAG Insert 1 R: CGGAATTCCAACAACTCCATCAATTGATGTTACTGAT Insert 2 F: GGACTAGTCACAATATTTAATTATAACGATTGCA Insert 2 R: ATAAGAATGCGGCCGCAATTAAATTATTTTCCAACTCTATAGATC
Generation of knock-in cell		
slc15A GFP and RFP knock-in	pDM1355	Insert 1 F: GGACTAGTCGTACCAATCTTTGAATATGGT Insert 1 R: GGACTAGTATTTGATTTTTCATGATCATAACC Insert 2 F: GGAGCCGGCTCAATTTAAAAATAAAAACACTTTGTA Insert 2 R: GGAGCCGGCATGCAAATTCTCCATTACCTCA
Expression in *E.coli*		
mCherry	pET-15b	F: CGCCATATGATGGTGAGCAAGGGCGAGGAG R: CCGCTCGAGGGGCCCCTTGTACAGCTCGTCC
GST-GFP	pGEX 4T-1	F: CCGGAATTCATGGTGAGCAAGGGCGAGGAG R: CCGCTCGAGCTTGTACAGCTCGTCCATGC



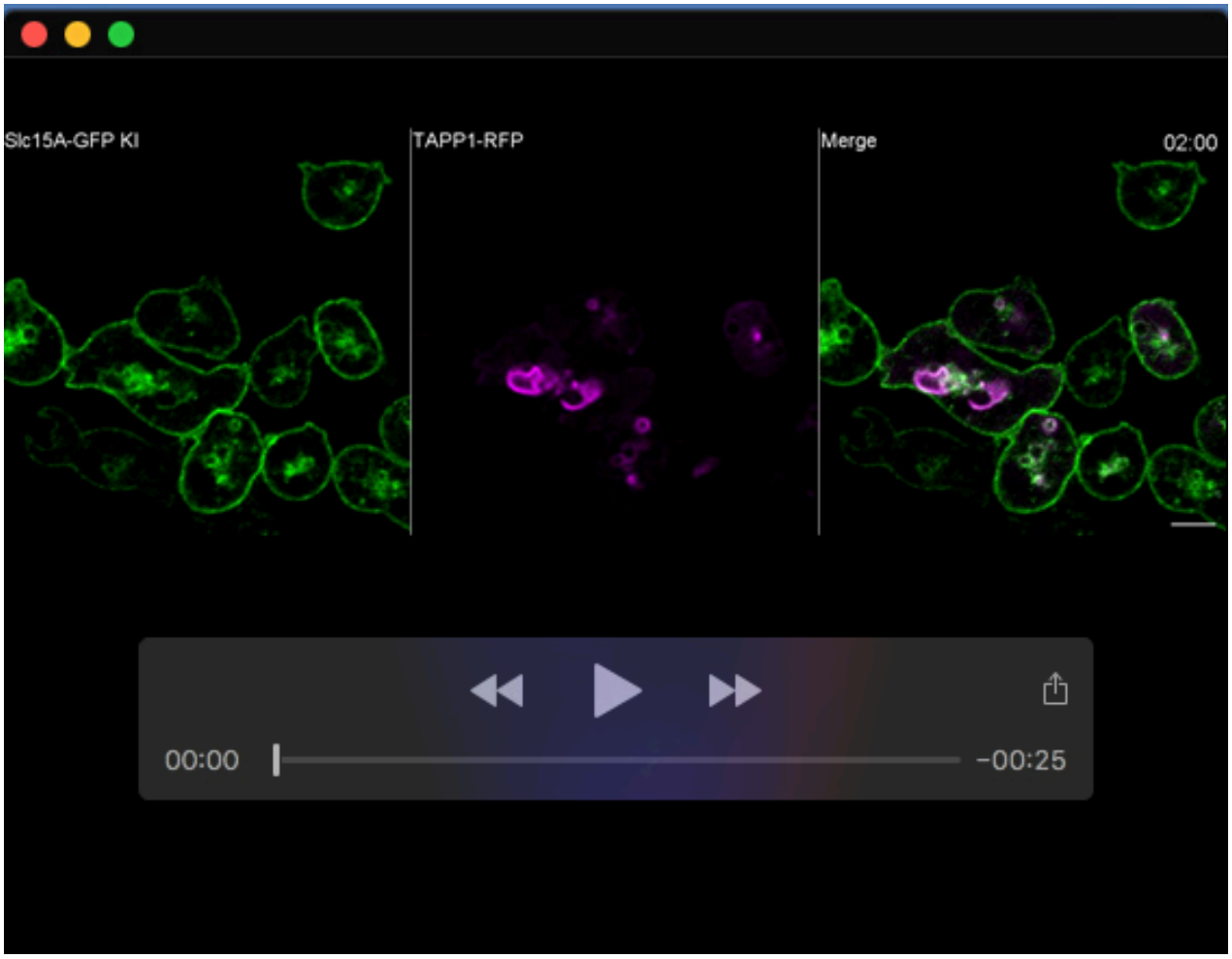
Movie 1. Time-lapse imaging of PHcrac-GFP dynamics in Ax2 and *slc15A*$^{-}$ cells. Corresponds to figure 4A. Images were captured at 3 sec per frame and played back at 15 frames per second. Scale bar, 5 µm.



Movie 2. Time-lapse imaging of GFP-Atg8a in Ax2 cells cultured in HL5 medium, SIH medium, or non-nutrient development buffer (DB). Corresponds to figures 4E-F and 5G. Images were captured at 6 sec per frame and played back at 1 frame per second. Scale bar, 5 μm.



Movie 3. Time-lapse imaging of GFP-Atg8a in *slc15A*$^{-}$ cells cultured in HL5 medium, SIH medium, or non-nutrient development buffer (DB). Corresponds to figures 4E-F and 5G. Images were captured at 6 sec per frame and played back at 1 frame per second. Scale bar, 5 µm.



Movie 4. Colocalization of Slc15A-GFP^{KI} with the PI(3,4)P_2 sensor TAPP1-RFP. Corresponds to figure 7A. Images were captured at 15 sec per frame and played back at 6 frames per second. Scale bar, 5 µm.