Supplemental information

Starvation-induced phosphorylation of the exchange factor DENND3 by Unc-51-like kinase activates Rab12 inducing autophagy

Running title: ULK phosphorylates DENND3 inducing autophagy

Jie Xu, Maryam Fotouhi and Peter S. McPherson

Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada

Address correspondence to:
Dr. Peter S. McPherson
James McGill Professor
Dept. Neurology & Neurosurgery
McGill University
3801 University Street
Montreal, QC H3A 2B4
Canada
phone: (514) 398-7355
Email: peter.mcpherson@mcgill.ca

Jie Xu
Ph.D. candidate
Dept. Neurology & Neurosurgery
McGill University
3801 University Street
Montreal, QC H3A 2B4
Canada
phone: (514) 398-6644 ext 00209
Email: jie.xu3@mail.mcgill.ca

Supplemental Materials and methods

cDNA constructs

Mouse DENND3 cDNA was from Imagenes. The coding sequence was amplified by PCR and cloned into pCMV vector for creating Flag-DENND3. The linker region between the DENN domain and WD40 repeats (amino acid 538-973) was cloned into pGEX vector for generating GST-DENND3-538-973. Flag-DENND3 S554A or S572A was created by Quikchange site-directed mutagenesis kit from Stratagene. Human Rab12 cDNA was purchased from Origene. Its coding region was amplified by PCR and cloned into pCMV or pEGFP vector for Flag-Rab12 or GFP-Rab12, respectively. Rab12 S56N or Q101L mutants were generated by Quikchange site-directed mutagenesis kit from Stratagene. GFP-RILPL1 was from Origene. The RILPL1 coding region was subcloned into pGEX vector for generating GST-RILPL1. HA-ULK1 WT (#22896), HA-ULK1 K46N (#22897), HA-ULK2 WT (#22898), HA-ULK2 K39T (#22899) and mCherry-LC3B (#40827) were from Addgene.

Constructs for siRNA-mediated knockdown

The following siRNA sequences were used: ULK1: 5'-cgcgcggtacctccagagcaa-3'; ULK2: 5'-cagcctgagatacgtgcctta-3'. Rab12-1: 5'-cagcattacctcagcttatta-3'; Rab12-2: 5'-aacctagtacttctaatatga-3'. DENND3-1: 5'-cgacggtttagttctgataaa-3'; DENND3-2: 5'-cagtcggaggaggacagaata-3'; DENND3-3: 5'-ctccaagtccacggacgataa-3'; DENND3-4: 5'-cagctgcgtcttgttggtgat-3'. For the rescue experiments following knock down, since the siRNA targets human

sequence, the constructs with mouse sequence were used in the re-expression rescue experiments.

Immunofluorescence

Cells were plated on coverslips coated with poly-L-lysine. The cells were washed with 37°C PBS and fixed with 3% PFA in 37°C PBS for 15 min, followed by standard protocols of immunofluorescence.

Live cell imaging

Cells were grown on MatTek dishes (MatTek Corporation). Live imaging was performed on an Andor laser confocal microscope system (Andor Technology) based on a spinning disk confocal scanning head CSU-X1 (Yokogawa Electric Corporation). The images were acquired on an Olympus IX-83 microscope (Olympus Corporation).

Supplemental Figure legends

Supplemental Figure S1.

Characterization of phosphospecific antibodies to the 14-3-3 binding sites in DENND3.

(A) HEK-293 cells were transfected with Flag-DENND3, wild type (WT) or S554A or S572A mutants. Lysates were subsequently processed for Western blot with the indicated antibodies

(B) HEK-293T cells were transfected with Flag-DENND3 and lysates were processed for immunoprecipitation with anti-Flag antibody. The immunoprecipitated proteins were treated with vehicle (control) or calf intestinal alkaline phosphatase (CIP) and processed for Western blot with anti-Flag, anti-pS554, or anti-pS572 antibodies as indicated

(C) HeLa cells were transfected with control siRNA or siRNAs targeting DENND3 and were subsequently processed for Western blot with the indicated antibodies.

Supplemental Figure S2.

DENND3 binds to and is phosphorylated by ULK1/2.

(A) HEK-293T cells were transfected with a HA vector, HA-ULK1 wild-type (WT) or a kinase inactive mutant, or HA-ULK2 wild-type (WT) or a kinase inactive mutant. Lysates were processed for Western blot with antibodies recognizing HA tag, GAPDH, and phosphospecific DENND3 S554 or S572 as indicated.

(B/C) Relative DENND3 phosphorylation at S554 (B) and S572 (C) was determined from 3 experiments as in (A). Bars represent mean ± SEM. Statistical analysis employed one-way ANOVA followed by Tukey's post-test. $^{**}p < 0.01$.

(D) HEK-293T cells were processed as in (A), followed by qPCR (n = 4 repeats). Bars represent mean ± SEM. Statistical analysis employed one-way ANOVA followed by Tukey's post-test. NS: Not Statistically Significant.

(E) Lysates from HEK-293T cells transfected with Flag-DENND3 and HA-ULK1 were incubated with protein G beads alone or protein-G beads coupled to anti-Flag monoclonal antibody (IP-Flag). Proteins bound specifically to the beads were processed for Western blot with antibody recognizing HA. An aliquot of the cell lysate (starting material, SM) equal to 5% of that added to the beads was analyzed in parallel.

(F) As in (D) except that the co-transfection was Flag-DENND3 and HA-ULK2.

(G) Lysates from HEK-293T cells transfected with HA-ULK1 were prepared and incubated with GST, GST-WD40 repeats of DENND3 coupled to glutathione-Sepharose beads. Protein specifically bound to the beads was processed for Western blot with antibody recognizing HA-ULK1. An aliquot of the lysate (starting material; SM) equal to 10% of that added to the beads was analyzed in parallel (n = 3 repeats).

Supplemental Figure S3.

Starvation activates Rab12.

(A) HEK-293T cells were transfected with Flag-Rab12 T56N inactive mutant or

Flag-Rab12 Q101L active mutant and lysates were incubated with GST-RILPL1 (Rab-interacting lysosomal protein-like 1) coupled to glutathione-Sepharose beads. Protein specifically bound to the beads was processed for Western blot with anti-Flag antibody. An aliquot of the lysate (starting material; SM) equal to 5% of that added to the beads was analyzed in parallel.

(B) HeLa cells were transfected with Flag-Rab12 and were then left unstarved or were starved with EBSS for the indicated time. Lysates were then prepared and incubated with GST-RILPL1 coupled to glutathione-Sepharose beads. Protein specifically bound to the beads was processed for Western blot with antibody recognizing Flag-Rab12. GST-RILPL1 is indicated in the ponceau stained transfer. Dashed line denotes discontinuous lanes from the same gel.

(C) HeLa cells were transfected with Flag-Rab12 and were treated with control siRNA or with siRNA for DENND3 or ULK1 and 2. The cells were subsequently starved with EBSS for 10 min and then lysates were prepared and incubated with GST-RILPL1 coupled to glutathione-Sepharose beads. Protein specifically bound to the beads was processed for Western blot with antibody recognizing Flag-Rab12. GST-RILPL1 is indicated in the ponceau stained transfer.

Supplemental Figure S4.

The perinuclear pool of Rab12 localizes at recycling endosome. HeLa cells transfected with Flag-Rab12 were processed for immunocytochemistry. The top 6 panels show localization of Flag-Rab12 and transferrin receptor labeled by internalized transferrin. The middle row of panels is magnified views of the

perinuclear region labeled by the white box on the lower magnification image on the top row of panels. The bottom row of panels shows colocalization of Flag-Rab12 and Rab11 at the perinuclear region. The scale bar = 2 μm.

Video S1.

Rab12 co-traffics with LC3-positive autophagosomes. HeLa cells transfected with GFP-Rab12 and mCherry-LC3 were starved with EBSS for 1 h before live cell imaging by spinning disk confocal microscope (n = 3 repeats). The arrow points to one of the structures in which Rab12 and LC3 co-traffic. The scale bar = 20 μm.

Video S2.

DENND3 and Rab12 regulate trafficking of LC3-positive autophagosomes. HeLa cells transfected with control siRNA were subsequently transfected with mCherry-LC3 and then starved with EBSS for 1 h before live cell imaging by spinning disk confocal microscopy (n = 3 repeats). At each time point color-coded LC3 vesicle tracks are shown from the previous 20 frames. The color scale bar represents the time line (from 0 to 60 seconds).

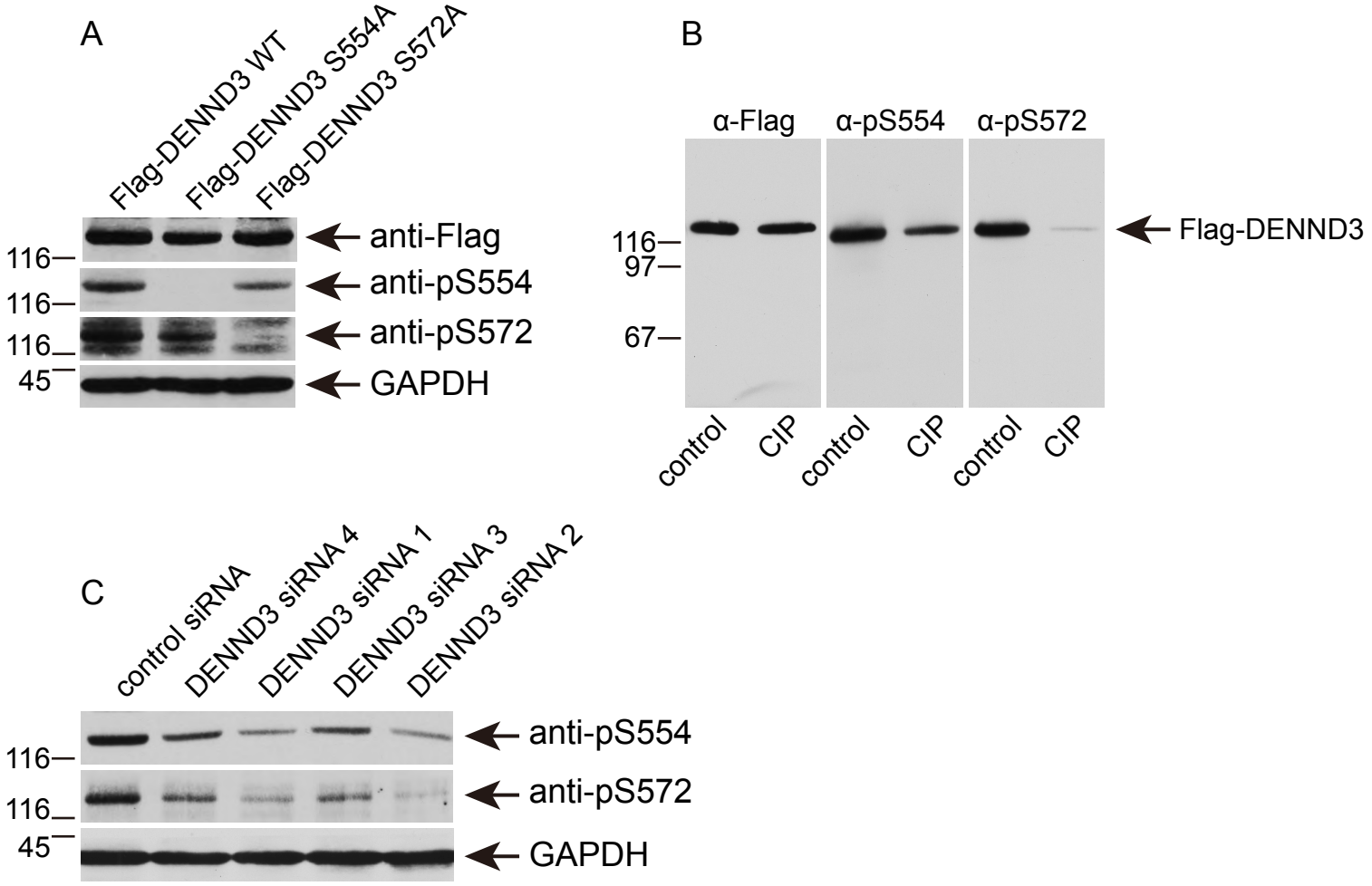
Video S3.

DENND3 and Rab12 regulate trafficking of LC3-positive autophagosomes. HeLa cells transfected with Rab12 siRNA were subsequently transfected with mCherry-LC3 and then starved with EBSS for 1 h before live cell imaging by

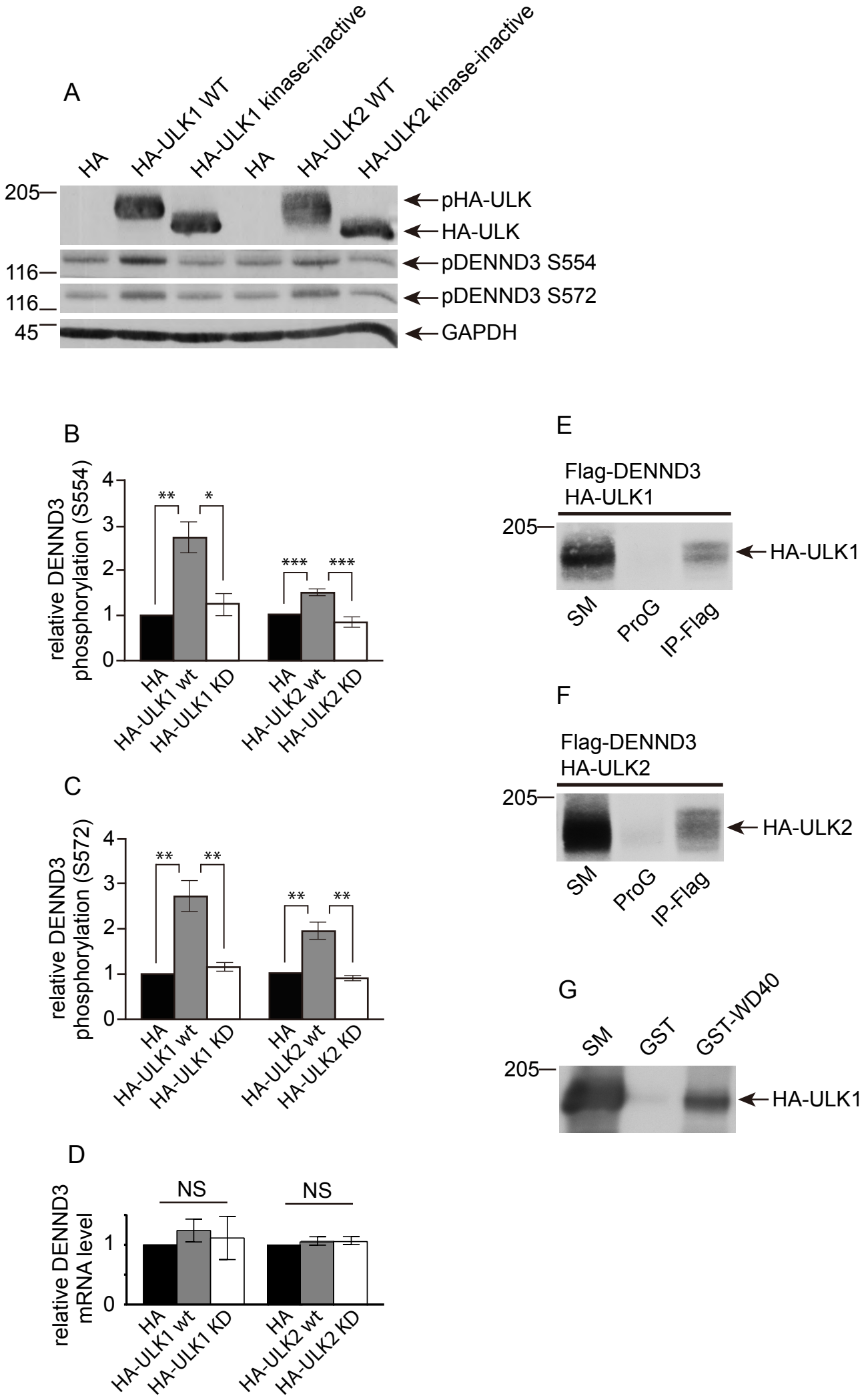
spinning disk confocal microscopy (n = 3 repeats). At each time point color-coded LC3 vesicle tracks are shown from the previous 20 frames. The color scale bar represents the time line (from 0 to 60 seconds).

Video S4.

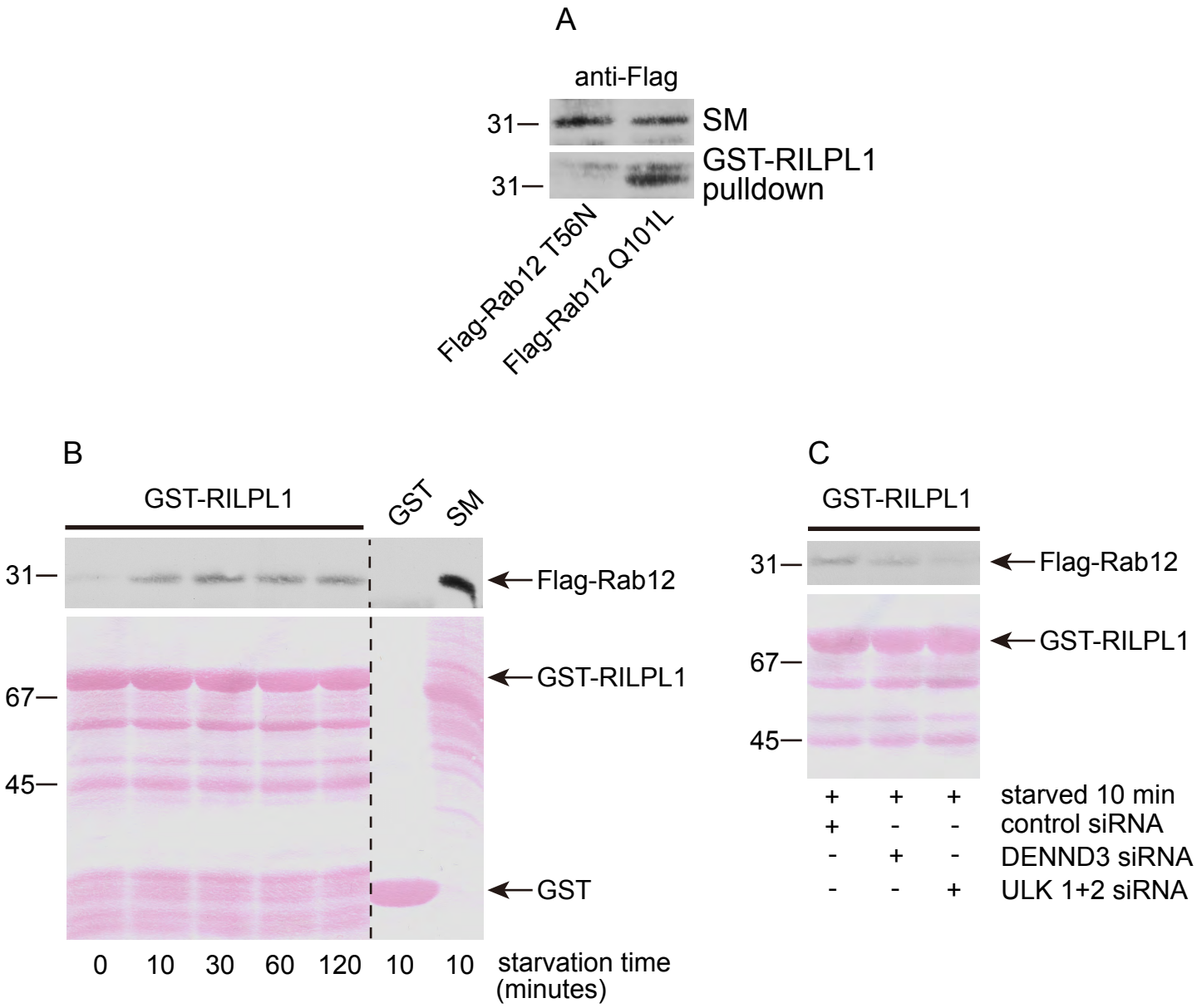
DENND3 and Rab12 regulate trafficking of LC3-positive autophagosomes. HeLa cells transfected with DENND3 siRNA were subsequently transfected with mCherry-LC3 and then starved with EBSS for 1 h before live cell imaging by spinning disk confocal microscopy (n = 3 repeats). At each time point color-coded LC3 vesicle tracks are shown from the previous 20 frames. The color scale bar represents the time line (from 0 to 60 seconds).



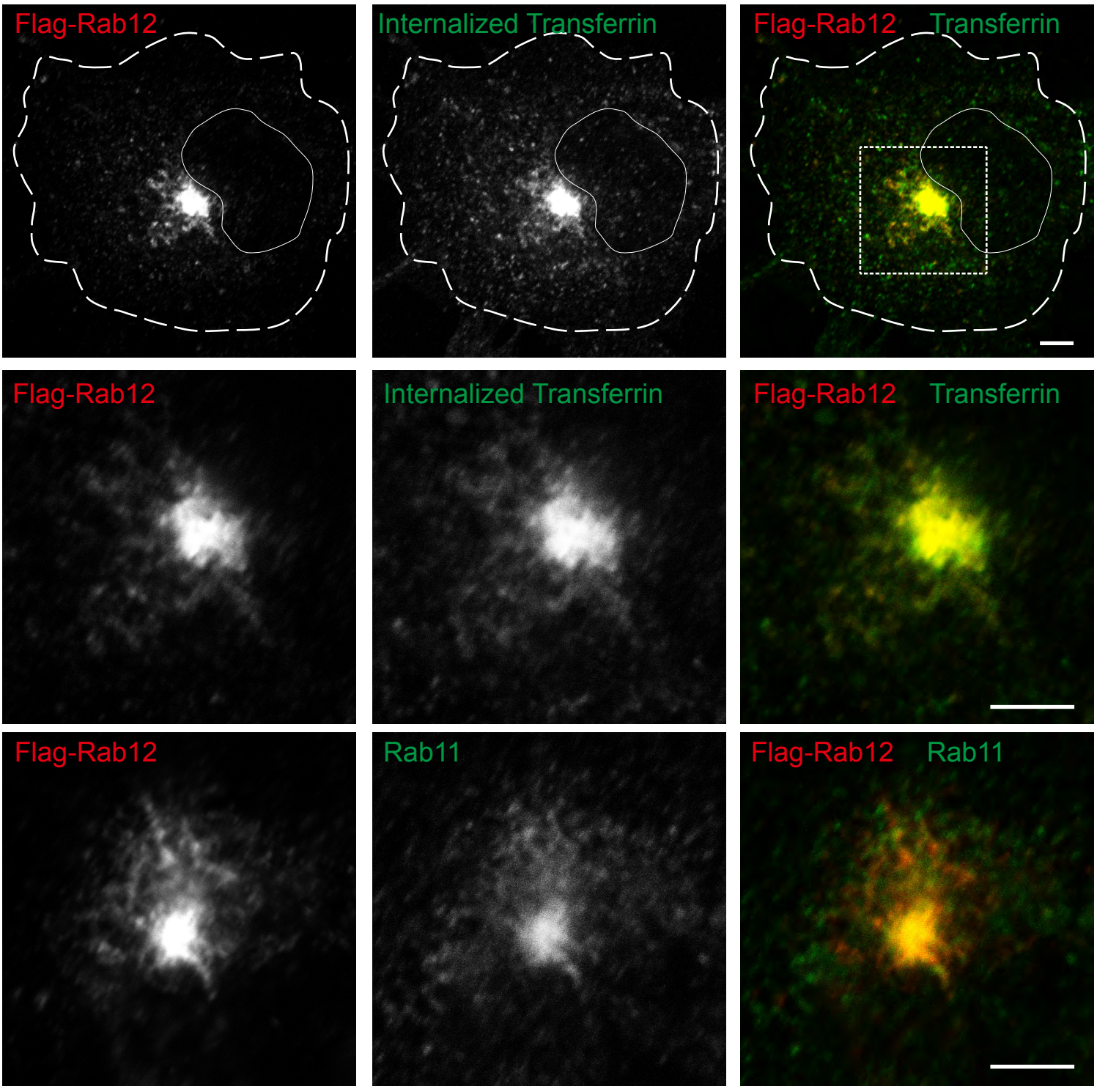
Xu et al., Supplemental Figure S1



Xu et al., Supplemental Figure S2



Xu et al., Supplemental Figure S3



Xu et al., Supplemental Figure S4