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Supplemental Information for

The connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes

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SUPPLEMENTAL DATA

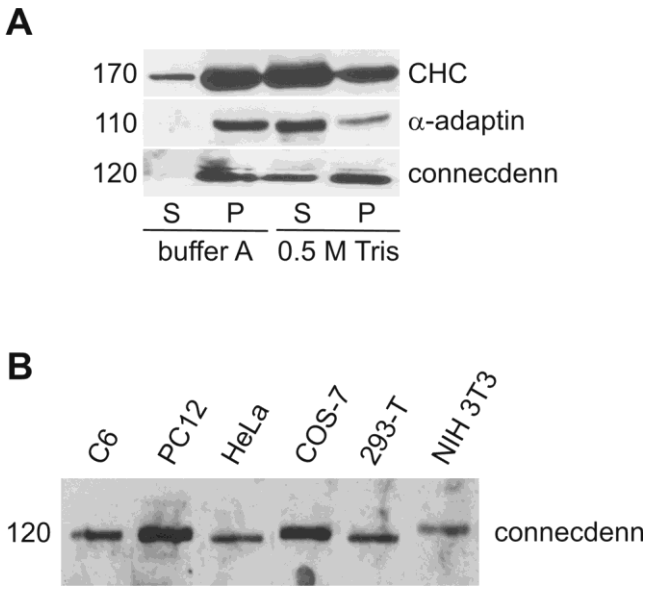

Figure S1 – Connecdenn is associated with CCV membrane, related to Figure 1. A. Equal aliquots of purified rat brain CCVs were incubated with buffer A or 0.5M Tris pH 8.0 (1:1 buffer A, 1M Tris pH 9.5) to strip the proteinous coat off the CCV membrane. The fractions were separated by centrifugation and equal volumes of resuspended pellets (P) and supernatants (S) were analyzed for protein partitioning of clathrin heavy chain (CHC), AP-2 (α-adaptin), and connecdenn by Western blot. **B.** Equal amounts (200 μg) of lysates from various cell lines as indicated were analyzed by Western blot for connecdenn expression levels.

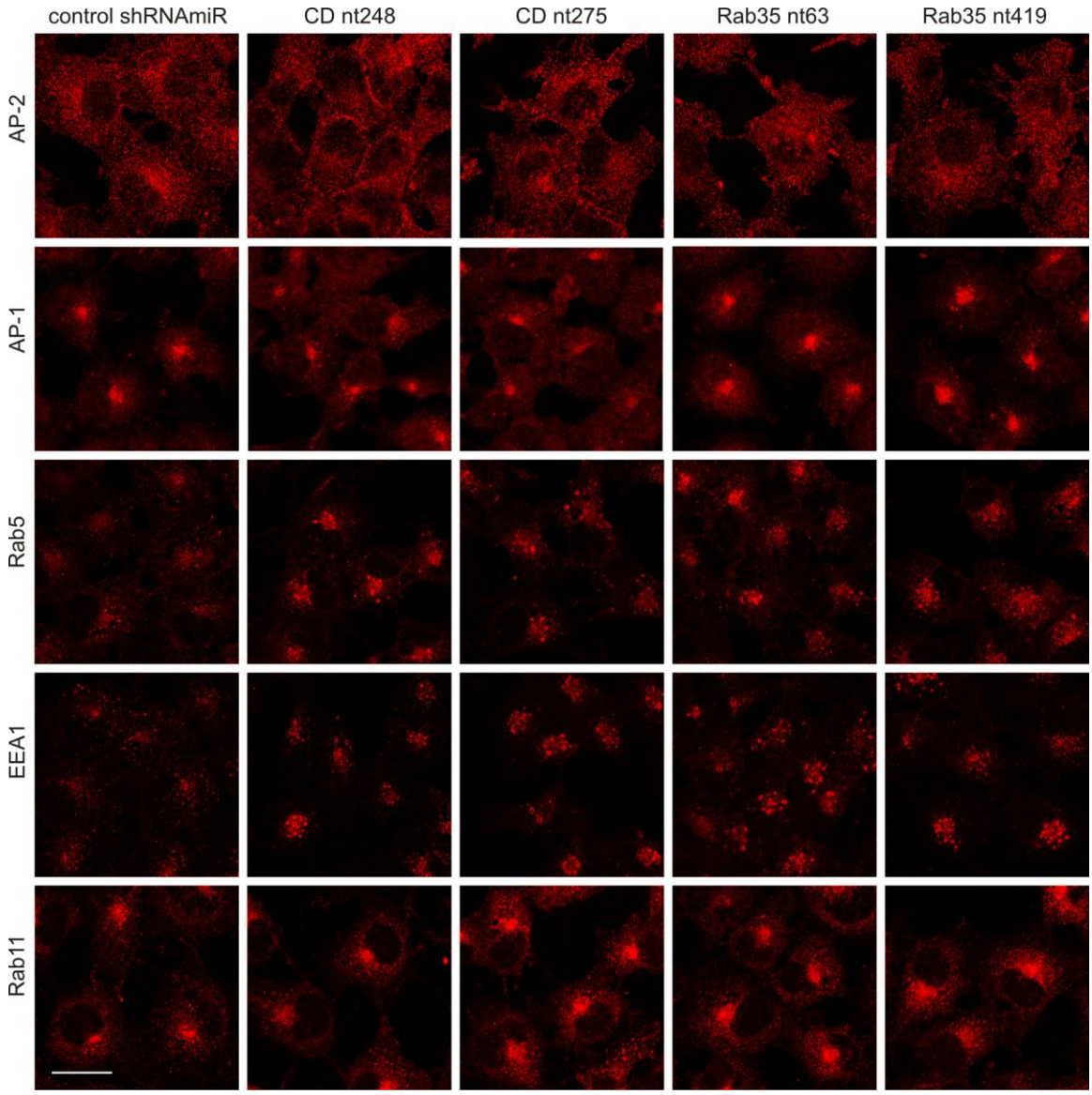

Figure S2 – KD of connecdenn or Rab35 affects early endosome morphology and localization, related to Figure 4. COS-7 cells were transduced with lentiviruses for expression of a non-targeting control shRNAmiR or shRNAmiRs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) and processed for immunofluorescence to reveal the localization of endogenous markers as indicated. The bar represents 20 μm.

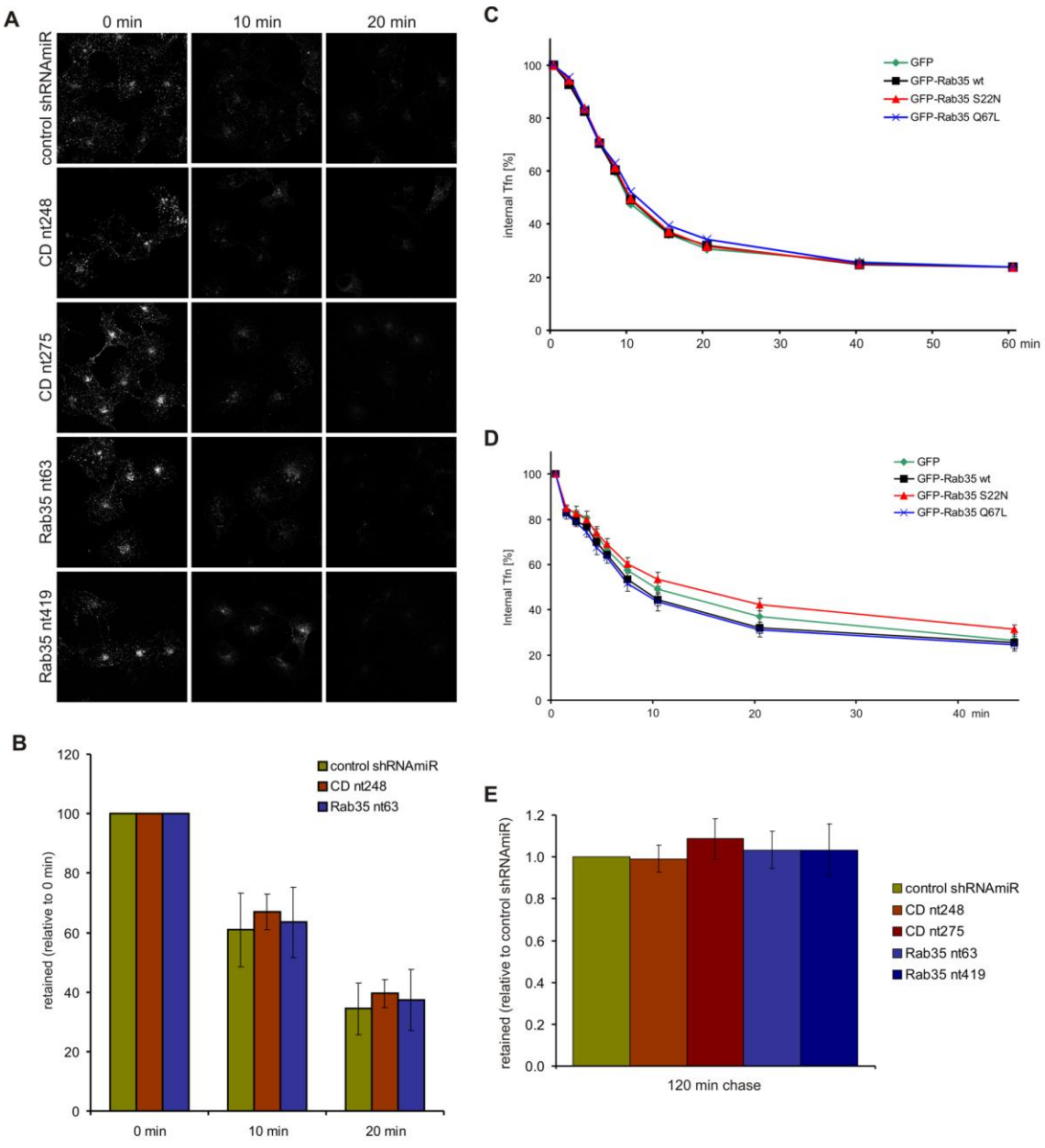

Figure S3 – No effect of connecdenn and Rab35 KD on transferrin or β1-integrin recycling, related to Figure 5. (A) COS-7 cells transduced with lentiviruses for expression of a non-targeting control shRNAmiR or shRNAmiRs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) were incubated with Cy3-labeled transferrin for 45 min at 16°C, acid washed to remove surface-bound label, chased at 37°C for the times indicated, acid washed, fixed, and images were collected using constant settings. **(B)** Quantification of the

transferrin signal intensity in cells imaged as described for (A) using ImageJ. The graph represent the mean (±SEM) of four independent experiments, plotted as percent of the initial internal label (set to 100%) and statistical analysis by Repeated Measure Two-Way ANOVA followed by Bonferroni posttests revealed no significant differences between control and KD cells. HeLa **(C)** or COS-7 cells **(D)** were transfected with GFP alone or with GFP-tagged Rab35 wt, Rab35 S22N, or Rab35 Q67L, continuously labeled with AlexaFluor647-transferrin at 37ºC for one hour, chased at 37ºC for the indicated times, and intracellular transferrin was measured by flow cytometry. The graph represent the mean (±SEM) of four independent experiments, plotted as percent of the initial internal label and statistical analysis by Repeated Measure Two-Way ANOVA followed by Bonferroni posttests revealed no significant differences between control and KD cells. **(E)** COS-7 cells transduced with lentiviruses for expression of a non-targeting control shRNAmiR or shRNAmiRs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) were incubated for one hour with antibodies against β1-integrin and analyzed by flow cytometry either directly or following a two hour chase. The graph represent the mean fluorescence (±SEM) after the two hour chase of four independent experiments, plotted as relative ratio with the control shRNAmiR set to 1. Statistical analysis by Repeated Measure One-Way ANOVA followed by Dunnett's posttests revealed no significant differences between control and KD cells.

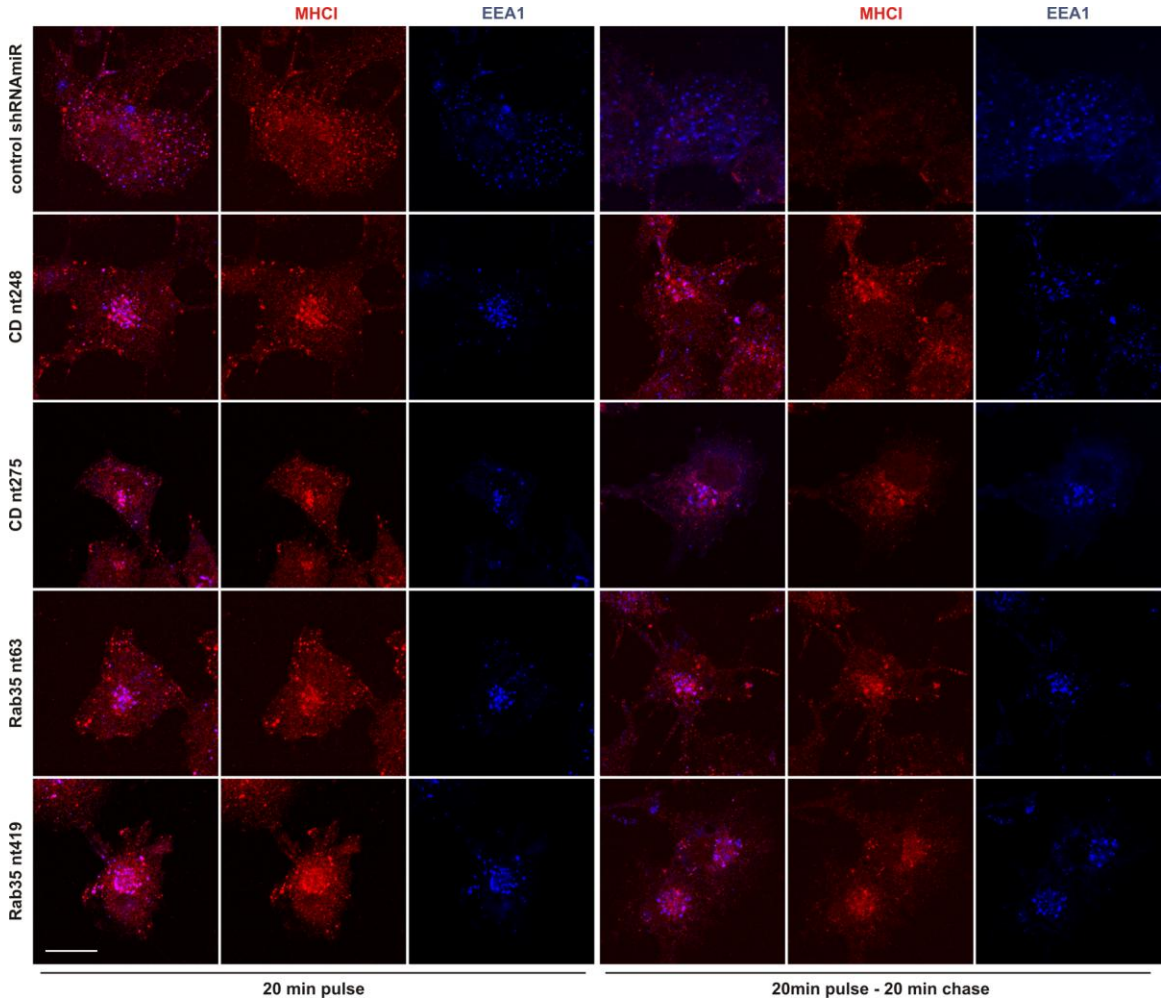

Figure S4 – Connecdenn and Rab35 KD affects MHCI trafficking, related to Figure 6. COS-7 cells transduced with lentiviruses for expression of a non-targeting control shRNAmiR or shRNAmiRs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) were incubated for 20 min with antibodies against MHCI and processed by immunofluorescence either directly or following a 20 min chase to reveal the localization of the intracellular antibodies and endogenous EEA1. The bar represents 20 μm.

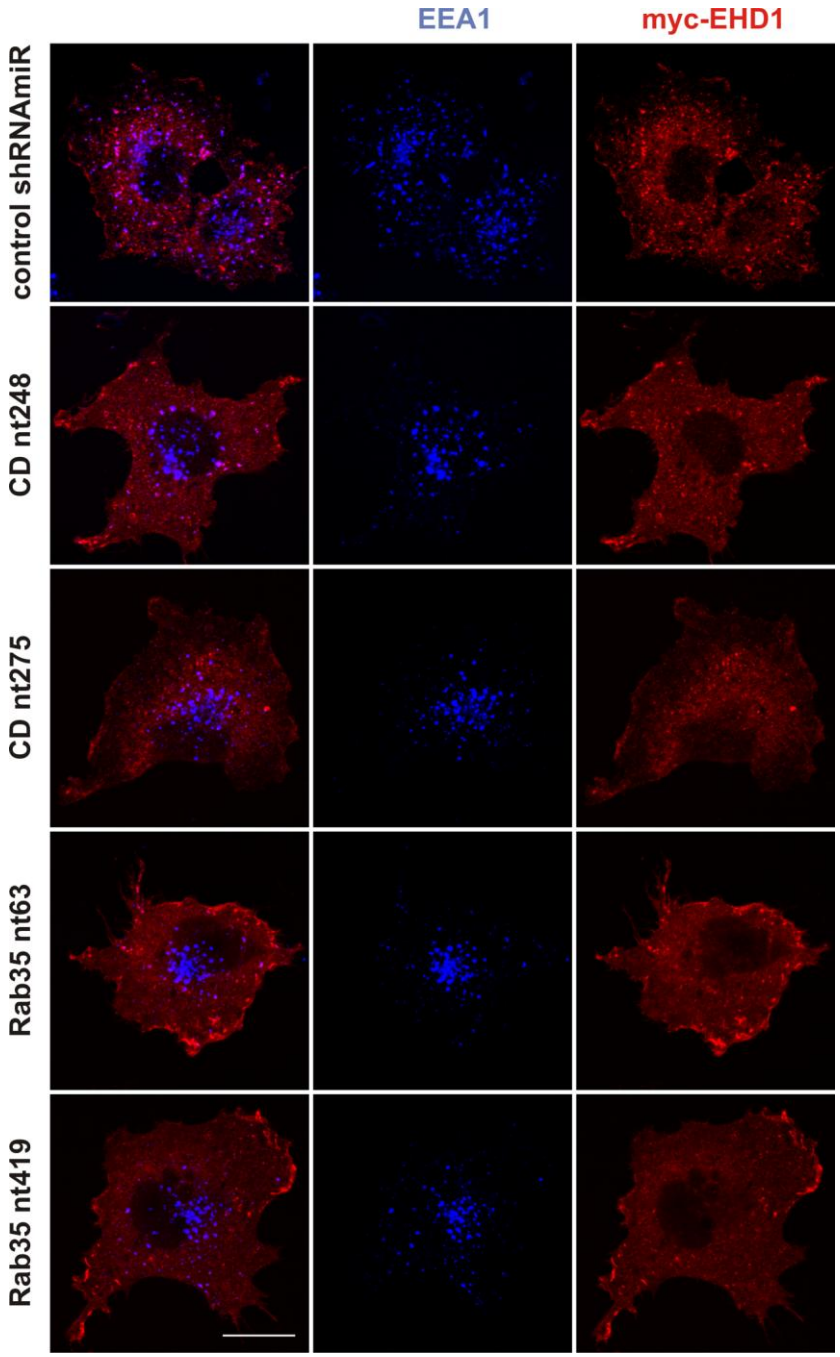

Figure S5 – Connecdenn and Rab35 control recruitment of EHD1 to early endosomes, related to Figure 6. COS-7 cells transduced with lentiviruses for expression of a non-targeting control shRNAmiR or shRNAmiRs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) were transfected with myc-EHD1 and processed by immunofluorescence to reveal the localization EHD1 and endogenous EEA1. The bar represents 20 μm.

EXPERIMENTAL PROCEDURES

Oligo nucleotides

CD aa1 F-BglII	GCGCAGATCTATGGGCTCCAGGATCAAGCAAAAC
CD aa403 R-EcoR1	GCCCGAATTCTCACTGATGGTACAGTTTATCACTG
CD aa372 F-BglII	GCGCAGATCTCTAGACCTTCTCAATTCCGGTGAAGGT
CD aa1016 R-BglII	GCGCGAATTCTCACTCAAAGGTCTCCCACTGTCTGCG
CD nt248 s	TGCTGATAAGCGGCAGAACCCGAATCGTTTTGGCCACTGACTGACGATTCGGGCTGCCGCTTAT
CD nt248 as	CCTGATAAGCGGCAGCCCGAATCGTCAGTCAGTGGCCAAAACGATTCGGGTTCTGCCGCTTATC
CD nt275 s	TGCTGTACAGAAGCAGCTCTTCGCTCGTTTTGGCCACTGACTGACGAGCGAAGCTGCTTCTGTA
CD nt275 as	CCTGTACAGAAGCAGCTTCGCTCGTCAGTCAGTGGCCAAAACGAGCGAAGAGCTGCTTCTGTAC
pcDNA3-myc s	AGCTTGCCACCATGGCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTGG
pcDNA3-myc as	GATCCCAGGTCCTCCTCTGAGATCAGCTTCTGCATTGATGCCATGGTGGCA
Rab3 aa1 F-BamH1	GCGCGGATCCATGGCATCCGCCACAGACTCG
Rab3 aa220 R-EcoR1	GCGCGAATTCTCAGCAGGCGCAGTCCTGGTG
Rab4 aa1 F-BamH1	GCGCGGATCCATGTCGCAGACGGCCATGTC
Rab4 aa218 R-EcoR1	GCGCGAATTCCTAACAACCACACTCCTGAG
Rab5 aa1 F-BamHI	GCGGGATCCATGGCTAATCGAGGAGCAACAAG
Rab5 aa215 R-NotI	GCGGCGGCCGCTTAGTTACTACAACACTGACTCCTG
Rab11 aa1 F-BamH1	GCGCGGATCCATGGGCACCCGCGACGACGAG
Rab11 aa216 R-EcoR1	GCGCGAATTCTTAGATGTTCTGACAGCACTG
Rab35 aa1 F-BamH1	GCGCGGATCCATGGCCCGGGACTACGACCACC
Rab35 aa201 R-EcoR1	GCGCGAATTCTTATTAGCAGCAGCGTTTCTTTC
Rab35 nt63 s	TGCTGAAACGCAACAGTAAACTGCTCGTTTTGGCCACTGACTGACGAGCAGTTCTGTTGCGTTT
Rab35 nt63 as	CCTGAAACGCAACAGAACTGCTCGTCAGTCAGTGGCCAAAACGAGCAGTTTACTGTTGCGTTTC
Rab35 nt419 s	TGCTGACAACTGGATGCCCATCTGCCGTTTTGGCCACTGACTGACGGCAGATGCATCCAGTTGT
Rab35 nt419 as	CCTGACAACTGGATGCATCTGCCGTCAGTCAGTGGCCAAAACGGCAGATGGGCATCCAGTTGTC
Rab35 S22N R	AGTAAACTGTTCTTGCCCACAC
Rab35 Q67L R	AGCGCTCCAGCCCCGCTG
Rabex5 aa1 F-BamHI	GCGCGGATCCATGAGCCTTAAGTCTGAACGCCGAGG
Rabex5 aa399 R-SalI	GCGCGTCGACTCACCAACTCTCAGCTTCTTGCTTCCTGGG

Antibodies and reagents

Mouse monoclonal antibodies against CHC (clone 23), α-adaptin (clone 8), and EEA1 (clone 14) were from BD Transductions (Lexington, KY, USA), against Flag (M2) from Sigma

(St-Louis, MI, USA), and against MHCI (W6/32) and β1-integrin (TS2/16) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against GFP (A6455) and Rab11 (71-5300) were from Invitrogen (Carlsbad, CA, USA), against, c-myc (C-3959) from Sigma, against Rab5 (Ab18211) from Abcam (Cambridge, MA, USA), and against EEA1 (C45B10) from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibodies against CLCs and connecdenn (3776) have been described previously (Allaire et al., 2006; Poupon et al., 2008). Polyclonal antibodies against Rab35 were raised in rabbits against GST-tagged full-length human Rab35. AlexaFluor 633-conjugated human transferrin (T-23362) was purchased from Invitrogen and Cy3-conjugated human transferrin (009-160-050) was from Jackson ImmunoResearch (West Grove, PA, USA).

Expression constructs

Human Rab35 (NM_006861) was obtained from Invitrogen, human Rab3A (NM_002866), Rab4A (NM_004578), Rab11A (NM_00663) were obtained from Origene (Rockville, MD, USA), and human Rabex5 (BC015330) was obtained from OpenBiosystems (Huntsville, AL, USA). The coding sequences were amplified by PCR and cloned into pGEX-6P1, pEBG, peGFP-C1, and pcDNA3-Flag (Ritter et al., 2004). Rab35 S22N and Q67L were produced by MEGA primer approach and cloned into pEGFP-C1, pcDNA3-Flag or -myc (generated as described in (Ritter et al., 2004)), and pGEX-6P1. The constructs for the GST-tagged DH/PH domain of intersectin 1-l, Flag-tagged connecdenn and the C-terminal region of NECAP 1 were previously described (Allaire et al., 2006; Hussain et al., 2001; Ritter et al., 2003), for the Flag-tagged C-terminal region of connecdenn, parts of the open reading frame encoding amino acids 372-1016 were cloned into pCMV-Tag2B. For expression of the DENN domain, the nucleotide sequence encoding amino acids 1-403 were amplified by PCR using an antisense oligo that introduced a restrictions site between the codon for amino acid 403 and the stop codon and cloned into pEBG and pcDNA3-Flag. The restriction site following the codon for

amino acid 403 was then used to insert the region of NECAP 1 encoding amino acids 129-275 for generation of the chimera expression construct in pcDNA3-Flag. GFP-Rab5 wt, S22N, and Q67L expression constructs were a generous gift from Dr. Presley (McGill University, Canada), the expression construct for myc-tagged Rabex5 was generously provided by Dr. Bonifacino (NIH, USA), and the expression construct for myc-tagged EHD1 was a generous gift from Dr. Caplan (University of Nebraska, USA). All plasmid were verified by sequencing.

Cell culture

HEK 293-T and COS-7 cells were maintained in DMEM High Glucose (Invitrogen, Grand Island, cat. no. 11995) containing 10% heat-inactivated FBS (PAA Laboratories Inc., Etobicoke), 100 U/ml penicillin, and 100 μg/ml streptomycin (both Invitrogen).

GST affinity-selection assays

Flag- and GFP-tagged proteins were expressed in HEK 293-T cells that were lysed in HEPES buffer (10 mM HEPES-OH, pH 7.4, 100 mM NaCl, 0.83 mM benzamidine, 0.23 mM phenylmethylsulphonyl fluoride, 0.5 μg/ml aprotinin and 0.5 μg/ml leupeptin) in the absence or presence of 10 mM EDTA as indicated. Triton X-100 was added to a final concentration of 1% and lysates were rocked for 5 min at 4°C before centrifugation at 205,000 x*g* to remove cell debris. Aliquots of the supernatant were incubated for 1 h at 4°C with GST fusion proteins pre-coupled to glutathione-Sepharose and then washed with the appropriate buffer. Samples were separated by SDS-PAGE and binding was detected by Western Blot.

Co-immunoprecipitation assays

HEK 293-T cells were transfected with GFP-tagged expression constructs for Rab35 wt, Rab35 S22N, or Rab35 Q67L using calcium phosphate. The next day, cells were lyzed in HEPES

buffer, cell debris were removed by cewntrifugation, and equal volumes were incubated with antibodies against GFP and protein A-agarose (α-GFP IP) or protein A-agarose alone (mock IP) for 1 h at 4°C. Beads were then washed with HEPES buffer, bound proteins were resolved by SDS-PAGE and binding was detected by Western blot using protein A-HRP in place of the secondary antibody.

Liposome preparation and lipid binding assay.

Liposome binding assays were performed using liposome suspensions (1 mg/ml) containing 90 mol% PtdChol and 10 mol% of each of the following phospholipids: PtdIns, PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 (Echelon Bioscience, Inc.) and PtdA, PtdSer, PtdChol (Avanti Polar Lipid Inc.). Lipid mixtures were resuspended in Lipid Buffer (25 mM HEPES pH 7.5, 150 mM KCl, 1 mM EDTA). To remove protein aggregates, purified proteins were centrifuged at 55,000 rpm for 45 min at 4ºC in a TL100 rotor (Beckman). Two micrograms of protein were incubated with 100 μg liposomes for one hour at room temperature and centrifuged at 55,000 rpm for 1 hour at 4ºC. Protein inputs and pellets were subjected to SDS-PAGE and stained with Coomassie brilliant blue. The intensity of proteins bands was measured using Odyssey Image software (LI-COR biosciences).

shRNAmiR lentivirus production and KD

Target sequences for human connecdenn and Rab35 were designed using the Block-iT RNAi Designer (Invitrogen) (CD nt248 GATTCGGGTTCTGCCGCTTAT, CD nt275 GAGCGAAGAGCTGCTTCTGTA, Rab35 nt 63 GAGCAGTTTACTGTTGCGTTT, Rab35 nt 419 GGCAGATGGGCATCCAGTTGT) and subcloned into pcDNA6.2/GW-EmGFP-miR (Invitrogen) or pcDNA6.2/GW-RFP-miR following manufacturer's instructions to yield shRNAmiR knock down constructs. The target sequences are designed as shRNA sequences that

fully match the mRNA target sequence, thus ensuring degradation of the target. Within the plasmid, the shRNA is integrated into stem sequences of the microRNA miR-155, which is located within the 3'-UTR of EmGFP or RFP. As such, transcription driven by the CMV promoter yields a pre-miRNA expression cassette that can either be translated for expression of the fluorescent reporter protein or that is recognized by the microRNA-processing machinery. In the latter case, the pre-miRNA is cleaved by drosha in the nucleus to release the shRNA structure, which is then transported into the cytosol, where it is processed by dicer and incorporated into the RISC complex to mediate degradation of the target mRNA.

For virus production, the EmGFP-shRNAmiR or RFP-shRNAmiR cassette was amplified by PCR, subcloned into the pRRLsinPPT vector, and VSVG pseudotyped virus was produced in HEK 293-T cells. Viral particles were concentrated by centrifugation and titered using HEK 293-T cells. The control shRNAmiR virus was described earlier (Thomas et al., 2009). For KD studies in COS-7 cells, cells were plated on the day of transduction. For transduction, the culture medium was replaced by DMEM High Glucose (Invitrogen) supplemented with 2% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 6 µg/ml polybrene (Sigma) and viruses were added at an MOI of 10. The next day media was replaced with fresh culture medium and the cells were cultivated until assays were performed five days after transduction.

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