SUPPLEMENTAL MATERIALS FOR:

Endosome-ER contacts control actin nucleation and retromer function through VAP-dependent regulation of PI4P

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Extended Experimental Procedures

Supplemental References

EXTENDED EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

HeLa and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37ºC and 5% CO2. Transfection of plasmids was carried out with Lipofectamine 2000 (Life Technologies). For siRNA experiments, HeLa cells were transfected with control or target siRNA oligos by using Lipofectamine RNAi MAX (Life Technologies) and cultured for 72 hours before analysis.

Generation of VAP Knockout Cell Lines with TALENs

TALEN construction and validation of targeted disruption of VAP genes

TALEN binding sites were chosen specific to exon 2 of VAPA and VAPB, respectively (**Figure 1A**). The VAPA and VAPB genomic sequences targeted by each pair of TALENs are: VAPA-TALEN-Left: 5'-TAGTCACTACAAATCTTAAA-3'; VAPA-TALEN-Right: 5'-TCACTTTGAAACACACTTTT-3'; VAPB-TALEN-Left: 5'-TTTTAAGGTGAAGACTACAG-3'; VAPB-TALEN-Right: TGATTCCGCTGTTGGGCCTC-3'. TALENs were constructed using the Golden-Gate ligation method as described (Miller et al., 2011; Sanjana et al., 2012). Assemblies of monomeric DNA-binding domains (designated by repeat variable diresidues, RVDs) and the TALEN cloning vectors used for each TALEN are:

VAPA-TALEN-Left: (NG) NI NN NG HD NI HD NG NI HD NI NI NI NG HD NG NG NI NI (NI), pTALEN_v2 (NI);

VAPA-TALEN-Right: (NG) HD NI HD NG NG NG NN NI NI NI HD NI HD NI HD NG NG NG (NG), pTALEN_v2 (NG);

VAPB-TALEN-Left: (NG) NG NG NG NI NI NN NN NG NN NI NI NN NI HD NG NI HD NI (NN), pTALEN_v2 (NN);

VAPB-TALEN-Right: (NG) NN NI NG NG HD HD NN HD NG NN NG NG NN NN NN HD HD NG (HD), pTALEN_v2 (HD).

HeLa cells were transiently transfected with plasmids expressing VAPA- and VAPB-specific TALENs respectively. After 72 hours, cells were harvested to extract genomic DNA. Regions surrounding the TALEN target sites of both loci were PCR amplified (with primers for VAPA: 5'_VAPA_DSB_400, GACACTGGTTTGCAGACAACTGG, 3'_VAPA_DSB_300, CACACTCCCTACACAGAAGATGAGTG; primers for VAPB: 5'_VAPB_DSB_345, GTGAGGTTTCAATGAGATGCCATATGTTAG, 3'_VAPB_DSB_255, GAAAAAAACAGGCGACTGGGACCCAGCTTTC). The PCR products were then melted and cross-hybridized such that wild type and TALEN-modified PCR products form homo- and heteroduplexes. Treatment with Surveyor Nuclease (Transgenomic) demonstrated TALEN cleavage efficiency of 11.5% for VAPA TALENs and 16.5% for VAPB TALENs (**Figure S1A**).

FACS enrichment of TALEN-modified cells

To enrich for gene-modified cells, a surrogate reporter system was used involving reporter plasmids that contain the TALEN target sequence framed between the coding sequences of mCherry and an out-of-frame EGFP (Kim et al., 2011) (**Figure S1B**). In this system mCherry is

constitutively expressed whereas EGFP is only expressed if the intervening TALEN target sequence is modified, so that expression of EGFP represents a proxy of TALEN-mediated gene modification of the endogenous sequence via the generation of double strand breaks followed by non-homologous end joining (NHEJ). To generate surrogate reporters for VAPA and VAPB, PCR amplicons containing the TALEN target sites were amplified (using the primers for VAPA: 5'_BglII_VAPA_Surro, GAGCTGTACAAGAGATCTGATGTAGTCACTACAAATCTTAAATTGCGAAATCCATCGTAC 3'_BamHI_VAPA_Surro, CGTCTCCTGGATCCATCACTTTGAAACACACTTTTGGTACGATGGATTTCGCAATTTAAG; and primers for VAPB: 5'_BglII_VAPA_Surro, GTACAAGAGATCTTGTTTTAAGGTGAAGACTACAGCACCACGTACGTACTGTGTGAGGCC, 3'_BamHI_VAPA_Surro, GTGGCGCCGGTGGATCCATGATTCCGCTGTTGGGCCTCACACAGTACGTACGTGGTGCTG) and ligated between BglII and BamHI in mCherry-E-Syt2-EGFP (Giordano et al., 2013) to generate mCherry-VAPA Surro-EGFP and mCherry-VAPB Surro-EGFP, respectively.

72 hours after transfection, cells co-expressing the TALEN pairs and the corresponding surrogate reporter were subjected to flow cytometry to enrich for cells double positive for mCherry and EGFP expression and thus likely to harbor TALEN-mediated VAP mutations.

Clonal isolation, verification of targeted gene disruption by DNA sequencing

Sorted cells were individually cloned. For either VAPA or VAPB TALENs, about 40 clones were analyzed by PCR genotyping of ~500 bp fragments surrounding the TALEN target sites, using one 6-carboxyfluorescein (6-FAM)-labeled primer and one regular primer. Names, sequences and 6-FAM label (if applicable) of these primers are:

5'_6FAM_VAPA_DSB_223, GACTTGTTTTTAAGTCTGCCACCTCG,

3'_VAPA_DSB_258, GCAGAGTACTGTAACAAGACATC,

5'_VAPB_DSB_243, CCTCAGCTCATCTCTTTCATCCATTGGC,

3'_6FAM_VAPB_DSB_179, TGGGGGTGGGGTGGAGAGAATTCTATCATCTTC.

Fragment analysis of the fluorescent PCR products with capillary electrophoresis (Genewiz) was then performed to resolve the length of the PCR amplicons of each clone. Clones carrying biallelic frameshift mutations were chosen. PCR amplicons of each such clone were ligated into pCR2.1 TOPO vectors using the TOPO-TA cloning kit (Life Technologies) and sequenced (Genewiz) for confirmation.

Three independent VAP double knockout (DKO) cell lines (DKO-1, -2, and -3) were generated. DKO-1 resulted from the single-round transfection of the VAPB-targeting TALENs which led to frameshift deletions in VAPB alleles. These TALEN pairs however, also resulted in the deletion of exon 2 in VAPA alleles due to an off-target-effect on its very similar, although not identical, sequence to exon 2 of VAPB (**Figure S1C**). Off-target effects of TALEN pairs at homologous sites are known to occur (Dahlem et al., 2012). DKO-2 and DKO-3 cell strains were generated by sequentially disrupting first the VAPA gene and then the VAPB gene. In both strains biallelic frameshift deletions on both VAPA and VAPB loci were detected.

Generation of Sac1 Knockout Cells with CRISPR/Cas9

The genomic sequence surrounding exon 5, which encodes the amino acid stretch at the beginning of the phosphatase domain of human Sac1, was analyzed for potential CRISPR/Cas9 targets *in silica* using the Cas9 design target tool (http://crispr.mit.edu) (Hsu et al., 2013). The Sac1 genomic sequence targeted by the predicted CRISPR gRNA with the highest "on-target score" is (with the PAM sequence underlined):

Sense strand: 5'-CCTTCCTAGCGATGCTAAACCAT-3',

Anti-sense strand: 5'-ATGGTTTAGCATCGCTAGGAAGG-3'.

The two sequences were synthesized and sub-cloned into a human codon-optimized Cas9 and chimeric gRNA expression plasmid that carries puromycin resistance, pSpCas9(BB)-2A-Puro (PX459), obtained from Addgene (Plasmid 48139) (Ran et al., 2013).

HeLa cells were transiently transfected with the Sac1 CRISPR/Cas9 plasmid. 24 hours after transfection, cells were supplemented with growth media containing puromycin (1.5 μg/mL) and incubated for 72 hours. Cells resistant to puromycin selection were then incubated with puromycin-free medium for 24 hours before harvesting for immunoblotting and imaging-based analysis.

DNA Plasmids

Sources of plasmids were as follows: M1 muscarinic acetylcholine receptor (M1R) (Bertil Hille, University of Washington, WA), EGFP-Rab7 (Bo van Deurs, University of Copenhagan), CH_{UTR}-mCherry (William Bement, University of Wisconsin-Madison), GFP-SNX9 (Kai Erdmann, University of Sheffield), GFP-GOLPH3 (Christopher Burd, Yale University), TGN46-GFP (Vas Ponnambalam, Leads University), GFP-CD-M6PR (Antonella De Matteis, Telethon Institute of Genetics and Medicine), VPS29-mCherry (Mark von Zastrow, University of California at San Francisco), YFP-FAM21 and YFP-WASH (Daniel Billadeau, Mayo Clinic), ST-mRFP (Jack Rohrer, University of Zurich), GFP-PH_{OSBP} (Tim Levine, UCL Institute of Ophthalmology), GFP-2xPH_{OSH2} (Tamas Balla, NIH), GFP-$P4C_{SidC}$ (Yuxin Mao, Cornell), Ruby-LifeAct (Roland Wedlich-Söldner, Max Planck Institute of Biochemistry), GFP-$PH_{PLC\sigma1,}$ iRFP-$PH_{PLC\sigma1}$, GFP-PIPK1γ-87, EGFP-Rab5, mRFP-Rab5, GFP-N-WASP, GFP-PI4KIIα, N-PH_{ORP5}-EGFP, N-PH_{ORP8L}-EGFP (our lab). The following plasmids were obtained from Addgene: GalT-EGFP (Plasmid 11929), mCh-Rab7A (Plasmid 61804), GFP-$P4M_{SidM}$ (Plasmid 51469).

To clone fluorescence-tagged VAP constructs, cDNA of VAPA and VAPB were PCR amplified from a total human brain cDNA library and sequence validated to correspond to the following GenBank entries: VAPA: NM_194434.2, VAPB: NM_004738.4. The following primers were used: EcoRI_VAPA_Fw, CAAGCTTCGAATTCGATGGCGTCCGCCTCAGGGGCCATGGCGAAG, BamHI_Stop_VAPA_Rv, GATCCGGTGGATCCCTACAAGATGAATTTCCCTAGAAAGAATCCAATG, EcoRI_VAPB_Fw, CAAGCTTCGAATTCGATGGCGAAGGTGGAGCAGGTCCTGAGCCTC, BamHI_Stop_VAPB_Rv, GATCCGGTGGATCCCTACAAGGCAATCTTCCCAATAATTACACCAAC. PCR products were ligated between EcoRI and BamHI for VAPA and VAPB in the pEGFP-C1 vector (Clontech) to generate EGFP-VAPA and EGFP-VAPB, and in the pmCherry-C1 (Clontech) to generate mCherry-VAPA and mCherry-VAPB. *P56S* mutation and *K87D M89D* double mutations in

VAPB were generated using a mutagenesis kit (QuikChange II XL, Agilent Technologies), with the following primers, respectively (targeted nucleotides are shown in lower case): VAPB_P56S_Fw, CAGCACCACGTAGGTACTGTGTGAGGtCCAACAGCGGAATCATCGATGCAGGGG, VAPB_P56S_Rv, CCCCTGCATCGATGATTCCGCTGTTGGaCCTCACACAGTACCTACGTGGTGCTG; VAPB_K87D-M89D_Fw, CCCAATGAGAAAAGTAAACACgacTTTgacGTTCAGTCTATGTTTGCTCC, VAPB_K87D-M89D_Rv, GGAGCAAACATAGACTGAACgtcAAAgtcGTGTTTACTTTTCTCATTGGG. The MSP domain construct was generated by PCR amplification from EGFP-VAPB using primers flanking the MSP domain of VAPB (KpnI_MSP-VAPB_Fw, GTCGACGGTACCGCGGTCCTGAGCCTCGAGCCGCAGCACG, BamHI_Stop_MSP-VAPB_Rv, CCGGTGGATCCCGGTCATTCAAACACACATCTAAG), and cloned into pEGFP-C1 vector between KpnI and BamHI.

GFP-PI4KIIβ was generated by PCR amplification from HA-PI4KIIβ (a kind gift from Tamas Balla) using the following primers: 5'_XhoI_PI4K2B, CTCAGATCTCGAGCTCATGGAGGATCCCTCCGAG, 3'_PstI_NonStop_PI4K2B, CCGTCGACTGCAGACCACCAGCACTCCAGGAGGAAAAAAATGGCTTCCTGC. PCR products were ligated between XhoI and PstI in the pEGFP-C1 vector (Clontech). To generate EGFP-SNX6, cDNA of SNX6 were PCR amplified from the total human brain cDNA library and sequence validated to correspond to the GenBank entry NM_152233.2, using the following primers: KpnI_SNX6_Fw, CGACGGTACCGCGGGCATGATGGAAGGCCTGGACGACG, BamHI_Stop_SNX6_Rv, CGGTGGATCCCGGTTATGTGTCTCCATTTAACACTGCC. PCR products were ligated between KpnI and BamHI in the pEGFP-C1 vector (Clontech). EGFP-SNX5 were generated by PCR amplified from cDNA clone of human SNX5 (Open Biosystems, clone ID: 7939468) using the following primers: KpnI_SNX5_Fw, CGACGGTACCGCGGGCATGGCCGCGGTTCCCGAGTTGCTGCAG, BamHI_SNX5_Rv, CCGGTGGATCCCGGTCAGTTATTCTTGAACAAGTCAATACAG). PCR products were ligated between KpnI and BamHI in the pEGFP-C1 vector (Clontech). OSBP-EGFP was generated by PCR amplified from cDNA clone of human OSBP (Open Biosystems, clone ID: 4560111) using the following primers: HindIII_OSBP_Fw, CTCGAGCTCAAGCTTATGGCGGCGACGGAGCTGAGAGGAGTGGTGGGGCCAG, BamHI_OSBP_Stop_Rv, CGACCGGTGGATCCCCGAAAATGTCCGGGCATGAGCTCCAGTCCTG. PCR products were ligated between HindIII and BamHI in the pEGFP-N1 vector (Clontech).

F18A, F28A and *F74A* mutations in SNX2 were generated using a mutagenesis kit (QuikChange II XL, Agilent Technologies), from wild type SNX2 as template with the following primers, respectively (targeted nucleotides are shown in lower case): SNX2_F18A_Fw, GCTGGGGGACGGGAAGCCCACCGACgcTGAGGATCTGGAGGACGGAGAGG, SNX2_F18A_Rv, CAGGTCCTCTCCGTCCTCCAGATCCTCAgcGTCGGTGGGCTTCCCGTCCCCC; SNX2_F28A_Fw, CTGGAGGACGGAGAGGACCTGgcCACCAGCACTGTCTCCACCCTAGAGTC, SNX2_F28A_Rv, CTTGACTCTAGGGTGGAGACAGTGCTGGTGgcCAGGTCCTCTCCGTCCTCC; SNX2_F74A_Fw, GATGATGACAGAGAAGATCTTgcTGCAGAAGCCACAGAAGAAGTTTC,

SNX2_F74A_Rv, CCAAAGAAACTTCTTCTGTGGCTTCTGCAgcAAGATCTTCTCTGTCATC). N-terminal alone truncation of SNX2 (YFP-SNX2$^{1-139}$) was generated by introducing a stop codon after the sequencing encoding the first 139 residues via mutagenesis, with the following primers (targeted nucleotides are shown in lower case): SNX2_NtermStop_Fw, GCAAATGGAGACATTTaaGACATAGAAATTGGTGTATCAGATCCAG, SNX2_NtermStop_Rv, CACCAATTTCTATGTCttAAATGTCTCCATTTGCTTCTTCTTCAATCTC). To generated iRFP-SNX2, cDNA of SNX2 was PCR amplified from YFP-SNX2, using the following primers: BspEI_SNX2_Fw, GAAGAGTCCGGAATGGCGGCCGAGAGGGAACCTCCTC, KpnI_Stop_SNX2_Rv, GATCCCGGGCCCGCGGTACCCTAGGCAATGGCTTTGGCTTCAGG. PCR products were ligated between BspEI and KpnI in the iRFP-FRB-Rab7 vector obtained from Addgene (Plasmid 51613).

siRNAs

Double-stranded siRNA were purchased from Integrated DNA Technologies with the following references: WASH (human WASH HSC.RNAI.N182905.12.1 from IDT), FAM21 (human FAM21 HSC.RNAI.N015262.12.2 from IDT), PI4KIIα (human PI4K2A HSC.RNAI.N018425.12.8), PI4KIIβ (human PI4K2B HSC.RNAI.N018323.12.1), OSBP (human OSBP HSC.RNAI.N002556.12.2 from IDT), SNX2 (HSC.RNAI.N003100.12.1 from IDT), Control (NC1 negative control duplex from IDT).

Antibodies and Chemicals

Antibodies obtained from the commercial sources include: rabbit polyclonal anti-VAPA (HPA009174, Sigma-Aldrich), anti-VAPB (HPA013144, Sigma-Aldrich), sheep polyclonal anti-TGN46 (AHP500GT, AbD Serotec), goat polyclonal anti-VPS35 (ab10099, Abcam), mouse monoclonal anti-EEA1 (clone 14, BD Tranduction Laboratories), mouse monoclonal anti-GM130 (clone 35/GM130, BD Tranduction Laboratories), rabbit polyclonal anti-p34 (07-227, EMD Millipore Corporation), mouse monoclonal anti-M6PR (IGF-IIR, clone 2G11, Santa Cruz), mouse monoclonal anti-clathrin heavy chain (clone TD1, American Type Culture Collection), mouse monoclonal anti-SNX2 (clone 13/SNX2, BD Transduction Laboratories), mouse monoclonal anti-c-Myc (clone 9E10, Santa Cruz), mouse monoclonal anti-GFP conjugated to HRP (Miltenyi Biotec), rat monoclonal anti-HA (clone 3F10, Roche), and mouse monoclonal anti-tubulin (clone B-5-1-2, Sigma-Aldrich). Alexa fluor phalloidin and Alexa fluor conjugated secondary antibodies were from Life Technologies. Rabbit polyclonal anti-PI4KIIα and anti-Sac1 antibodies were generated by our lab as previously described, respectively (Guo et al., 2003; Nemoto et al., 2000).

The following antibodies were kind gifts: rabbit anti-WASH and anti-FAM21 antibodies (Daniel Billadeau, Mayo Clinic), rabbit anti-PI4KIIβ (Helen Yin, UT Southwestern), rabbit anti-OSBP antibody (Hiroyuki Arai, University of Tokyo), rabbit anti-GRASP55, rabbit anti-Golgin97, mouse anti-P230, and rabbit anti-GRASP65 (James Rothman, Yale University),

Chemicals purchased from commercial sources include: puromycin, Oxo-M and Atropine (Sigma-Aldrich), Latrunculin B (Calbiochem), and PIK-93 (Selleckchem). VPS34 inhibitor VPS34-IN1 was obtained from Dr. Dario Alessi (University of Dundee). Compound A1 is a kind gift from Dr. Tamas Balla (NIH).

Fluorescence Microscopy

Fixed cells

Cells grown on glass coverslips (Neuvitro) were fixed with 4% PFA, washed in PBS, permeabilized with PBS containing 0.1% Saponin and 1% BSA, immunostained with designated antibodies in the same buffer and mounted using ProLong Gold anti-fade reagent (Invitrogen). Fixed cell samples were imaged by spinning disc confocal (SDC) microscopy. Images from a mid focal plane are shown.

Live cells

Cells were plated on 35 mm glass bottom dishes (MatTek Corp) at low density allowing attachment overnight, transfected and imaged with a SDC microscope 16-20 h after transfection. Before imaging, cells were transferred to imaging buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, and 3 mM D-glucose with pH adjusted to 7.4 with NaOH.

Imaging

Spinning disc confocal (SDC) microscopy was performed using the Improvision UltraVIEW VoX system (Perkin-Elmer) built around a Nikon Ti-E inverted microscope and a Hamamatsu C9100-50 camera, equipped with PlanApo objectives (60 x 1.49-NA) and controlled by Volocity (Improvision) software. Total internal reflection fluorescence (TIRF) microscope was performed on a setup built around a Nikon TiE microscope equipped with 60 x 1.49-NA objectives. Excitation light was provided by 488-nm (for GFP and blue-light activation), 561-nm (mCherry and mRFP) and 640-nm (iRFP) DPSS lasers coupled to the TIRF illuminator through an optical fiber. The output from the lasers was controlled by an acousto-optic tunable filter and fluorescence was detected with an EM-CCD camera (Andor iXon DV-887). Acquisition was controlled by Andor iQ software.

Image Quantification

Fluorescence signal was quantified using Fiji (http://fiji.sc/wiki/index.php/Fiji). Data were processed with Excel (Microsoft) and plotted with Prism6 (GraphPad). Each dot represents value from a single cell with the black bar as the mean.

Colocalization analysis

Colocalization analysis was carried out by manually selecting an area of 15 x 15 μm^2, correcting background, and measuring the Pearson's correlation coefficient using the Fiji colocalization plug-in Coloc2.

Quantification of the endosomal localization of PI4P probes

Cells were co-transfected with a Golgi complex marker (ST-mRFP) and with PI4P probes that recognize with high affinity the Golgi and endosomal PI4P (GFP-$P4C_{SidC}$) (Luo et al. 2015). The fluorescence intensity of the mRFP channel, which produced the most intense signal in the Golgi complex area, was set to define the Golgi area of each cell. A region covering the entire cell, but excluding the Golgi complex area and the cell edge, was then manually selected (ROI) and the mean fluorescence pixel intensity in the ROI was calculated. Data is normalized by dividing the fluorescence intensity of the ROI of each cell by the average fluorescence intensity of the ROI of control cells.

Quantification of the plasma membrane localization of the PI4P probe via line scans

For this analysis, the N-PH_{ORP8L}-GFP probe was used. This is a low affinity "sensor" of plasma membrane PI4P that remains primarily in the cytosol and even more in the nucleus under control conditions, but relocates to the plasma membrane in response to the increase of PI4P in this membrane (Chung et al., 2015). A line of 5 µm in length was manually drawn perpendicular to the plasma membrane (see dashed lines in **Figure 2F**, **2L**). Fluorescence pixel intensity along the line was calculated and normalized by dividing the fluorescence of each pixel by the average fluorescence intensity of the line in the cytosolic region.

Quantification of WASH and actin on endosomes

Cells were immunostained for WASH and EEA1 and stained with fluorescent phalloidin. Images were background corrected, and EEA1 fluorescence was used to define the area (ROI) occupied by early endosomal compartments. The fluorescence intensity of WASH immunoreactivity and of phalloidin within the ROI was then measured (see examples in the right fields in **Figure S7G**, **S7H**, and **S7I**). Data were normalized by dividing the fluorescence intensity value of each pixel by the average fluorescence intensity observed in the ROI of control cells.

Quantification of the traffic of internalized antibody directed against CI-MPR

Cells were incubated at 37ºC in serum-free DMEM containing 10 µg/mL mouse anti-CI-MPR mAb for up to 60 min, quickly rinsed with PBS, and then stained for the internalized antibodies by immunofluorescence as described above. Images were background corrected, and the total intracellular (internalized) fluorescence intensity was measured by manually selecting an area covering the entire cell. The fluorescence intensity within a 10 x 10 μm^2 region centered on the Golgi complex was then measured (see dashed boxes in **Figure 2H**). The non-Golgi fluorescence intensity was obtained by subtracting the fluorescence intensity in the Golgi complex from the total fluorescence intensity. Data was presented as the non-Golgi/Golgi CI-MPR fluorescence ratio from each cell.

Phosphoinositide Analysis

WT and VAP DKO cells at 60% confluency on 10 cm dishes were metabolically labeled with [^{3}H]myo-inositol (MP Biomedicals) in inositol-free DMEM (MP Biomedicals) for 48 h. Lipid extraction and HPLC analysis was performed as described previously (Nakatsu 2012; Chung 2015b). Briefly, cells were washed twice with PBS, lysed with 4.5% perchloric acid, collected by scraping and centrifuged into pellets. The pellets were rinsed with ice-cold 0.1 M EDTA, deacylated with a mixture of methylamine/water/*n*-butanol/methanol (36:8:9:47) for 1 h at 50ºC and dried in a SpeedVac (Savant). The residue was extracted using a mixture of *n*-butanol/petroleum ether/ethyl formate (20:40:1) and water. Deacylated phosphoinositides were then separated using high performance liquid chromatography (Shimadzu Scientific Instruments) and detected by an online flow scintillation analyzer (B-RAM, IN/US).

Immunoblotting

HeLa cells were lysed in buffer containing 2% SDS, 150 mM NaCl, 10 mM Tris (pH 8.0), and incubated at 60ºC for 20 min followed by incubation at 70ºC for 10 min. The lysates were treated with Benzonase Nuclease (Novagen) for 30 min at room temperature. Cell lysates were

processed for SDS-PAGE and immunoblotting with standard procedure. All immunoblotting were developed by chemiluminescence using the SuperSignal West Dura reagents (Thermo Fisher Scientific).

Immunoprecipitation

HeLa cells expressing the indicated constructs were washed in cold PBS and lysed on ice in lysis buffer [50 mM Tris, 150 mM NaCl, 1% digitonin, 0.5 mM EDTA, 10% glycerol, pH 7.4 and protease inhibitor cocktail (Roche)]. Cell lysates were then centrifuged at 21,000 g for 20 min at 4ºC. For anti-GFP immunoprecipitation, supernatants were incubated with Chromotek GFP-trap agarose beads (Allele Biotech) for 2 h at 4ºC under rotation. Subsequently, beads were washed in lysis buffer containing 1% digitonin once and 0.2% digitonin twice. Afterwards, immunoprecipitated proteins bound to the beads were incubated in PAGE sample loading buffer (containing 2% SDS) and then incubated at 60ºC for 20 min and 70ºC for 10 min. Immunoprecipitates were processed for SDS-PAGE and immunoblottings were carried out as described above.

Mass spectrometry analysis of VAP and SNX2 interactions

Plasmids

ORF clones of VAPB WT, VAPB FFAT, and SNX2 were sequentially cloned into the pDONR223 entry vector and the lentiviral destination vectors pHAGE-N-FLAG-HA-PURO and pHAGE-N-GFP-BLAST using λ-recombinase (Gateway system).

Cell culture and generation of stable cell lines

HEK293T and HeLa cells (ATCC) were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone) at 37°C/5% CO_2. Plasmid DNA and viral helper constructs (VSVG, TAT1B, MGPM2, CMV-Rev1B) at a 4:1 ratio (transgene:helper) were diluted in OptiMEM (Gibco), and combined with 1µg/µl polyethylenimine (PEI) (Polysciences Inc.) in a 3:1 ratio (PEI:total DNA). The PEI/DNA mix was added to HEK293T cells followed by incubation at 37°C/5% CO_2 for 16 hours. The DMEM media was refreshed and cells were incubated for an additional 24 hours for virus production. Viral supernatant collected from these cells supplemented with 1µl polybrene (8mg/mL) was used to infect HeLa cells. Stable HeLa cell lines were generated 48-72 hours post infection through the addition of selection media containing puromycin (1µg/mL) and blasticidin (10µg/mL).

Immunoprecipitation and CompPASS proteomic analysis

AP-MS and CompPASS analysis (using the Comparative Proteomics Analysis Software Suite) were performed as previously described (Sowa et al., 2009). For each immunoprecipitation, stable cell lines from 4x15cm dishes at 80% confluence were harvested in lysis buffer (50 mM Tris-HCl/pH 7.5, 150mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium glycerol 2-phosphate, 1mM sodium orthovanadate, 0.27M sucrose, 1% (v/v) NP-40, 1 mM DTT) supplemented with protease inhibitors (Roche) to produce whole cell extracts. Whole cell extracts were sonicated, clarified by centrifugation (16000xg for 10 minutes/4°C), and filtered through 0.45m filters (Sartorius). Immunoprecipitation was performed with 60µL/50% slurry anti-HA agarose resin (SIGMA) for 4 hours at 4°C. Beads were washed with lysis buffer (3x) and with phosphate buffered saline/pH

7.2 (PBS) (2x). The immunoprecipitated bait was eluted twice (30 minutes at 30°C each) with HA peptide (250 μg/ml) (Bio-Synthesis Inc) diluted in PBS. Eluates were reductively carboxymethylated and precipitated using 20% trichloroacetic acid (TCA).

TCA-precipitated proteins were resuspended in 200mM HEPES buffer with 200ng of sequencing grade trypsin (Promega) and incubated overnight at 37°C. Digestion was quenched with 5% formic acid (FA)/5% acetonitrile (ACN) and de-salted using C18 stage tips. Peptides were eluted with 5% FA/75% ACN, dried using a speedvac, and resuspended in 5% FA/5% CAN. Samples were processed in technical duplicate on an LTQ Velos (Thermo) mass spectrometer, and spectra search with Sequest prior to target-decoy peptide filtering, and linear discriminant analysis. Protein Assembler was used to convert spectral counts to average protein spectral matches (APSMs), which takes into account peptides that match more than one protein in the database. Peptides were identified with a false discovery rate of < 1.0%. The following MS2 conditions were used: Activation Type – Collision induced dissociation; Minimum Signal Required - 2000.0; Isolation width (m/z) - 1.00; Normalized Collision Energy - 35.0; Default Charge State – 2; Activation Q - 0.250; Activation Time (ms) - 10.000. Peptide data (APSM) were uploaded into the CompPASS algorithm. The CompPASS system identifies high confidence candidate interacting proteins (HCIPs) based on the normalized weighted D (NWD)-score, which incorporates the frequency with which they identified within the stats table, the abundance (APSMs, average peptide spectral matches) when found, and the reproducibility of identification in technical replicates, and also determines a z-score based on APSMs (Sowa et al., 2009).

Immunoprecipitation and quantitative/TMT proteomic analysis

For quantitative proteomic analyses, AP-MS was performed using three independent biological replicate samples. Stable HeLa cell lines were harvested as described in the previous section. Each immunoprecipitation was performed using 60μL/50% slurry anti-FLAG magnetic beads (SIGMA) for 4 hours at 4°C. Beads were washed with lysis buffer (3x) and with elution buffer (50mM Tris/pH 8.0, 150mM NaCl) (2x). The immunoprecipitated bait was eluted twice (10 minutes at 25°C each) with 3xFLAG peptide (250 μg/ml) diluted in elution buffer. Eluates were precipitated and digested as previously described. Peptides derived from the various samples (triplicate independent immunoprecipitation per bait) were subjected to 6-plex TMT analysis using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a Proxeon EASY-nLC II liquid chromatography (LC) pump (Thermo Fisher Scientific), using multinotch MS3 (McAlister et al., 2014; Ting et al., 2011) with synchronous-precursor-selection (SPS) where precursor ions (n=10) were collected. For quantification, a 0.03 *m/z* window centered on the theoretical *m/z* value of each of six reporter ions and the closest signal intensity from the theoretical *m/z* value was recorded. Total signal to noise values for all peptides were summed for each TMT channel, and all values were adjusted to account for variance in sample handling. The peptide intensities were normalized to the bait. For each peptide, a total minimum signal to noise value of 100 was required.

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