Supplementary Information for

**Functional characterization of 67 endocytic accessory proteins using multi-parametric quantitative analysis of CCP dynamics**

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This PDF file includes:

- Materials and Methods
- Figures S1 to S8
- Tables S1 to S2
- Legends for Datasets S1 and S2
- SI References

Other supplementary materials for this manuscript include the following:

- Supplemental Dataset S1 (separate file)
- Supplemental Dataset S2 (separate file)
Materials and methods

Cell line generation

ARPE19 cells were obtained from ATCC and cultured in DMEM:F12 (1:1) medium (Gibco 11330-032) supplemented with heat inactivated 10% FBS (Sigma F0926). To generate a stable cell line expressing nuclease deficient Cas9, ARPE19 cells already expressing the CCP fiduciary marker eGFP-CLCa (1), were transduced with mammalian lentiviral expression vector pMH0001 (Addgene plasmid #85969) encoding the dCas9-BFP-KRAB fusion protein downstream of a SFFV promoter. The BFP signal was used as selection marker for dCas9-KRAB expressing cells. The minimal UCOE (ubiquitous chromatin opening element) sequence (2) was also included to effectively prevent silencing of juxtaposed heterologous promoters (Muller-Kuller et al., 2015). eGFP and BFP positive cells were selected by FACS. Cells with medium expression levels of BFP and eGFP were then expanded for 2 weeks and frozen in multiple aliquots to be used for imaging so as to avoid variations originating from passage number.

sgRNA Production and cell transduction

CRISPRi knockdown was achieved using two different sgRNA sequences targeting individual EAPs. Target sequences with the highest confidence score were obtained from a human CRISPRi library (3). All sgRNA target sequences localize in the range of -50 to +50 of the transcript start site. The list of sgRNA sequences for EAPs is included in Sup. Table 1. Scrambled sgRNA sequences used were: 5’TTGGCTATGAAGAGATACTC3’ and 5’AATTCTCCGAACGTGTCACG3’. sgRNA were cloned into a modified pU6-sgRNA-EF1α-puroT2A-BFP viral expression vector (#60955, Addgene). Sequences corresponding to T2A and BFP in the original vector were removed by seamless cloning using two primers: 5’GGTGCCGGATCGGGATAAGAATTCGTCGAGGGA3’, and its reverse complement. The Puromycin cassette was retained to be used as a selection tool. sgRNA sequences targeting EAPs were introduced at the BstXI/BspI enzyme sites in the pU6 modified vector following the format: 5’ U6 -> target sequence 1 -> SunCas9gRNA scaffold -> CCTTCAC spacer -> U6 -> target sequence 2 -> SunCas9gRNA scaffold 3’. The cloning of guide sequences was outsourced from Genepharma Co. Ltd. After constructs were received, each was re-transformed, purified, and checked by PCR for presence of the sgRNAs cassette. Scrambled sgRNA control vector was generated by gBlock (IDT) and inserted by ligation using the same BstXI/BspI sites. As an extra assurance, 10 random sgRNA constructs were sequenced using the following primers to verify the presence of expected target sequences: 5’TAGAGATCCGACGCACCACATC3’ and 5’TGCATGGCGGTAATACGGTT3’. A subset of sequenced vectors was then used for preliminary viability, and protocol development experiments (data not shown).

Virus production was done following standard protocols. Briefly, HEK cells were transfected (Lipofectamine 2000, Invitrogen) with individual sgRNA-carrying transfer plasmid along with packaging and envelope plasmids. After 24 hrs, medium was replaced, and virus collected after 48 h. To reduce variability, 10 mls of each virus was produced in sets of 10 EAP targets per production, and frozen in 1 ml aliquots for single use in experiments to avoid loss of viral infectivity due to repeated freeze-thaw cycles. All negative control (sgScrambled) and positive control (sgCALM) viruses were produced in one batch.

For transduction, double labeled eGFP-CLCa;dCas9-BFP-KRAB cells were thawed and allowed to recover for 1 week before starting experiments and used for a maximum of 3.5 weeks to avoid any potential loss of dCas9-BPF-KRAB expression and to avoid previously observed cleavage of eGFP-CLC. For pU6 lentivirus transduction, 180,000 cells were seeded into 6-well plates and allowed to attach for 14-16 hrs. 500 µl of fresh media was then added to the cells followed by 500 µl of virus, and 1 µg/mL of Polybrene. Cells were then spin-infected for 30 minutes
at 800 RPM. Another 1 ml of fresh media was added and transduced cells were incubated for 24 hrs at 37°C. Then, media was replaced with fresh media containing 2 µg/ml puromycin and cells were maintained under selection for 48 hrs. The cells surviving puromycin selection were then seeded on ultra-clean gelatin-coated coverslips treated as described below.

**siRNA-mediated knock down**

siRNAs sequences used in this study are listed in Table S2. siRNAs were purchased as Dharmacon ON-TARGETplus smart pools or as individual siRNAs. Lipofectamine RNAi Max (ThermoFisher Scientific 13778150) was used as transfection reagent according to companies' instructions. In brief, 180,000 cells were seeded/well in a 6-well plate for each condition the day before adding siRNA (day 0). Cells were subjected to a double round siRNA treatment on day 1 and day 2, including an “Allstarts” negative control sequence (Qiagen 1027281). 30 min prior to transfection, culture media was replaced with 2 ml of fresh medium. Concomitantly, 5.5 µl of 20 µM siRNA (diluted in 100 µl of OptiMEM) was added to a tube containing 6.5 µl of transfection reagent (diluted in 100 µl OptiMEM). The mixture was gently vortexed and incubated at RT for 20-30 min. The mixture was then gently pipetted up and down before adding dropwise to the cells. On day 3, cells were trypsinized and seeded on gelatin coated coverslips for TIR-FM imaging on day 4.

**Western blotting**

EAPs that failed to show any phenotype after performing CRISPRi-mediated knockdown were checked for their knockdown efficiency by western blot. Briefly, cells were washed with cold PBS and lysates were collected using ice-cold lysis buffer (50 mM Tris, 2 mM EGTA, 150 mM NaCl, 1% NP40, 10 mM NaF, 2 mM Na vanadate, 0.25% Na deoxycholate, 1 tablet of complete protease inhibitor cocktail/50ml lysis buffer, pH 7.4). Cell lysates were then denatured and loaded on Mini-PROTEAN® TGX Stain-Free™ Protein Gels and probed with the relevant antibody following semidry transfer onto nitrocellulose membranes. Antibodies used were as follows: anti-CALM (Abcam #ab172962), α-adaptin (Thermo Fisher #MA3-061), Amph1 (Santa Cruz #SC376397), dynamin2 (Santa Cruz #SC6400), EPS15 (Santa Cruz #SC-534), intersectin2 (Abnova #H00050618-A01), Snx9 (Atlas antibodies #HPA031410), Myo6 (Santa Cruz #SC50461), cofilin1 (Cell Signaling #5175S), cortactin (Millipore #05-180), AAK1 (Abcam #ab134971), DENND1A (Thermo Fisher #PA5-70324), DENND1B (Abcam #ab187903), Arp3 (Santa Cruz #SC48344), intersectin1 (Abcam #ab118262), GAK (Abnova #H00002580-M01). Loading controls were either western blots for actin or prominent cell lysate proteins visualized with the help of trihalo UV gels (Stain-Free gels BioRad). Signals were detected using chemiluminescence or infrared fluorescence.

**Transferrin receptor internalization**

In cell ELISA-based assays for TfnR internalization were performed using D65-anti-TfnR mAb as ligand, exactly as previously described (4).

**Coverslip preparation and cell seeding**

To prevent any potential problems of cell attachment due to protein kd as well as to minimize the presence of clathrin plaques, cells were seeded onto gelatin-coated cover glass for imaging. Briefly, 22x22 mm glass coverslips (#1.5; Corning #2850-22) were incubated at 50-60°C in 1M HCl for 16 hrs and allowed to cool to RT. After removing HCl by multiple ddH2O washes, coverslips were immersed in ddH2O and sonicated 2-3 times for 30 min each. Water was gradually replaced with increasing concentration of EtOH (50%-->70%-->95%) while sonicating in each EtOH solution for 30 min. Finally, cleaned coverslips were stored in 95% EtOH. Before use, coverslips were coated with gelatin. Briefly, coverslips were dried by flaming and placed into 6-well plates. After washing with PBS to remove any residual EtOH, coverslips were treated with sterile
0.01% poly-L-lysine (Sigma #P8920) for 10 min at RT. After washing 3 times with PBS, coverslips were then incubated with a gelatin solution (2% sucrose/0.2 mg/ml gelatin (Sigma #G6650) in PBS) for 5 min at RT. The gelatin solution was removed, and coverslips were allowed to air dry for 5 min at RT. Gelatin was then fixed with 0.5% formaldehyde (Electron Microscopy Sciences #15710) for 15 min at 4°C. The coverslips were thoroughly washed with PBS and incubated in the presence of FBS-containing DMEM:F12 media for ~2 hrs at 37°C to neutralized residual PFA, if any. Approximately 300,000 cells were seeded on gelatin coated coverslips at least 12-16 hrs before TIR-FM imaging.

**TIR-FM imaging**

On the day of TIR-FM imaging, cells were checked for appropriate spreading and density. Fresh media was added at least 30 min before imaging each slide-mounted coverslip. To minimize experiment-to-experiment variation during image acquisition, all microscope components were primed by a 30-min mock acquisition: pre-determined laser and camera settings were used for acquisition without a sample on stage. These settings were defined prior to the screen to allow for sufficient signal intensity over background while keeping photobleaching <10% over the course of the acquisition time. For DASC analyses it is important to image with the highest possible intensity, while still avoiding photobleaching. During imaging, cells were maintained at 37°C in the same culture media.

Two TIR-FM systems were used for acquisition of live cell data: #1 fully motorized Nikon Eclipse Ti-E inverted microscope with integrated second-generation Perfect Focus System, 60x Nikon 1.49NA TIRF DIC objective, coupled to an Andor “Diskovery TIRF/ Borealis widefield illuminator” equipped with an additional 1.8x tubelens (yielding a final magnification of 108x), motorized laser incident angle adjustment, set to 80 nm penetration depth, and an Andor laser launch. Images were acquired with a PCO-Edge 16 bit, 2560x2160px sCMOS camera. Temperature was maintained via an OKO lab custom built full body environmental chamber with temperature control and CO₂ stage incubator operated by Bold Line controller and OKO-Touch with SmartBox for data logging. Components were controlled via MetaMorph v.7.7 (Molecular Devices). TIR-FM system #2 is closely related, but uses an Andor Zyla 4.2 16 bit, 2048x2048 px sCMOS camera.

Time-lapse movies for all conditions were acquired for 7.5 min with a 1 sec interval between consecutive frames. Given the storage size of a single movie (up to 3.6Gb), image stacks were split into two along the largest xy dimension using a custom-written Fiji macro, yielding a total of 22-24 datasets per condition.

**Quantitative analysis of CCP dynamics**

CmeAnalysis and DASC are freely available for download at: [https://github.com/DanuserLab/cmeAnalysis](https://github.com/DanuserLab/cmeAnalysis). Image analysis was carried out using our cmeAnalysis (1, 5, 6) and DASC (7) analysis pipelines as previously described.

**EAP data clustering**

Cluster analysis was based on the initiation densities of sCLs, initiation densities of *bona fide* CCPs, lifetimes of *bona fide* CCPs and their percentage relative to all valid traces. All computations were done using the factoextra package in R. APC clustering was calculated using the apcluster package in R, creating a pairwise protein similarity matrix based on negative distances (r=2). Finally, the K-means and APC clustering results were visually compared using an alluvial diagram generated by RAWgraphs ([https://rawgraphs.io/](https://rawgraphs.io/)). The script used for the EAP
phenotypic clustering is available at https://github.com/bioinformatics-jeonlee/EAPs_Phenotypic_Clustering.

Determination of relative levels of AP2α1 and AP2α2 isoforms

Mass spectrometry was performed exactly as previously described (4). Relative abundance of AP2α1 vs AP2α2 was calculated based on the recovery of 3 unique peptides.
Figure S1: Experimental and analytical pipeline (A) ARPE-19 cells stably expressing eGFP-CLCa, as a fiduciary marker to follow CCP dynamics, and dCas9-BFP-KRAB for sgRNA-mediated protein kd were transduced with lentiviruses coding for 2 sgRNA against the protein of interest, as well as a puromycin cassette for transduced cell selection. (B) A strict experimental timeline was followed, starting with fresh cells seeded in the morning of day 0. 24 hrs later, cells were transduced or not with lentivirus and puromycin selection was started the morning of day 2. On day 4, medium was replaced with standard culture medium. After confirming effective puromycin selection by comparing un-transduced cells with sgCALM lentivirus transduced cells (positive control), cells were seeded onto two gelatin-coated cover glasses in the afternoon of day 4 to be imaged the following day. Cover glasses were removed for imaging and cells that remained in wells were lysed and stored at -80°C. TIR-FM live cell data was acquired as described in Methods.
Figure S2: Knockdown efficiencies of representative EAPs. Western blots showing knockdown efficiencies of the indicated proteins. Either actin or a prominent band detected by trihalo stain free gels served as a loading control.
Figure S3: Validation of CALM as positive control. (A) Western blot visualizing the efficacy of sgRNA/dCas9-mediated kd of CALM in ARPE19 cells and (B) corresponding quantification. (C) Change in cell morphology upon CALM kd. Arrowheads indicate presence of large lamellipodia devoid of CCPs. (D) Lifetime distributions of bona fide CCPs in all CALM kd conditions (red) and their corresponding control cells, treated with scrambled sgRNAs (black). (E) Bar graph illustrating the fraction of bona fide CCPs with lifetimes above (green) or below (red) the full width half maximum (FWHM) of the corresponding control lifetime distribution. As shown, kd of CALM consistently shifts the lifetime distribution of bona fide CCPs towards longer lifetimes, leading to an increased fraction of CCP above the FWHM of the corresponding control lifetime distribution. (F) Initiation density of all valid traces in all CALM kd conditions (white) and their corresponding control cells, treated with scrambled sgRNAs (grey). Box and whiskers plots show median and 10 to 90 percentiles. Each box contains data from at least 14 (CALM) or 22 (scramble) datasets, each containing 2-5 cells. p-values: ***: p<0.001.
Figure S4: cmeAnalysis vs DASC. (A) Intensity profile of a representative persistent/non-terminal trace. (B) Montage of clathrin-coated structure shown in (A). (C) cmeAnalysis results are based solely on valid traces, whereas DASC draws data from a larger pool of intensity traces to calculate the initiation density of clathrin structures (CS). However, %ACs (abortive coats) and CCPs are determined from valid traces. (D) Trace-classes overlaid to raw data (zoom in). (E) Comparison of the effect of EAP kd on the percentage of CCPs as determined by cmeAnalysis and DASC. A subset of conditions was analyzed using DASC to compare with the corresponding results from cmeAnalysis. The conditions are plotted as black dots on a panel with x and y axis corresponding to the percentage difference in CCP% (ΔCCP%) obtained from DASC and cmeAnalysis, respectively. The correlation between the two ΔCCP% results is interpreted as a linear regression fitting (blue solid line). Conditions that are inconsistent (fitting residue above 80th percentile; dotted lines) are marked in red.
Figure S5: Effect of EAP kd on the percentage of valid traces and *bona fide* CCPs. Bar graphs sorted from strongest decrease to strongest increase effect of EAP kd on (A) the percentage of valid traces relative to all detected traces, and (B) on the percentage of all *bona fide* CCP traces relative to all valid traces.
Figure S6: Effect of EAP kd on the initiation densities of valid traces, sCLSs and CCPs Bar graphs sorted from strongest decrease to strongest increase effect of EAP kd on (A) initiation density of valid traces, (B) initiation density of sCLSs, and (C) initiation density of bona fide CCPs.
Figure S7: Effect of EAP kd on the mean lifetimes and maximum intensities of CCPs. Bar graph sorted from strongest decrease to strongest increase effect of EAP kd on (A) the mean lifetimes of bona fide CCPs, and (B) maximum intensities of CCPs.
Figure S8: Differential role of AP2 subunits during early stages of CCP formation (A) Amino acid sequence comparisons of AP2α1 and AP2α2 as determined using using LALIGN, https://www.ebi.ac.uk/Tools/psa/lalign/. Initiation densities of (B) sCLSs and (C) bona fide CCPs. (D) Percentage of CCPs relative to all valid traces. (E) Mean lifetimes of bona fide CCPs. AP2α1 and AP2α2 were individually targeted by sgRNAs and depleted using CRISPRi. (F) Lifetime distribution of bona fide CCPs after CRISPRi kd of AP2α1 and AP2α2 a and siRNA kd of µ2. As for siRNA kd of both α isoforms, kd of µ2 resulted in a sharpening of the lifetime distribution of CCPs. Bona fide CCP analyzed: controls for (AP2α1, AP2α2) and AP2 µ2: (13,410) and 22,897, respectively; AP2 α1: 7,442; AP2 α2: 15,627; AP2 µ2: 25,046.
Figure S9: Effect of single- and double-round siRNA-mediated kd of GAK on CCP dynamics measured by cmeAnalysis and DASC. (A) Western blot showing kd efficiency of GAK after single or double treatment with siRNA. (B- E) cmeAnalysis of the dose-dependent effects of GAK kd on (A) initiation densities of sCLSs and (B) bona fide CCPs. (D) Lifetime distribution of bona fide CCPs and (E) Percentage of bona fide CCPs scrambled: 212,004; GAK: 191,542 from 3 biologically independent experiments. (F) Effect of GAK kd on total surface-bound transferrin receptor (TfnR). Increased surface TfnR reflects a disproportionate defect in TfnR internalization vs. recycling. (G) Effect of GAK kd on rates of TfnR internalization.
Figure S10: A role for R-SNARE proteins and their adaptors during early stages of CCP formation. (A). Percent difference plot of initiation density of sCLSs, initiation density of *bona fide* CCPs, percent CCPs and mean lifetimes of CCPs. Results are expressed as %Δ relative to the experimental control. Initiation densities of (B) sCLSs and (C) *bona fide* CCPs. (D) Percentage of CCPs relative to all valid traces. (E) Lifetime distribution of *bona fide* CCPs. (F) Mean lifetimes of *bona fide* CCPs. *Bona fide* CCP analyzed: controls Vamp3, Vamp 7 and Vamp8: 95,384, 50,116 and 104,785, respectively; Vamp3: 79,070; Vamp7: 74,232; Vamp8: 80,769.
### Supplemental Table 1: sgRNA sequences

| EAP   | Gene name   | Sequence 1                  | Sequence 2                  |
|-------|-------------|-----------------------------|-----------------------------|
| AAK1  | AAK1        | GGGCCTGCAGCGACAGAGAAGAGG    | GCTGCAGCGACAGAGAAGAGG       |
| Abp1  | DBNL        | GCATGGCGCCAGACCTGAGC        | GCGGCAGACCTGAGCCGGGAAG      |
| Amphiphysin1 | AMPH1    | GCCATGGCCAGACATCAAGAC      | GCGAAGAGACAGAGCGCGAG        |
| APPL1 | APPL1       | GGGCCCGCCCTCTCCGGAGAAGG     | GCTGCAGCCCTTTGGCGGGAGA      |
| APPL2 | APPL2       | GGGCCCGCGGCGGCGGAGAGG      | GGGCGCGGCGGAGGCCGCT         |
| Arf6  | ARF6        | GCGGCCGGCCAGAAGAAGAAGG     | GCGGCGGAGGAAGGTTGGGGAGA     |
| Arp2  | ACTR2       | GGGGGGCGGGAGAGAAGAAGG     | GAAAAGTTGGGCGAAAGGAG        |
| Arp3  | ACTR3       | GACGGTGCAGGAGAGAAGG      | GTGCGAGAGAAGGTTGGGGAGA      |
| β-arrestin | ARRB1   | GACGGTGCAGGAGAGAAGG     | GTGCGAGAGAAGGTTGGGGAGA      |
| β2-adaptin | AP2B1   | GCTGGGTCGGAGACCCGGAGG     | GCGGAGGAAAGGGAAGAAGG        |
| CALM  | PICALM      | GTCGCGCTTCACTACAGTTCC      | GCTCGGATCACCCGGCGAGGAGT     |
| CLCa  | CLTA        | GGAAGCCCTACCCGGGTATC       | GCTGGTCTTCCCTCTCCAGT        |
| CLCb  | CLTB        | GGAGAGCAGGTGCCGGGAGG       | GGCTGTCAGCCGCGGAGGCCCGG     |
| cofillin1 | CFL1      | GTTCAGTCGGGTCCCCGGAGG     | GTCCGGAAAAAAGGAAAGGAG       |
| cortactin | CTTN      | GGCTGCGGCAGCGCGAATCCA     | GCGGAGACCAGCCAGCGCGG        |
| Dab2  | DAB2        | GCACTCCCGCGGAGAGAAGG     | GCGGCTCAGAGCTCCGGCGG        |
| DENND1A | DENND1A  | GCTGGAGACCCTAGTGCTCCC     | GACATGGGCGTTGGCGGCGG        |
| DENND1B | DENND1B  | GGGCCGTCGCGGGGAGAAGG      | GCGGCTAAGCGCGGAGGCCG        |
| dynamin-1 | DNM1      | GCCGCGGAGCGAGGAGGAGG      | GCCGCGGAGCGAGGAGGAGG        |
| dynamin-2 | DNM2      | GGATCTGCGGAGGAGGAGG      | GCCAGGTCAGGAGGAGGAGG        |
| endophilin A1 | SH3GL2  | GCGGCCGCGCGAGGAGGAGG      | GCTGGGAGCTAGCCCGGAGG        |
| endophilin A2 | SH3GL1  | GCGGCCGCGCGAGGAGGAGG      | GCGGAGCGTACGTGGCCGGAGG      |
| endophilin A3 | SH3GL3  | GCGGCCGCGCCGCCGGCGGCT     | GGTTGAGGTGGACGTGAGCAG       |
| Eps15 | EPS15       | GACCTGTGCAGAGAGAGG       | GACCGGCGGGCGCAAGCGCAG       |
| Eps15R | EPS15L1     | GCGAGTCCGCAGCGGAGATGG     | GCGGCCAGTCCCGCGGAGAAGAGA    |
| epsin1 | EPN1        | GGCACGCAGACGCGAAGAGG      | GTTGTCCTCCCTGCTCCCGG        |
| Gene      | Accession | Forward Sequence      | Reverse Sequence      |
|-----------|-----------|-----------------------|-----------------------|
| epsin2    | EPN2      | GAGGTGAGCCACAGCCCGTG  | GAGGCCAGAGCGAGTCCCG   |
| epsin3    | EPN3      | GAGCGCCAGGCACACTCGG   | GGGAGGTCAGACCCCTCAT   |
| FCHO1     | FCHO1     | GCAGCAGCCGACAGCCAGG   | GTCAGCAGCAAGCGCCGAGG  |
| FCHO2     | FCHO2     | GTGCTGTCTCCGCGACCGG   | GCGAGCCGGAGATCCCTAT   |
| GAK       | GAK       | GTCAGCAACCAGCGGCAGG   | GGAGCTGACCCGAGCGGAG   |
| Grb2      | Grb2      | GTGCTGTCTCCGCGACCGG   | GCGAGCCGGAGATCCCTAT   |
| Hip1      | HIP1      | GAGAGCCGTGGGATCCGGG   | GAGCCACGAGGGCAGGAGG   |
| Hip1R     | HIP1R     | GGACTGACAGCTCACGGG    | GCTGGGCTGCGGACGGGAT   |
| HSC70     | HSPA8     | GCAGGCGAGGTCTAAGAG    | GGTATCGTGAGGCAGGGAG   |
| intersectin1 | ITSN1   | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| intersectin2 | ITSN2   | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| Myosin6   | MYO6      | GAGCCCGTGAGGAGGAGGAG  | GCGAGCCGGAGATCCCTAT   |
| Myosin1e  | MYO1E     | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| NECAP1    | NECAP1    | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| NECAP2    | NECAP2    | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| Nme1      | NME1      | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| Numb      | NUMB      | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| OCRL1     | OCRL      | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| PIK3Cα    | PIK3CA    | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| PIK3Cβ    | PIK3CB    | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| PIK3Cδ    | PIK3CD    | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| PIK3Cγ    | PIK3CG    | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| PIP5Kα    | PIP5K1A   | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| PIP5Kβ    | PIP5K1B   | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| PIP5Kγ    | PIP5K1C   | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| Rab35     | RAB35     | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| Rab4A     | RAB4A     | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
| Rab5A     | RAB5A     | GAGCTGAGAGGAGGAGGAG   | GCGAGCCGGAGATCCCTAT   |
### Supplemental Table 2: List of siRNAs

| Protein            | Product code | Source                          |
|--------------------|--------------|---------------------------------|
| Abp1/DBNL          | L-016867-00-0005 | Dharmacon SMARTpool            |
| ARH/LDLRAP1        | L-013025-00-0005 | Dharmacon SMARTpool            |
| INPPL1             | L-004152-00-0005 | Dharmacon SMARTpool            |
| OCRL1              | L-010026-00-0005 | Dharmacon SMARTpool            |
| AAK1               | L-005300-02-0005 | Dharmacon SMARTpool            |
| HIP1               | L-005001-00-0005 | Dharmacon SMARTpool            |
| VAMP7              | L-020864-00-0005 | Dharmacon SMARTpool            |
| HIP1R              | L-027079-00-0005 | Dharmacon SMARTpool            |
| AP2 α-adaptin      | 5'-GAGCAUGUGCAACGCUGGCCA-3' | Sigma                       |
| HRB/ArfGap1        | L-011873-00-0005 | Dharmacon SMARTpool            |
| GAK                | L-005005-00-0005 | Dharmacon SMARTpool            |
| DENND1B            | L-016923-02-0020 | Dharmacon SMARTpool            |
| GRB2               | J-019220-07-0002 | Dharmacon ON-TARGETplus-Individual siRNA |

| Protein            | Product code | Source                          |
|--------------------|--------------|---------------------------------|
| Rab5B              | RAB5B        | GACAGGCTTGGGGCGACGG             |
| Rab5C              | RAB5C        | GGGGGCGCGGGCGGTTACTTG           |
| SHIP2              | INPPL1       | GGCCCGGGCTTTGAGGATCCCG         |
| Snx18              | SNX18        | GAGTCGGCGCTGCGAACTTG           |
| Snx9               | SNX9         | GGGAGTAGCCGAGCGCCAGAAG         |
| Synaptojanin1      | SYNJ1        | GCTGCTGCGCCAGACACCAC          |
| Synaptojanin2      | SYNJ2        | GGCAGCGACGCGCCGGAGGAG          |
| Syntaxin4          | STX4         | GCCCGAAAGGTTTGACTACAGGT       |
| Syntaxin7          | STX7         | GTGAGGGCTCCGGGGATTAGGGT       |
| TTP                | SH3BP4       | GGGCTCCCGGGACTACCGG          |
| VAMP3              | VAMP3        | GCGGCAGCGCGCCACGAGAGA         |
| VAMP8              | VAMP8        | GGTGAACGTGAGGCCACCTT           |

**Source:** Sigma
| SYNJ2  | L-012624-00-0005 | Dharmaco SMARTpool |
|--------|-----------------|-------------------|
| Dab2   | SASI_Hs01_00161135  
  SASI_Hs01_00161136 | Sigma |

**Supplemental Dataset 1 (separate file):** Excel spread sheet providing raw cmeAnalysis results for controls and each EAP knockdown condition. Parameters measured include initiation density for all traces, initiation density for valid traces, percent valid traces, initiation density of sCLS, initiation density of CCPs, percent CCPs, mean CCP lifetimes, maximum intensities and density of persistent structures.

**Supplemental Dataset 2 (separate file):** Excel spread sheet providing percent difference values from cmeAnalysis for each EAP knockdown condition relative to scrambled sgRNA or siRNA control. Parameters measured include initiation density for all traces, initiation density for valid traces, percent valid traces, initiation density of sCLS, initiation density of CCPs, percent CCPs, mean CCP lifetimes, maximum intensities and density of persistent structures.

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