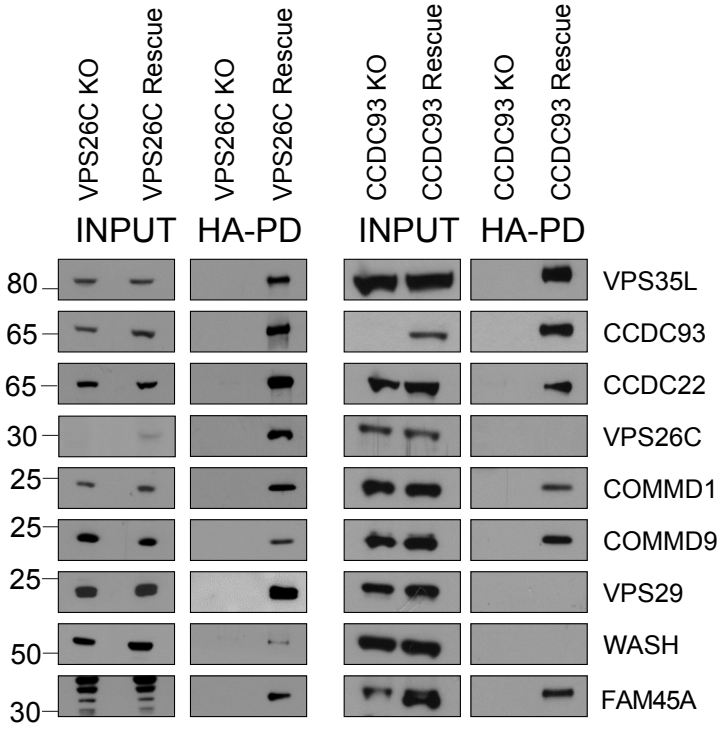
Supplementary information

Endosomal PI(3)P regulation by the COMMD/CCDC22/CCDC93 (CCC) complex controls membrane protein recycling

Singla, Fedoseienko et *al., 2019*

Supplementary figure 1

a



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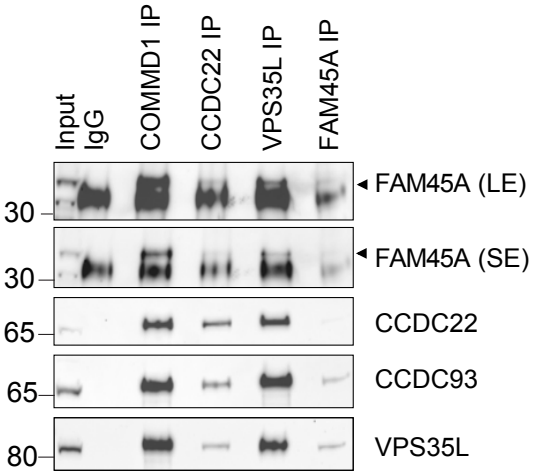
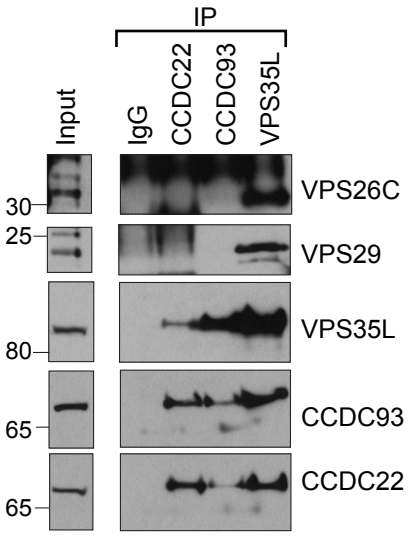


c

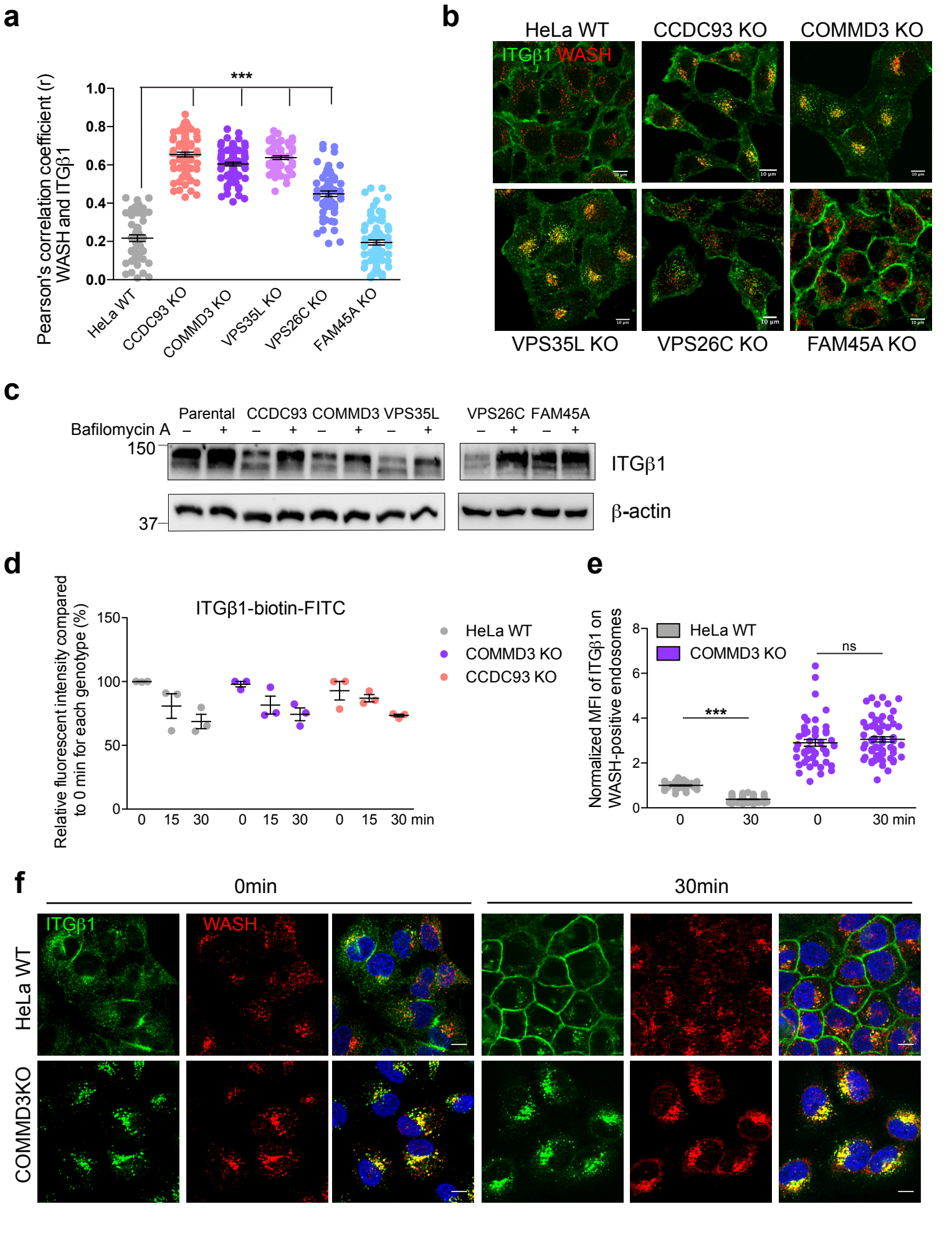
CCC component	Relative representation
COMMD10	100%
COMMD6	100%
COMMD8	100%
FAM45A	100%
COMMD1	87%
CCDC22	70%
COMMD2	70%
COMMD3	70%
COMMD7	67%
COMMD9	67%
COMMD4	65%
VPS29	65%
CCDC93	52%
KIF2B	44%
VPS35L	43%
COMMD5	43%
VPS26C	35%
FAM167A	31%
E2F6	25%
TRIM16L	25%
CTAGE5	19%
CCDC151	16%
TFDP1	16%
VPS33B	12%

d



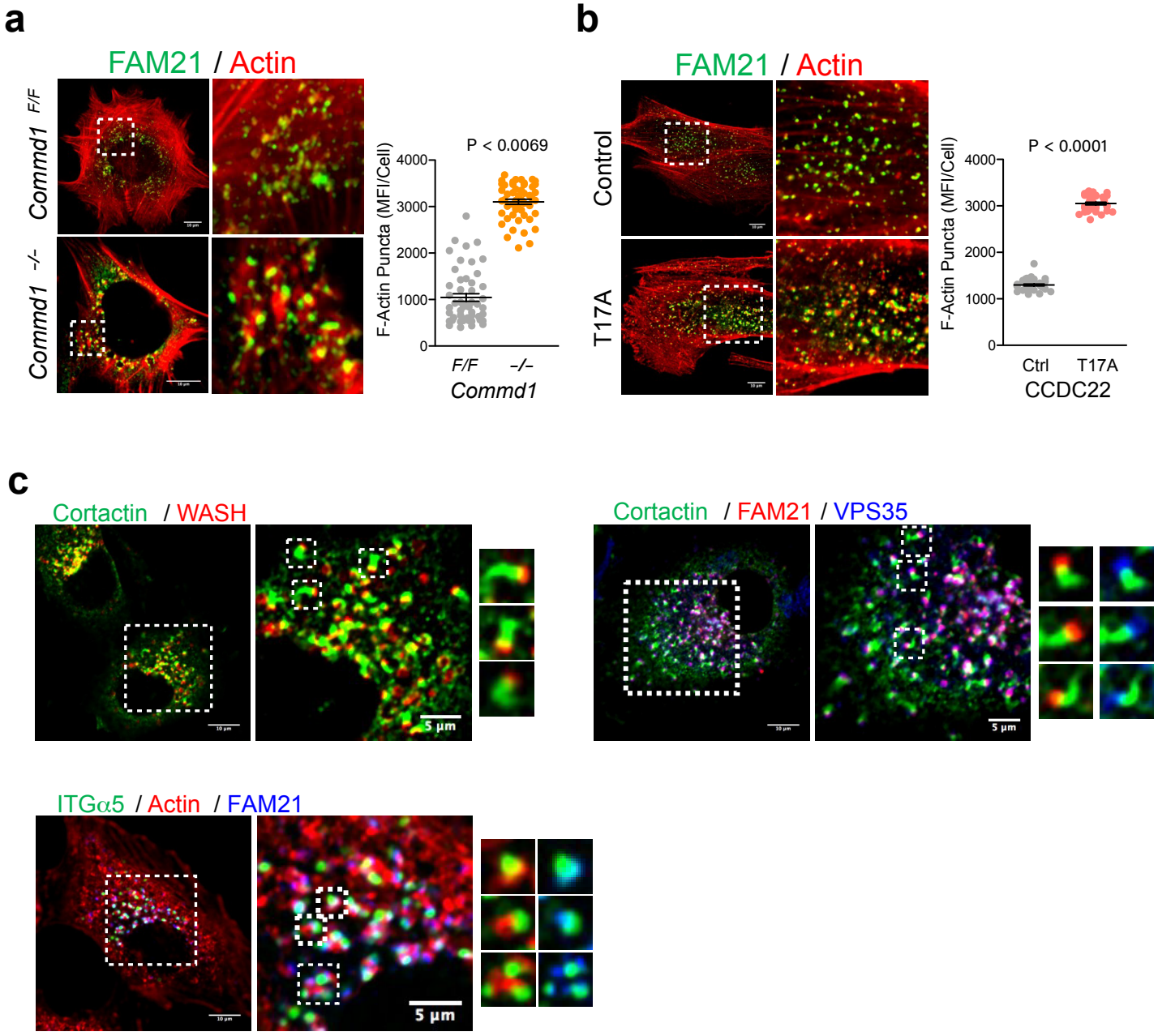
Supplementary figure 1. CCC and Retriever interactome analysis. **(a)** Immunoprecipitation and immunoblotting in VPS26C and CCDC93 CRIPSR/Cas9 KO lines, which were then stably transduced with an empty vector (EV) or HA-tagged versions of VPS26C or CCDC93. Immunoprecipitation of VPS26C or CCDC93 from cell lines was followed by immunoblotting for the indicated proteins. Representative of 2 independent iterations. **(b)** Immunoprecipitation of the indicated proteins (COMMD1, CCDC22, VPS35L, FAM45A) from HEK293T cell lysates, followed by immunoblotting. LE, long exposure; SE, short exposure. Arrows point to the band that corresponds to FAM45A, seen above the IgG light chain region. **(c)** Interactome results for components of CCC or retriever in previously reported protein-protein interaction studies in human cells and *Drosophila*. The number of times that any indicated protein was identified, was tabulated and normalized by the maximum theoretical occurrences in the examined gene matrix, which is presented in the form of percentage. **(d)** Immunoprecipitation of endogenous CCDC22, CCDC93 and VPS35L was followed by immunoblotting for the indicated proteins (representative of at least 3 iterations).

Supplementary figure 2



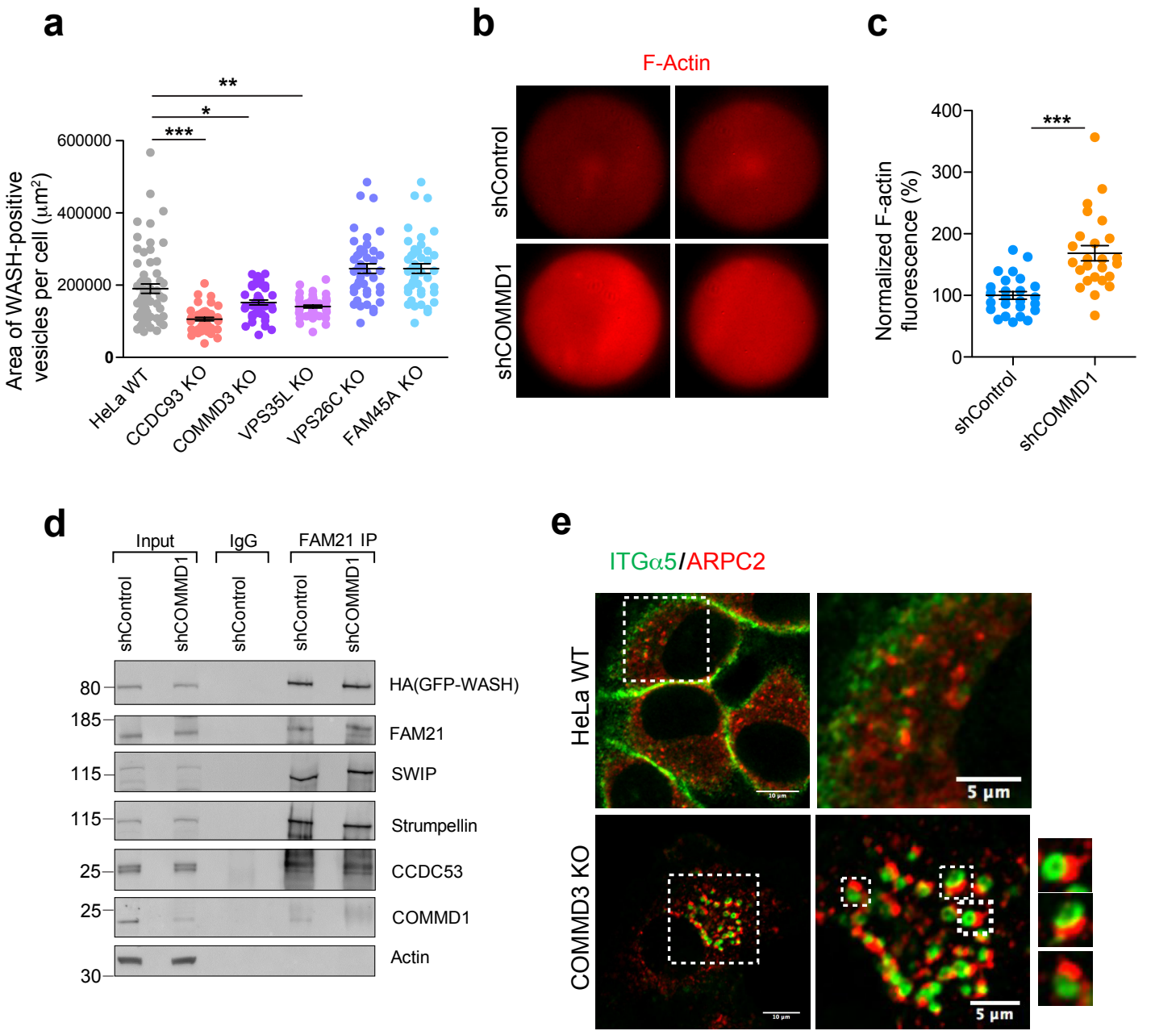
Supplementary figure 2. CCC deficiency leads to defective ITGβ1 recycling. **(a)** Pearson correlation coefficients for WASH and internalized integrin β1 (ITGβ1) following a recycling assay. Results for individual cells are plotted, along with the mean and s.e.m for each group (n=50 cells for WT, 75 for CCDC93 KO, 79 for COMMD3 KO, 57 for VPS35L KO, 61 for VPS26C KO, 77 for FAM45A KO); ****P*<0.0001 (one-way ANOVA and Dunnett's test to control). **(b)** Confocal microscopy imaging of ITGβ1 and WASH immunofluorescence staining quantified in (a). Representative images of 2 independent experiments are shown. Scale bars, 10 μm. **(c)** Knockout and parental HeLa cells were incubated with vehicle or lysosomal inhibitor Bafilomycin A prior to lysis. ITGβ1 protein levels analysed by western blotting. Representative blots from 3 independent experiments. **(d)** ITGβ1 endocytosis dynamics were determined by staining surface ITGβ1 in the indicated cell lines, using ITGβ1-biotin antibody. Internalization rate of ITGβ1-biotin-FITC was measured by FACS analysis (as described in the methods section) and normalized to 0 minutes time point for each genotype. The results are plotted as the mean and s.e.m for each group. Representative of 3 independent experiments. **(e)** Recycling of ITGβ1 was determined in parental and COMMD3 KO cells following ITGβ1 surface pulse labeling and subsequent stripping of remaining surface antibody (as described in the methods section). Normalized mean fluorescent intensity for ITGβ1 on WASH-positive areas was quantified. The results are plotted as the mean and s.e.m for each group (n=36 cells for WT 0 minutes, 107 for WT 30 minutes, 48 for COMMD3 KO 0 minutes, 60 for COMMD3 KO 30 minutes); ***P<0.0001; not significant, ns (unpaired two-tailed t-test to 0 minutes). **(f)** Confocal images depicting the subcellular localization of ITGβ1 and WASH in the recycling experiment quantified in (e) are shown. Representative images of 2 independent experiments. Scale bars, 10 μm.

Supplementary figure 3



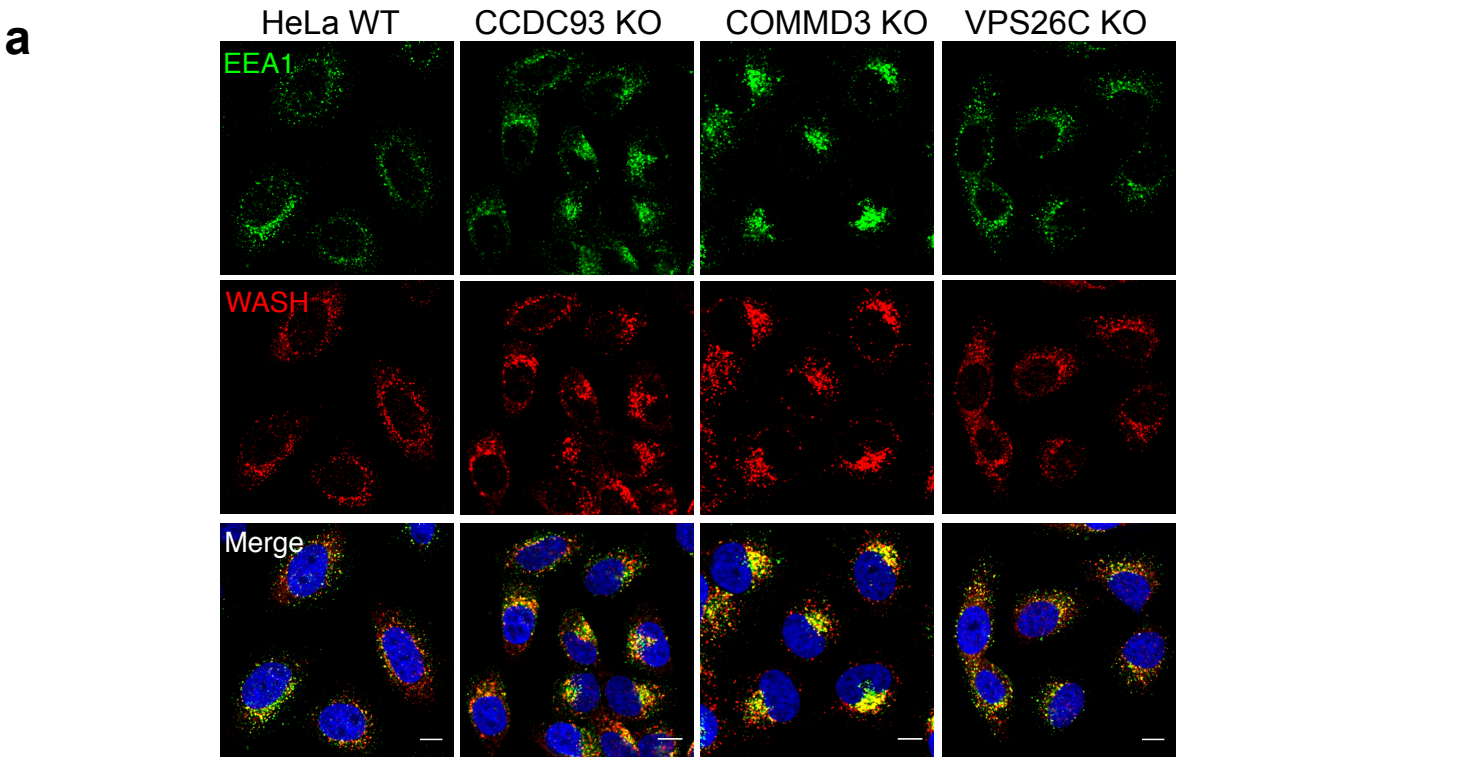
Supplementary figure 3. Cortactin and actin form comets in COMMD3 KO cells. (a) Representative fields of view from F-actin and WASH immunofluorescence staining in COMMD1 knockout (*Commd1*$^{-/-}$) mouse embryonic fibroblasts compared to control cells (*Commd1*$^{F/F}$). Scale bars, 10 µm**.** Mean fluorescence intensity of F-Actin on FAM21-positive vesicles was quantified in the indicated cell lines (n=50 cells). The results are plotted as the mean and s.e.m for each group (unpaired two-tailed t-test to control). **(b)** Representative fields of view from F-actin and WASH immunofluorescence staining in CCDC22 p.T17A fibroblasts compared to control fibroblasts. Scale bars, 10 µm**.** Mean fluorescent intensity of F-Actin on FAM21-positive vesicles was quantified in the indicated cell lines (n=34 cells). The results are plotted as the mean and s.e.m for each group (unpaired two-tailed t-test to control). **(c)** Confocal images of COMMD3 KO cells showing presence of WASH, FAM21 and VPS35 at the tips of cortactin tails, and ITGα5 with FAM21 at the tips of actin tails. Scale bars, 10 µm; 5 µm on zoomed images.

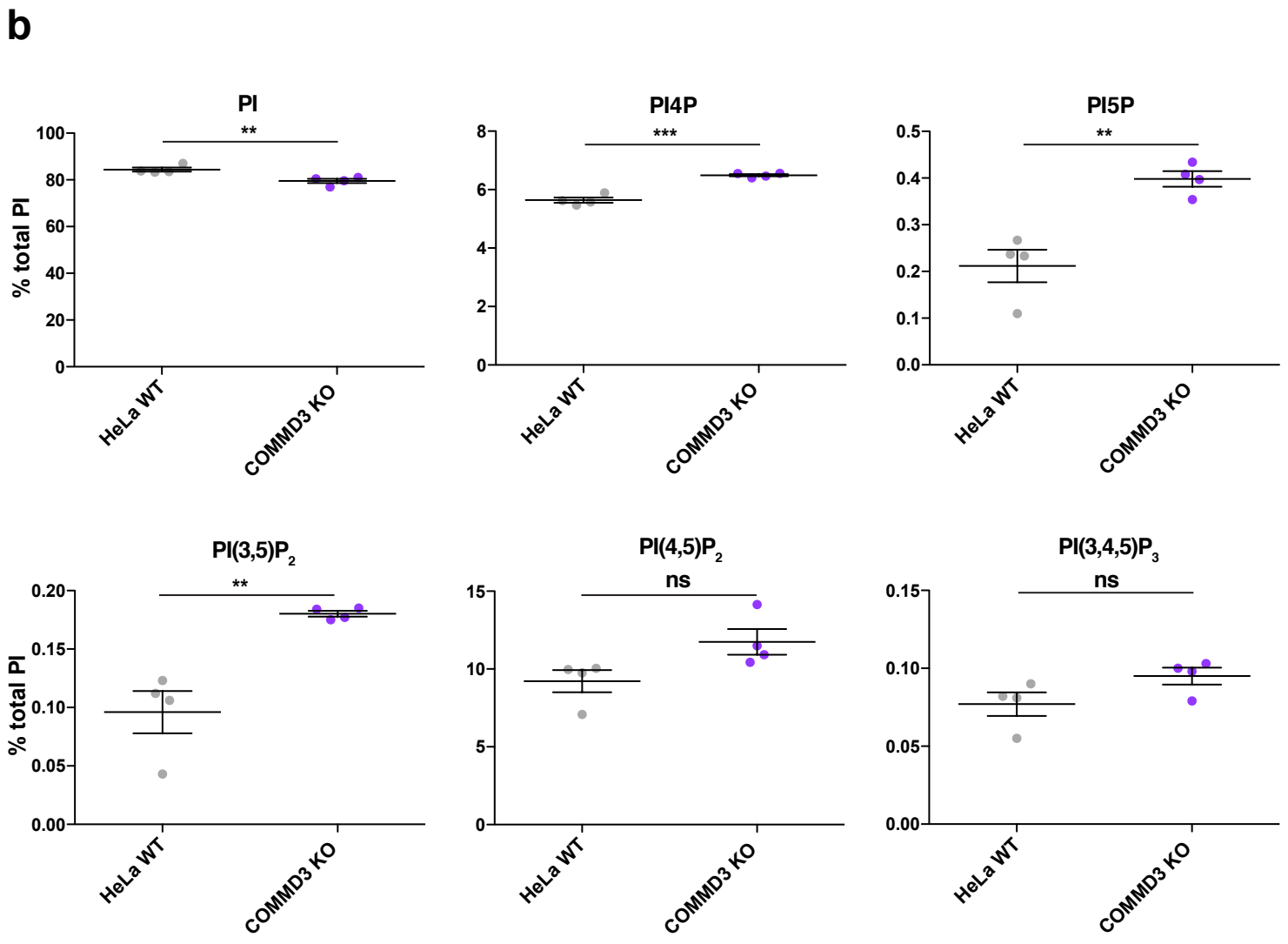
Supplementary figure 4



Supplementary figure 4. WASH activity and distribution is altered in CCC-deficient cells. (a) WASH-positive area was quantified (n=62 cells for WT, 45 for CCDC93 KO, 48 for COMMD3 KO, 50 for VPS35L KO, 43 for VPS26C KO, 48 for FAM45A KO) and plotted as the mean and s.e.m; ***P<0.0001; **P<0.005; *P<0.05 (one-way ANOVA and Dunnett's test to control). Representative images are shown in Figure 4b. **(b)** Representative images of agarose beads following in vitro F-actin polymerization in the indicated cell lines. The WASH complex was immunoprecipitated using a FAM21 antibody and incubated with G-actin and Arp2/3 complex. **(c)** Bar graph representation of the mean and s.e.m for F-actin fluorescence in (b); n=25 beads were quantified per group; ***P<0.0001 (unpaired two-tailed t test to control). **(d)** FAM21 was immunoprecipitated from HA-GFP-WASH shControl and shCOMMD1 cell lysates followed by immunoblotting for the indicated proteins. Actin serves as a loading control. **(e)** Representative confocal images of HeLa WT and COMMD3 KO cells costained for internalized ITGα5 and ARPC2. Scale bars, 10 µm; 5 µm on zoomed image.

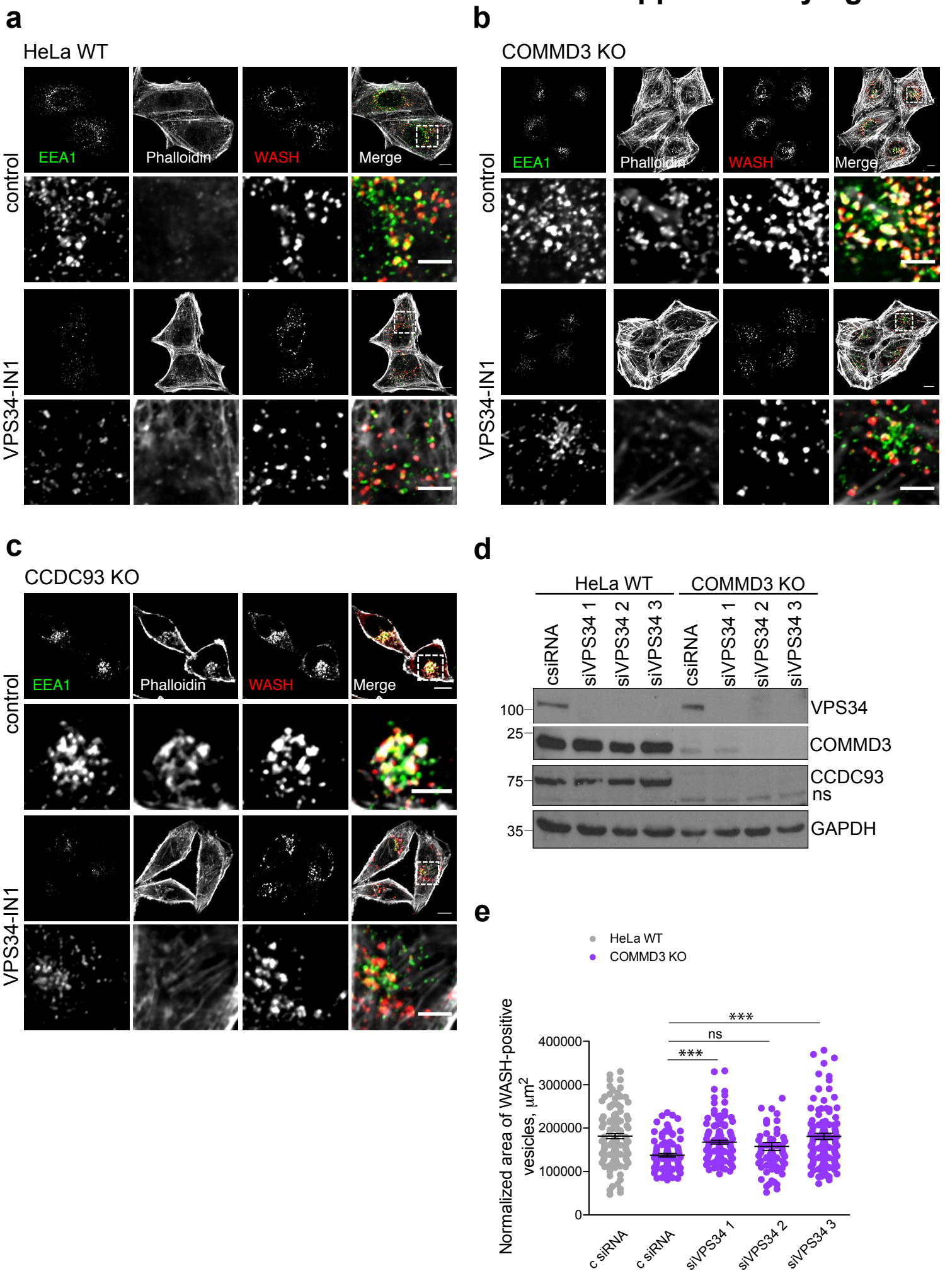
Supplementary figure 5





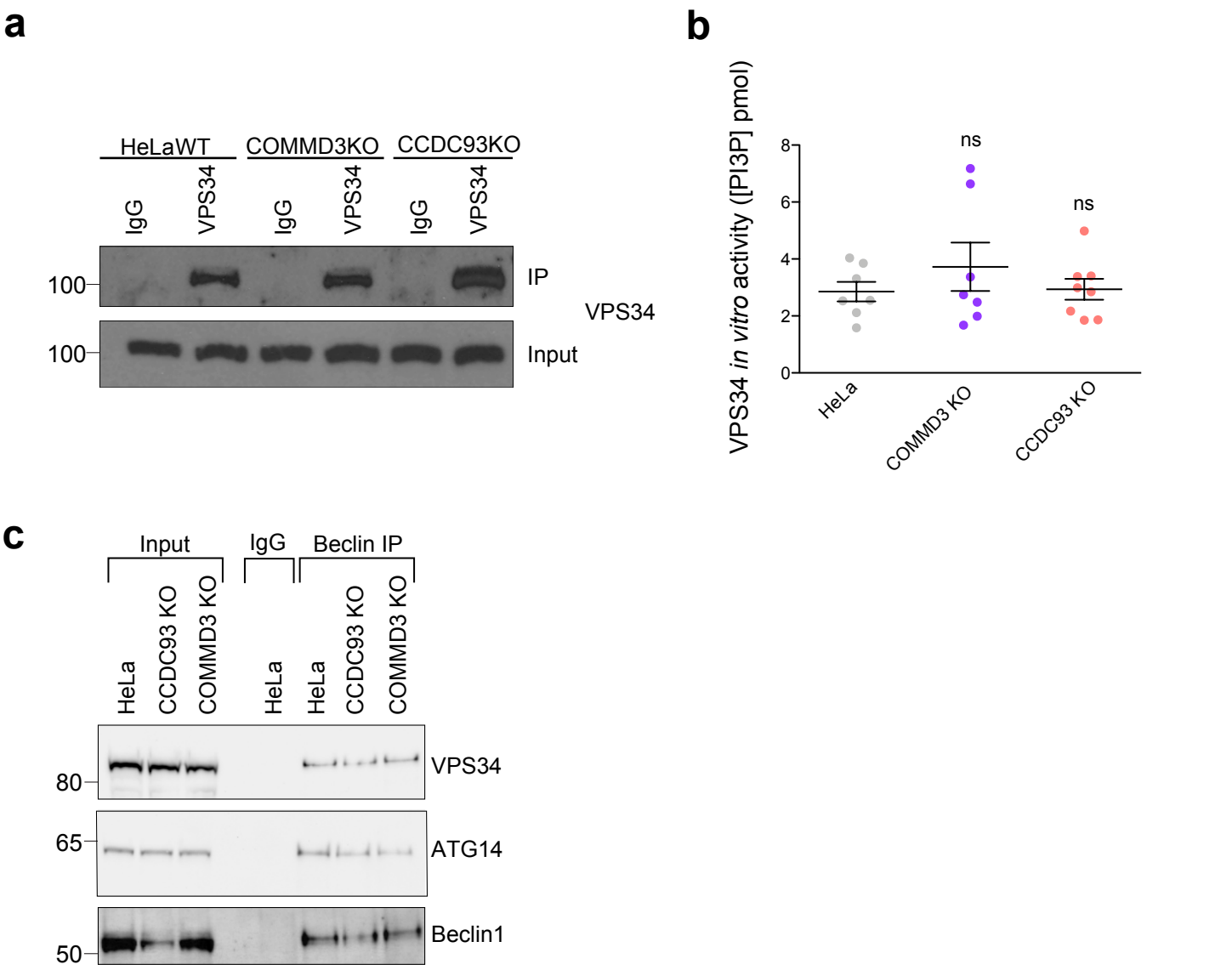
Supplementary figure 5. The levels of PI(3)P, PI(4)P and PI(3,5)P2 are increased in CCC-deficient cells. **(a)** Representative fields of view from EEA1 and WASH immunofluorescence staining experiment partially shown in Figure 5a. Scale bars, 10 μm. **(b)** Phosphorylated phosphoinositide levels were quantitatively measured in HeLa WT and COMMD3 KO cells by HPLC as described in the methods section. PI levels were significantly decreased (**p=0.0083), while PI(4)P (***p=0.0001), $PI(3,5)P_2$ (**p=0.0036) and PI(5)P (**p=0.0029) levels were significantly elevated in COMMD3 deficient cells as compared to HeLa WT. $PI(4,5)P_2$ and $PI(3,4,5)P_3$ levels were not significantly changed (p=0.0593 and p=0.1024, respectively). The results are plotted as the mean and s.e.m for each group. Statistical significance was analyzed using unpaired two tailed student t-test (n = 4 biological replicates).

Supplementary figure 6



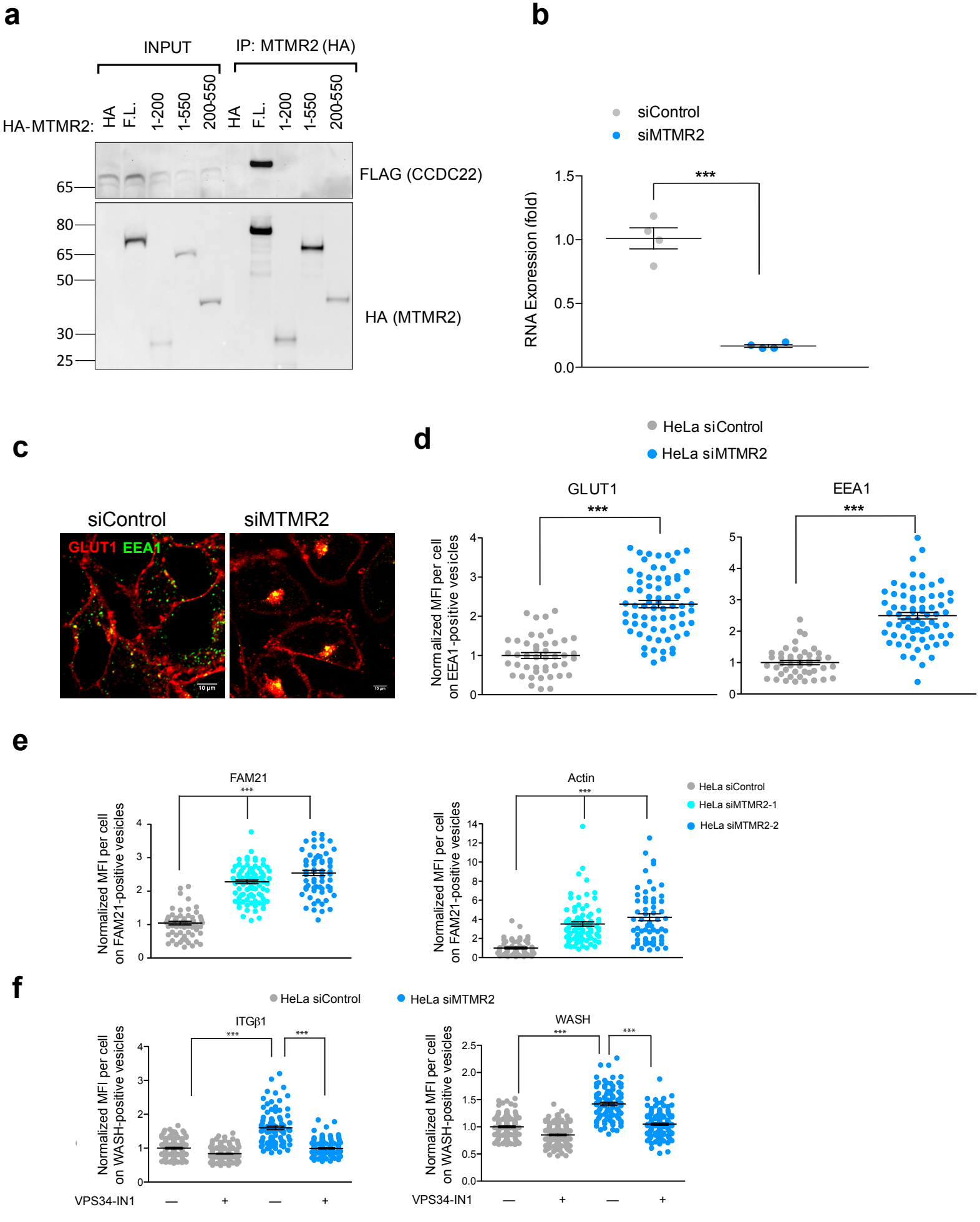
Supplementary figure 6. Accumulation of actin in CCC KO cells is PI(3)P-dependent. (a), (b), (c). Representative confocal images for EEA1, phalloidin (F-Actin), and WASH in WT (a), COMMD3 KO (b) and CCDC93 KO (c) HeLa cells treated with vehicle control or VPS34 inhibitor VPS34-IN1 (1 μM) for 1 h. Scale bars, 10 μm, zoom 5 μm. Normalized phalloidin and WASH fluorescence on EEA1-positive areas was quantified in Figure 6b. **(d)** HeLa WT and COMMD3 KO cells were treated with control siRNA or 3 independent siRNAs against VPS34 for 72 hours, cells were lysed, and levels of the indicated proteins were analyzed by immunoblot. **(e)** WASH-positive area was quantified in parental and COMMD3 KO cells, transfected with either control or 3 independent siRNAs targeting VPS34. Results for individual cells are plotted, along with the mean and s.e.m for each group (n=130 cells for HeLa WT control siRNA (csiRNA), 90 for COMMD3 KO csiRNA, 120 for COMMD3 KO siVPS34 1, 85 for COMMD3 KO siVPS34 2, 130 for COMMD3 KO siVPS34 3); ***P<0.0001, ns=not significant (one-way ANOVA and Dunnett's test to COMMD3 KO control siRNA). For representative images of WASH staining please see Figure 6c.

Supplementary figure 7



Supplementary figure 7. VPS34 kinase activity is not increased in CCC-deficient cells. (a) Endogenous VPS34 was immunoprecipitated from the indicated cell lines and used for *in vitro* ELISA-based VPS34 kinase activity assay. **(b)** VPS34 *in vitro* kinase activity measured in CCC-deficient cells compared to HeLa WT cells. Average of n=3 independent experiments is shown, each experiment was performed in triplicate. Values are quantified minus IgG control reaction values. The results are plotted as the mean and s.e.m for each group (one-way ANOVA and Dunnett's test to control). **(c)** Endogenous Beclin-1 was immunoprecipitated in CCC-deficient cells and the recovered material was probed for the components of VPS34 complex. Results are representative of 2 separate experiments.

Supplementary figure 8



Supplementary figure 8. MTMR2 role in endosomal recycling. (a) The indicated constructs of 2xHA tagged MTMR2 and FLAG-CCDC22 were transfected in HEK293T cells followed by immunoprecipitation of MTMR2 using HA antibody. The recovered material was immunoblotted for CCDC22 (FLAG) and MTMR2 (HA). **(b)** mRNA expression of *MTMR2* gene in siControl and siMTMR2-transfected HeLa cells used in (c) and (d) was assessed by qRT-PCR. The results are plotted as the mean and s.e.m for each group; n=4 biological replicates; ***P<0.0001 (unpaired two-tailed t test). **(c)** Immunofluorescence staining was performed to examine GLUT1 and EEA1 localization in MTMR2 knockdown cells. Representative images are shown from 2 separate experiments, scale bar, 10 μm. **(d)** On the cells shown in (c), normalized mean fluorescence intensity of GLUT1 and EEA1 on EEA1-positive vesicles was quantified in siControl (n=45) and siMTMR2-transfected HeLa cells (n=71) and plotted as the mean and s.e.m for each group.; ****P*<0.0001 (unpaired two-tailed t test). **(e)** Quantification of normalized MFI of FAM21 per cell (left panel) and F-actin MFI on FAM21-positive vesicles (right panel) for the experiment shown in Figure 7d. Results for individual cells are plotted, along with the mean and s.e.m for each group (n=58 cells for siControl, 87 for siMTMR2-1, 63 for siMTMR2-2); ****P*<0.0001 (one-way ANOVA and Dunnett's test to control). **(f)** Quantification of normalized WASH MFI per cell (left panel) and ITGβ1 MFI on WASH-positive endosomes (right panel), corresponding to the experiment shown in Figure 7e. Results for individual cells are plotted, along with the mean and s.e.m for each group (n=150 cells for siControl, 178 for siControl VPS34-IN1, 91 for siMTMR2, 132 for siMTMR2 VPS34-IN1); ****P*<0.0001 (unpaired two-tailed t test).

Supplementary figure 9

Figure 1e

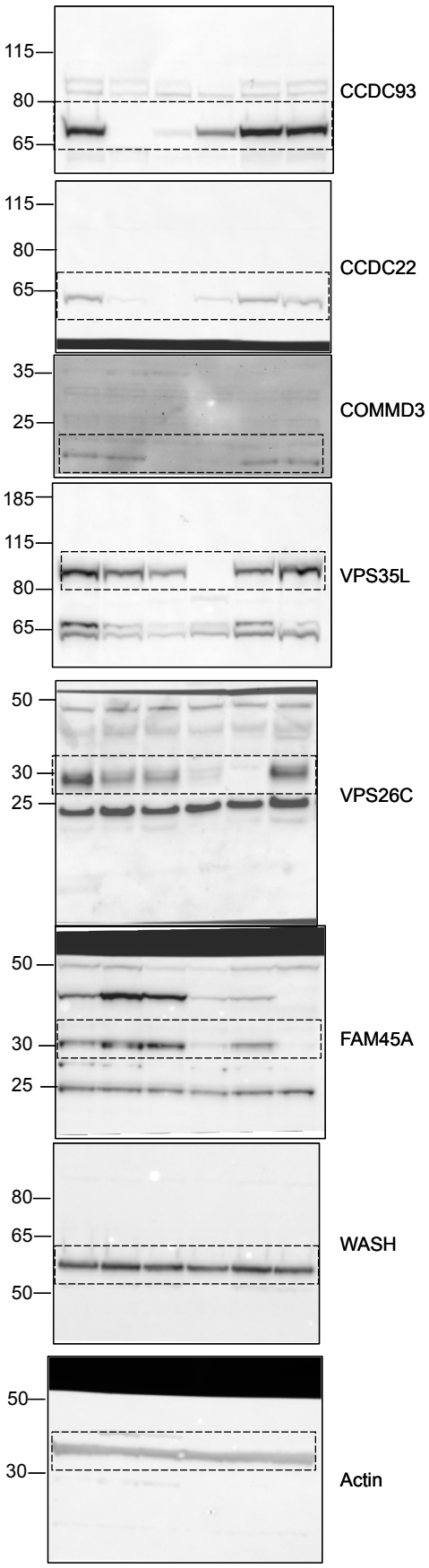


Figure 2b

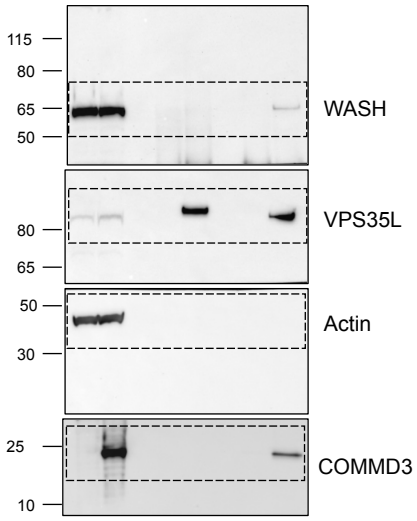


Figure 4e

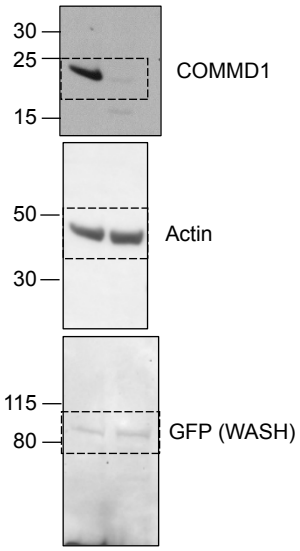


Figure 7a

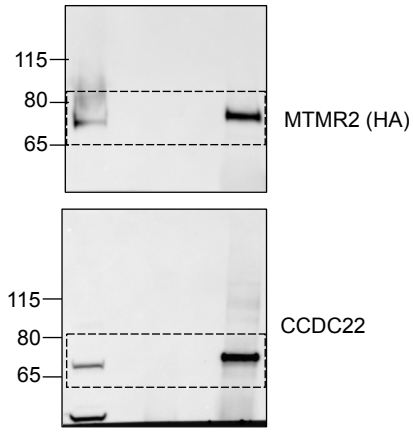


Figure 7b

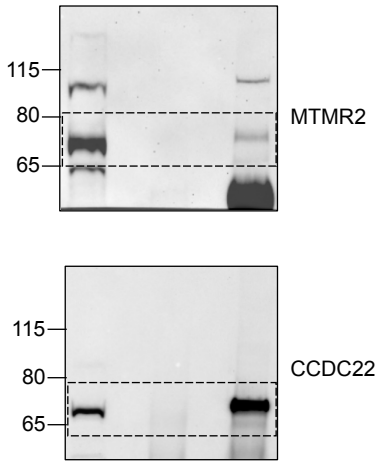


Figure 7c

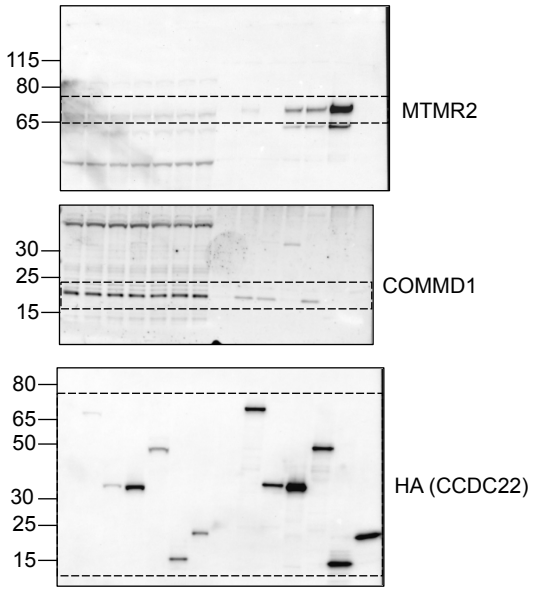
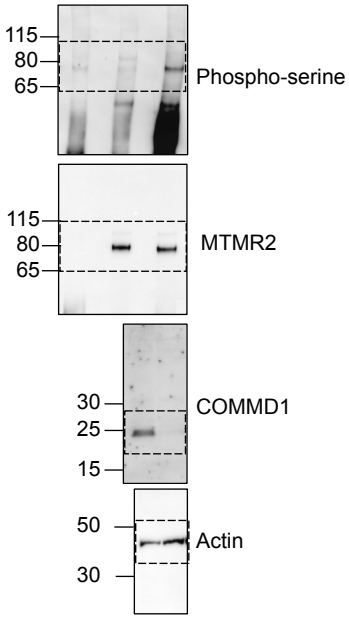
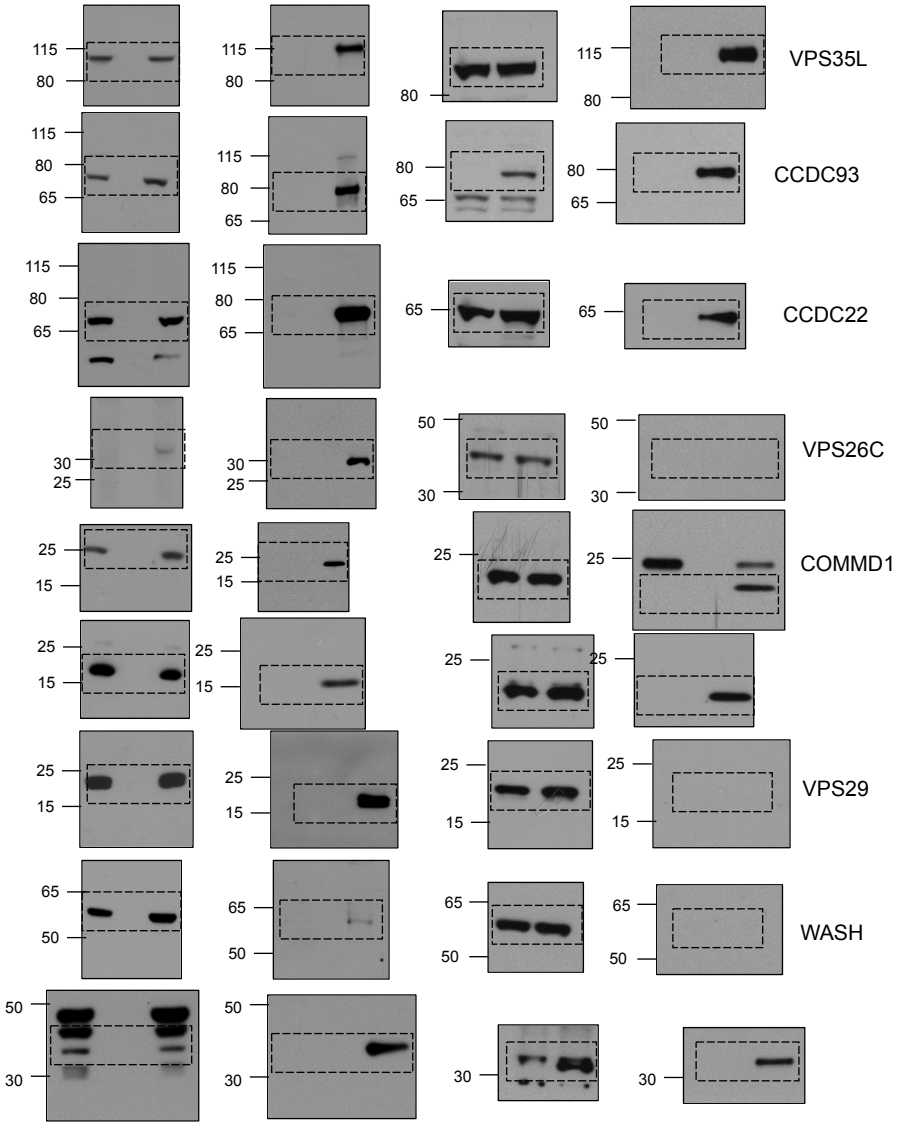


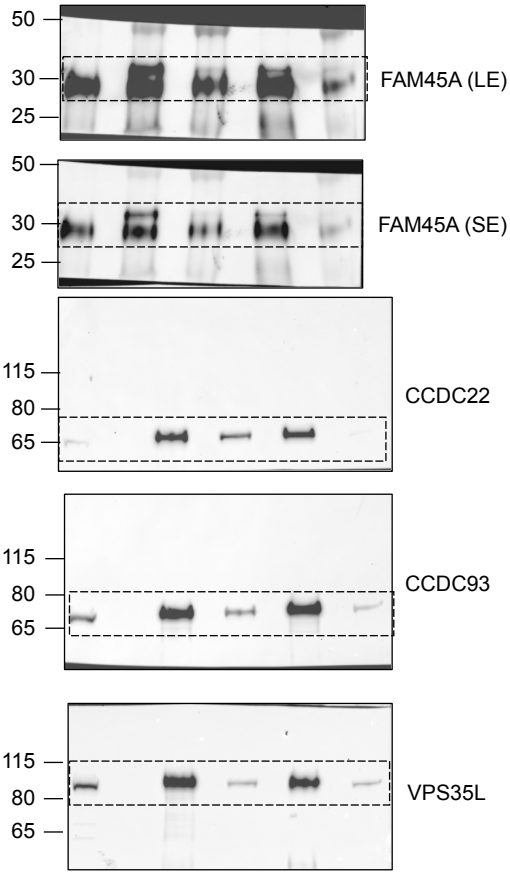
Figure 7f



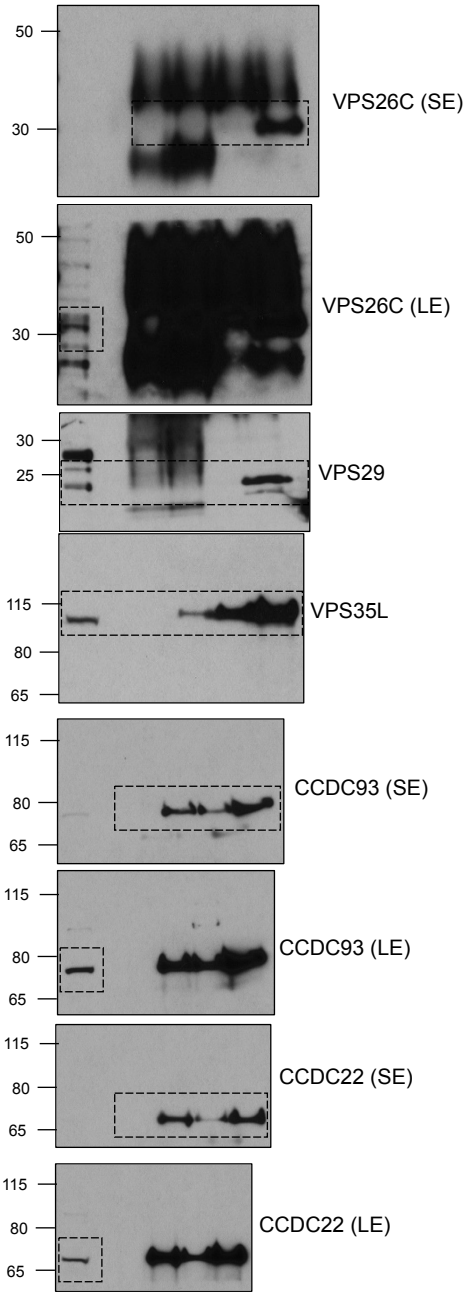
Supplementary Figure 1a



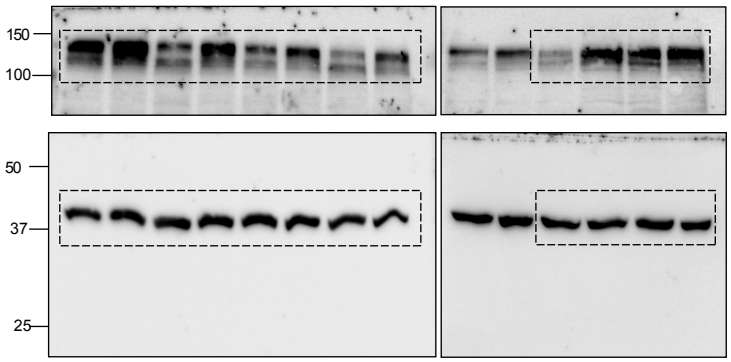
Supplementary Figure 1b



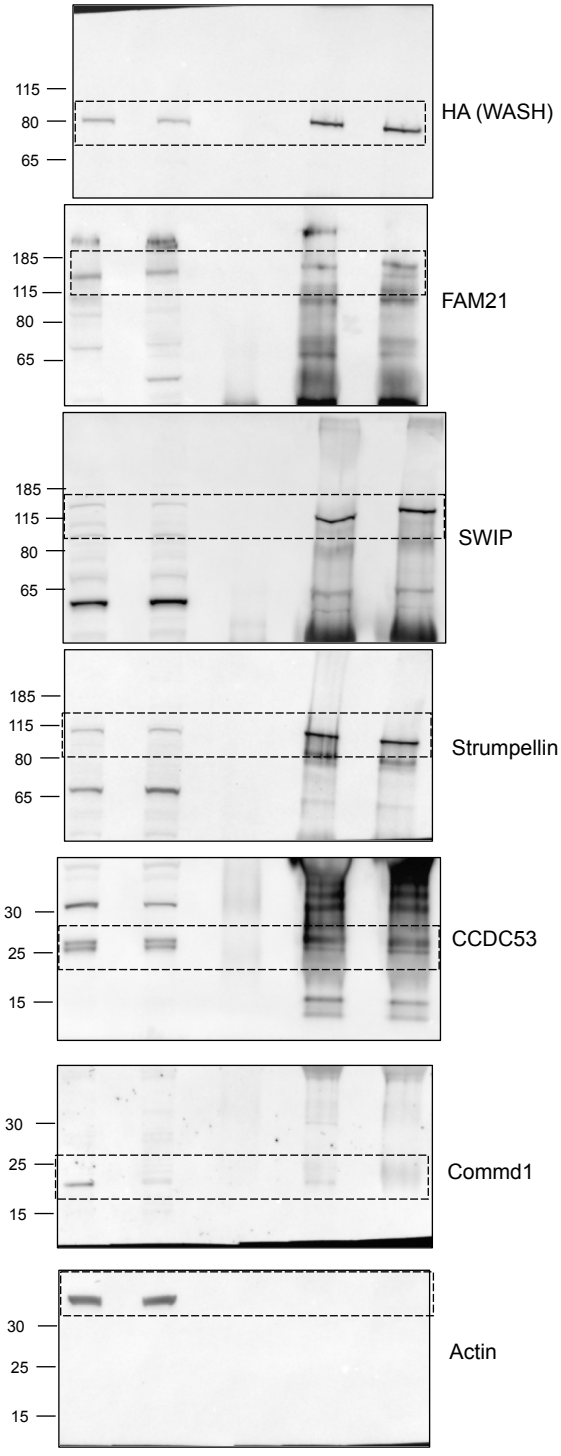
Supplementary Figure 1d



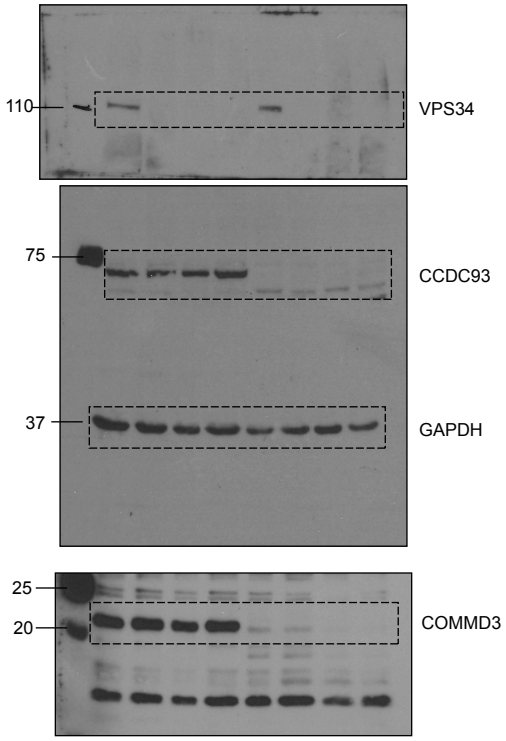
Supplementary Figure 2c



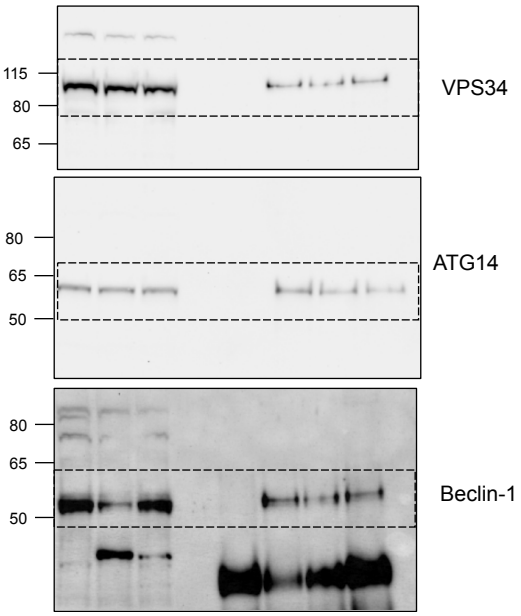
Supplementary Figure 4d



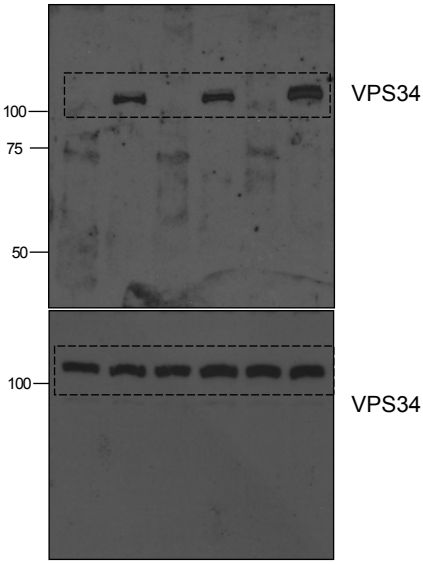
Supplementary Figure 6d



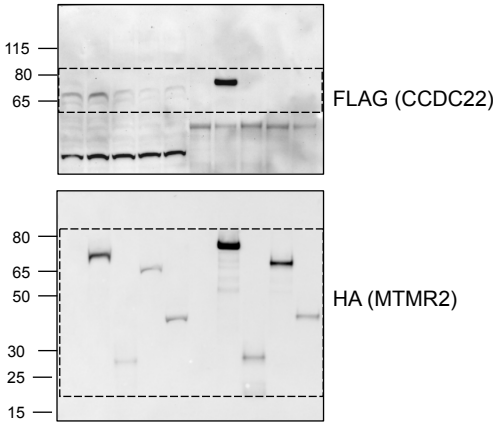
Supplementary Figure 7c



Supplementary Figure 7a



Supplementary Figure 8a



Supplementary figure 9. Uncropped blots. All western blot images used to derive data in this paper are shown here. SE, short exposure, LE, long exposure.

Supplementary table 1: Targeting sequences used in shRNA and CRISPR/Cas9 experiments

shRNA strategy			
Gene target (Human)	***Reference sequence***	***Target sequence***	***Reference***
COMMD1	NM_152516	GTCTATTGCGTCTGCAGACTT	van de Sluis et al., 2010
WASH	NM_182905	CCAGAGAACTACTTCTATGTG	Deng et al., 2015
Gene target (Mouse)	***Reference sequence***	***Target sequence***	***Reference***
Commd1	NM_144514	GATGAAGTTAAAGTCAAGCAA	This paper
CRISPR/Cas9 gRNA			
Gene target (Human)	***Guide construct***	***Target sequence***	***Reference***
CCDC93	Guide 1	GACTTTCCGGCGATGTAGCTGGG	Phillips-Krawczak et al., 2015
COMMD3	Guide 1	CTTCACGCTTCTCCTCCGGGGTTTTA	This paper
VPS35L	Guide 1	CACTAAGCTGAAGAACCCAC	This paper
VPS26C	Guide 1	CACCGAATAAAGTTTATCACGCCG	McNally et al., 2017
FAM45A	Guide 1	CACCGTGGAGTCGGGCTGATCGGTG	This paper

Supplementary table 2: Antibodies used in this study

Primary antibodies
WB, western blot; IP, immunoprecipitation; IF, immunofluorescence staining; FC, flow cytometry; aa, amino acid.

Target	**Source** (host species)	**Catalog or identifying number**	**Application** (dilution)
ARPC2	Upstate Biotechnology	07227	IF (1:250)
ATG14	MBL	M184-3	WB (1:1000)
β-Actin	Sigma Aldrich	A5441	WB (1:5000)
Beclin1	Cell signaling	3738S	WB (1:1000)
CCDC22	ProteinTech Group	16636-1-AP	WB (1:1000)
CCDC22	Custom made (rabbit)	711 (Ref 30)	IP (5µl per mg of protein)
CCDC53	Custom made (rabbit)	MC2211	WB (1:3000)
CCDC93	ProteinTech Group	20861-1-AP	WB (1:1000), IP (1µg per mg of protein)
CD29 Biotin (Integrin β-1)	Sigma	SAB4700396	FC (1:1000)
COMMD1	Custom made (rabbit)	709 (Ref 28)	WB (1:1000), IP (5µl per mg of protein)
COMMD1	Novus Biologicals	NBP2-03755	WB (1:500)
COMMD3	Abcam	ab176583	WB (1:1000)
COMMD3	Custom made (rat)	UT-R 43 (Ref 34)	WB (1:500)
COMMD9	Custom made (rabbit)	192-AP (Ref 30)	WB (1:1000)
Cortactin	Millipore	05-180	IF (1:250)
EEA1	BD Biosciences	610456	IF (1:250)
FAM21	Custom made (rabbit)	MC2188	WB (1:3000), IF (1:1000)
FAM45A	Custom made (rabbit)	95-110 (This study)	WB (1:1000)
FLAG	ThermoFisher Scientific	MA1-142	WB (1:1000)
FLAG	Sigma	F1804	WB (1:1000)
GAPDH	GeneTex	GT239	WB (1:5000)
GFP	Roche	11814460001	WB (1:1000)
GLUT1	Abcam	ab115730	IF (1:250)
HA	Sigma	H3663	IF (1:250)
HA	Biolegend	901502	WB (1:1000)
Integrin β-1	Santa Cruz	Sc-13590	IF (1:100)
Integrin α-5	BD Biosciences	555615	IF (1:100)
Integrin β-1	Cell signaling	4706S	WB (1:1000)
MTMR2	Santa Cruz	Sc-365185	IP (1µg per mg of protein)
MTMR2	Sigma	WH0008898M3	WB (1:5000)
Phospho-(Ser/Thr)	Cell Signaling	9631	WB (1:1000)
Strumpellin	Custom made (rabbit)	MC2186	WB (1:3000)
SWIP	Custom made (rabbit)	MC2193	WB (1:3000)

VPS26A	Abcam	Ab23892	WB (1:1000)
VPS26C	Millipore	ABN87	WB (1:5000)
VPS29	GeneTex	GTX104768	WB (1:1000)
VPS34	Santa Cruz	sc-365404	WB (1:1000)
VPS34	Echelon	Z-RO15	IP (1:125)
VPS35	Abcam	Ab10099	IF (1:1000)
VPS35L	ThermoFisher Scientific	PA5-28553	WB (1:1000), IF (1:250)
VPS35L	Custom made (rabbit)	MC2425 (This study)	IP (5µl per mg of protein)
WASH	Custom made (rabbit)	MC2058	WB (1:3000), IF (1:1000)
Secondary antibodies used for immunofluorescence			
Fluorophore	**Source** (target species)	**Catalog number**	
Alexa 488	Invitrogen (mouse)	A21202	
Alexa 488	Invitrogen (mouse)	A11029	
Alexa 488	Invitrogen (rabbit)	A21206	
Alexa 488	Invitrogen (goat)	A11055	
Alexa 555	Invitrogen (rabbit)	A21428	
Alexa 568	Invitrogen (mouse)	A10037	
Alexa 568	Invitrogen (rabbit)	A10042	