Supplementary material

for

Phosphatidylinositol 4-kinase IIα function at endosomes regulated by the ubiquitin ligase Itch

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Supplementary materials and methods

Plasmids

Full-length murine PI4KIIα was cloned into pGEX4T-1 (Amersham Biosciences) or pET28a+ (Novagen) for bacterial expression or a pcDNA3-based plasmid encoding an in-frame fusion with the HA epitope tag for expression in mammalian cells. Full-length human Itch was cloned into pET28a+. Itch-WW domains were cloned into pGEX4T-1 and pET28a+ for bacterial expression of GST and His tagged expression or into a custom-made pcDNA3-based plasmid encoding an in-frame fusion with a c-MYC epitope tag at its N-terminus. GST-fused tandemly repeated UBA domain (3x UBA) derived from human ubiquilin was created by multiple overlap-extension PCR reactions and cloned into pGEX4T-1. Human Fz4 was

cloned into a custom-made pcDNA3-based plasmid for expression of a C-terminally eGFP-tagged chimera in mammalian cells. HA-Dvl2 was derived from mouse cDNA by RT-PCR and cloned into a pcDNA3-based plasmid encoding an in-frame fusion with the HA epitope tag. Site-directed mutants were created using the QuickSite mutagenesis kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. All constructs were verified by dsDNA sequencing (MWG). FLAG-Ubiquitin and FLAG-Ubiquitin7R were kind gifts of Dr. Klaus-Peter Knobeloch (University of Freiburg, Germany). The plasmid encoding human Itch was a kind gift of Dr. Annie Angers (University of Montreal, Canada).

Antibodies

HA (clone HA.11, Babco/Convance), FLAG (M2, Sigma), actin (clone AC-15, Sigma), clathrin heavy chain (clone TD.1), GFP for western blot detection (Abcam, AB6556), GFP for coimmunoprecipitations (Stressgen, cat. no. SAB500E), Itch (BD transduction, cat. no. 611198), Gadkin (own), EEA1 (BD transduction, cat. no. 610456), AP-3 (SA4, Hybridomabank and dS40, a kind gift from Prof. Stefan Höning (Univ. of Cologne, Germany). Secondary antibodies were purchased from Molecular Probes (AlexaFluor^{430}, AlexaFluor^{488}, AlexaFluor^{568}, AlexaFluor^{647}) or Dianova (HRP-coupled antibodies).

Polyclonal antibodies against PI4KIIα were raised in rabbits by injecting 2 different peptides. Antisera were tested for specificity and affinity-purified on GST-PI4KIIα cross-linked to GST-beads.

siRNAs sequences

PI4KIIα1:GGAUCAUUGCUGUCUUCAA;

PI4KIIα2:AAGAGGACCUAUAUGAACUCUUCAAGA;

Itch: GGAGCAACAUCUGGAUUAA; Control: GUAACUGUCGGCUCGUGGU

Peptides

PI4KIIα PPxY : NH_2-PERAQPPEYTFPSG-COOH

Control: NH_2-CQPARQGQAAPAQAQSSQ-COOH

Cell culture and transfections

Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with fetal calf serum and antibiotics. Protein expression in stably transfected Hek293 Flip-in cell lines was induced by addition of 1µg/ml doxycyclin. Lipofectamine2000 was used for plasmid transfections, Oligofectamine for transfection of siRNA (both from Invitrogen Inc.).

Immunoprecipitation

Cells were lysed in extract buffer (20 mM Hepes pH 7.4, 100 mM NaCl or KCl, 2 mM $MgCl_2$, 1% Triton X-100) supplemented with 1 mM PMSF and protease inhibitor cocktail (Sigma Inc., St. Louis, USA). For the analysis of phosphorylated proteins, phosphatase inhibitor cocktails were added (Sigma Inc., St. Louis, USA). Lysates were centrifuged at 20,000 xg, supernatants were collected ultracentrifuged for 15 min at 180,000 xg. Extracts were incubated with antibodies immobilized on protein A/G PLUS-agarose (Santa Cruz Biotechnology). Beads were washed with extract buffer and bound proteins were eluted with SDS-PAGE sample buffer.

To identify proteins preferentially associating with unmodified or ubiquitinated PI4KIIα GST-PI4KIIα was ubiquitinated *in vitro* as described below. Reaction mixes containing His_6-Itch (WT) or His_6-Itch (D830A) were supplemented with 40 µl of agarose covalently coupled to anti-GST-IgG (SIGMA-Aldrich) and 1 ml of HeLa cell extracts and incubated for 4 h at 4°C. Beads were washed extensively and eluted with Laemmli sample buffer. Finally the eluates were separated by SDS-PAGE and analyzed by MS/MS-based mass spectrometry.

Tissue extracts and GST pulldowns

Tissue extracts were prepared as described before (Kahlfeldt et al, 2010). Detergent extracts were pre-cleared by ultracentrifugation at 180,000 xg for 15 min. The pre-cleared supernatant was incubated with purified GST-tagged proteins for 2 h at 4°C on a rotating wheel, washed with 20 mM Hepes pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100 and bound proteins were either eluted with high salt buffer (20 mM Hepes pH 7.4, 1 M NaCl, 2 mM $MgCl_2$) followed by TCA precipitation (large scale pulldown) or by addition of sample buffer.

Protein expression and purification

His_6-tagged or GST-tagged proteins were expressed in BL21 (DE3) and purified from *E. coli* using His-Select Nickel Affinity Gel (Sigma) or GST Bind Resin (Novagen) according to the manufacturer's instructions. For NMR GST-WW3 Itch was purified and subjected to thrombin cleavage. The GST tag was removed by collecting the flow through after running the GST-fused protein through a concentration spin column (10,000 MWCO, Vivaspin2, Sartorius Stedim Biotech). Purified WW3 was dialysed into a buffer containing 20 mM Tris pH 7 and 100mM NaCl.

PI 4-kinase activity assays

1 μg purified His-PI4KIIα was incubated with or without 1.5 μg His_6-WW1-4, 0.5 μg His_6-WW3, 0.5 μg His_6-WW4 of Itch, or 3 μg of His_6-Itch (FL) in kinase buffer (25 mM Hepes/KOH pH7.2, 25 mM KCl, 2.5 mM MgOAc, 150 mM K-glutamate, 10 μM $CaCl_2$, 0.2% CHAPS) containing 10 μg phosphatidylinositol (PI), 200 μM ATP and 10 μCi ^{32}P-γ-ATP for 5 min at 30° C. Lipids were extracted and analyzed by thin layer chromatography using appropriate standards. PI(4)P formation was quantified using phosphorimage analysis using a Cyclone Storage Phosphor Screen (Packard) and quantified using OptiQuant

Software.

Ubiquitination assays

For *in vitro* ubiquitination assays 5 μg His_6-Itch, His_6-Itch (D830A) or FLAG-NEDD4.1 (BPS Bioscience) was incubated with 2.5 μg GST/His_6-PI4KIIα or GST/His_6-PI4KIIαSF in 100 μl reaction buffer containing 100 mM KCl, 20 mM Hepes pH 7.4, 2 mM $MgCl_2$, 1 mM DTT, 4 mM ATP, 2 μg/ml E1 (UBE1, Boston Biochem), 2 μg/ml E2 (UbcH7, Boston Biochem) and 50 μg/ml mammalian ubiquitin (Boston Biochem). Samples were incubated for 30 min at 37° C and the reaction was stopped by addition of SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and staining with Coomassie Blue or by immunoblotting.

Fz4 internalization

HEK293 cells were transfected with siRNAs (two subsequent rounds on day 1 and day 3), followed by transfection with a plasmid encoding Fz4-eGFP and, if indicated, plasmids for rescue. Cells were seeded on glass coverslips o.n. and stimulated with media containing 400 ng/ml Wnt5a (R&D Systems) and 1 μM PMA (Calbiochem) for 40 min. Cells were processed for immunostaining and analyzed by spinning disc confocal microscopy under the control of Volocity software (Improvision Inc., UK). Fz4-eGFP uptake was quantified as the amount of Fz4-eGFP fluorescence accumulated in EEA1-positive endosomes normalized to the total amount of Fz4-eGFP present in the cell using Volocity software.

HA-Fz4 antibody uptake assay

Inducible HekFlpIn/TR cells were stably transfected with a luminally HA-tagged Fz4 construct. Indicated target genes were silenced for 2 consecutive rounds. After the second

round of knockdown, cells were seeded onto matrigel-coated coverslips and HA-Fz4 expression was induced for 24 h with 1 μg/ml doxycyclin. Next, coverslips were washed with medium and placed cell-side-down on parafilm on a drop of medium containing rat anti-HA antibody (Roche) for 1 h at 4 ^{o}C. Cells were washed and shifted to 37 ^{o}C in full medium for antibody internalization to occur for After 15 minutes, cells were washed and fixed with PFA. Non internalized (cell surface localized) rat-anti HA antibodies were blocked by incubation under non-permeabilizing with a saturating concentration of goat-anti-rat IgGs. After washing, cells were fixed once more with PFA and the internalized rat anti-HA IgGs were visualized with fluophore-conjugated anti-rat antibodies using standard immunofluorescence labeling protocols.

Fz4 degradation assay

Above described HA-Fz4 HekFlpIn/TR cells were silenced for 2 consecutive rounds and HA-Fz4 expression was induced by adding 5 ng/ml doxycylin. After 6 h, cells were washed once with full medium and further cultured in the presence of 10 μg/ml cycloheximide to prevent further HA-Fz4 synthesis. Cells were harvested at 0, 3, 6 and 9 h after doxycyclin removal. For harvesting, cells were washed once with PBS, placed on ice and lysis buffer (20 mM Hepes pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM PMSF, 0.3% protease inhibitor cocktail) was added. Cells were scraped, transferred to Eppendorf tubes and frozen till all time points were collected. Extracts were centrifuged for 30 min at 20,000 x *g*, the resulting supernatants subjected to Western blot analysis.

Transferrin uptake

Hek293 cells doubly transfected with siRNAs (on days 1 and 3) were seeded on glass coverslips 96 h post-transfection, starved for 1 h in serum-free media and stimulated for 10 min with media containing 25 μg/ml transferrin AlexaFluor^{488} (Molecular Probes). Cells were

washed, fixed and analyzed by quantitative epifluorescence microcscopy under the control of Slidebook software (3i Inc., Göttingen).

Immunofluorescence microscopy

Cells were seeded on coverslips and fixed with 4% PFA, 4% sucrose in PBS for 3 min followed by fixation with ice-cold methanol for 7 min. For colocalization studies using antibodies against AP-3 and PI4KIIα cells were fixed with ice-cold methanol only; for analysis of Itch and AP-3 cells were fixed with PFA (10 min) only. Standard protocols were used for immunostaining. Images were acquired on a Zeiss Axiovert 200M-based spinning disc confocal microscope (Perkin Elmer Inc.) under the control of Volocity software (Improvision Inc.) or in the case of transferrin by epifluorescence microcscopy under the control of Slidebook software (3i Inc., Göttingen).

Quantitative RT-PCR

RNA was isolated from PI4K2β-silenced Cos7 cells with TRIzol (Invitrogen) according to the manufacturer's protocol. 1 µg RNA was used for cDNA synthesis with SuperScript III reverse transcriptase (invitrogen) and poly d(T) primer (Roche). Product was diluted 1:10, 5 µl was combined with either Axin2 (forward: TTATGCTTTGCACTACGTCCCTCCAAG, reverse: GACGCAACATGGTCAACCCTCAAGAC) or GAPDH primers (forward: GCAAATTCCATGGCACCGTCAAG, reverse: GCATCGCCCCACTTGATTTTGGAG) and SYBR Fast universal mix (KAPA biosystems). The qPCR reaction was performed on a Mx3005P qPCR system (Agilent Technologies).

Quantification of protein levels

SiRNA-transfected Hek293 cell extracts were analyzed by immunoblotting using fluorescently labeled secondary antibodies for detection. Signals were detected using a Storm

Scanner (Molecular Dynamics) and quantified using ImageQuant5.2 software (Molecular Dynamics).

Protein identification by MS/MS-based mass spectrometry

Sample preparation, in-gel digestion, and liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis were done as described (Lange et al, 2010). In brief, Coomassie-stained gel bands were cut into slices and tryptic in-gel digestions were performed in the presence of $H_2{}^{18}O$ (Campro Scientific GmbH, 97% ^{18}O) and $H_2{}^{16}O$ for material purified by GST and GST-PI4KIIα preparations respectively. LC-MS/MS analyses of were performed on a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher) equipped with an Eksigent 2D nanoflow LC system (Axel Semrau GmbH). Mass spectra were acquired in a data-dependent mode. The dynamic exclusion time (for precursor ions) was set to 120 sec and automatic gain control was set to 1×10^6 and 20,000 for Orbitrap-MS and LTQ-MS/MS scans, respectively. The generated peak lists and the MASCOT server (version 2.2, Matrix Science) were used to search against the SwissProt database (version 56.2, contains 398,181 sequences, comprising 143,572,911 residues). A maximum of two missed cleavages was allowed, and the mass tolerance of precursor and sequence ions was set to 10 ppm and 0.35 Da, respectively. Acrylamide modification of cysteine and methionine oxidation were considered as possible modifications. Scaffold (version 2.02, Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Quantification was carried out using the Mascot Distiller Quantitation Toolbox (version 2.2.1.2, Matrix Science Ltd., London) and was based on calculations of $^{16}O/^{18}O$ isotope intensity ratios of at least two identified peptides.

For the experiment shown in suppl. table 1 sample preparation, in-gel digestion, and protein identification and relative quantification were done as described above with the

exception that LC-MS/MS analyses were performed on an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher, Bremen, Germany). Stable isotope labeling was performed during tryptic digestion in the presence of $H_2{}^{18}O$ (Campro Scientific GmbH, 97% ^{18}O) and $H_2{}^{16}O$ for proteins from the non-ubiquitinated and ubiquitinated pull-down respectively.

Nuclear Magnetic Resonance

NMR spectra were recorded on Bruker AV700 MHz equipped with triple-resonance cryoprobes. 1H-15N Heteronuclear Single Quantum Coherence (HSQC) spectra of 15N labeled Itch WW3 with or without peptide PPXY were measured at 300 K applying a protein concentration of 120 μM. Buffer conditions were 100 mM NaCl, 20 mM Tris and pH 7. Spectra were subsequently processed and overlaid using Topspin (Bruker) and CCPNMR Analysis software (Vranken et al, 2005). Assignment by Shaw et al. (Shaw et al, 2005) was downloaded from the Biological Magnetic Resonance Data Bank (Ulrich et al, 2008).

Statistics

Statistics were done using the unpaired t-test for all quantified experiments with the following exceptions. Kinase assays and qRT-PCR experiments were assessed with a paired t-test. For the data shown in Figs. 3B and 3J (no peptide vs. peptide -treated samples) a one-sample t-test was used.

Supplementary references

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Legends to supplementary figures

Supplementary figure S1: Identification of Itch as a PI4KIIα-binding protein by MS/MS-based proteomic analysis

(**A**) Coomassie gel of proteins purified from rat brain extract on a GST-PI4KIIα matrix. GST incubated with rat brain extract or GST-PI4KIIα in the absence of extract was used as control. (**B**) Itch protein sequence; peptides identified by MS/MS analysis are highlighted in yellow. (**C**) Selection of proteins identified in the large scale pulldown shown in A by MS/MS-based

mass spectrometry. **(D)** Cell extract of HEK293 cells stably expressing HA-PI4KIIα were incubated with a matrix containing either GST-fused WW1-4 domains of Nedd4.1 or Nedd4.2. WW1-4 domains of both Nedd4-variants bind to HA-PI4KIIα, but not to Gadkin (negative control). **(E)** Antibody against endogenous Itch co-immunoprecipitates HA-PI4KIIα from an extract prepared from HEK293 cells stably expressing HA-PI4KIIα. **(F)** Extract prepared from HEK293 cells stably expressing HA-PI4KIIα was incubated with individual GST-fused WW domains of Itch. WW1, WW2 and WW3 bind HA-PI4KIIα, whereas WW4 does not. Arrowhead indicates brain-specific background band.

Supplementary figure S2: PI4KIIα and Itch localization

(**A-F**) Confocal images of HeLa cells immunostained for the indicated endogenous proteins. Scale bar, 12 µm. (**G-L**) Fluorescence intensities of lines shown in (A-F) plotted over distance. (**M**) Pearson correlations of data shown in (A-F).

Supplementary figure S3: Itch negatively regulates PI4KIIα activity

Analysis of PI4KIIα-mediated PI(4)P synthesis using PI and γ-^{32}P-ATP as substrates. Lipid products were separated by TLC and analyzed by phosphoimaging. **(A)** Production of PI(4)P by His_6-PI4KIIα increases in a time-dependent manner. Data were normalized to the average amount of PI(4)P generated within 15, 20, and 25 min. Individual data points represent the mean of two independent experiments. **(B)** Linear correlation between the production of PI(4)P and the amount of His_6-PI4KIIα present during the assay. Data were normalized to the amount of PI(4)P generated by 1 µg of PI 4-kinase. **(C)** Mutation of the PPxY-motif does not impair the catalytic activity of His_6-PI4KIIα. **(D)** The activity of His_6-PI4KIIα is inhibited upon addition of His_6-tagged WW1-4 domains of Itch in a dose-dependent manner (molar ratios: 1:1, 1:5 and 1:15). Full-length His_6-Itch is almost as efficient in inhibiting His_6-

PI4KIIα as the WW-domains alone. Data were derived from two independent experiments and normalized to the amount of PI(4)P generated by His_6-PI4KIIα in absence of Itch. **(E)** Multi-ubiquitination of His_6-PI4KIIα by Itch does not affect its kinase activity. (*Left*) Western blot analysis of samples derived from *in-vitro*-ubiquitination experiments using a PI4KIIα-specific antibody. Ubiquitinated PI4K-IIα is only generated in presence of wild-type Itch, but not in presence of the catalytically inactive mutant C830A. (*Right*) Aliquots derived from the ubiquitination reactions were subjected to kinase activity assays. Data were normalized to the amount of PI(4)P synthesized by unmodified His_6-PI4KIIα, i.e. kinase preincubated in presence of His_6-Itch(C830A); n = 3.

Supplementary figure S4:

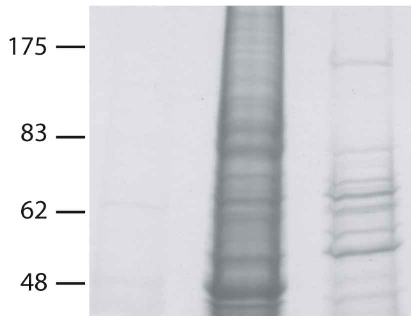
(**A**) Working model describing the function of PI4KIIα and Itch in degradative sorting of the Wnt receptor Fz4. See main manuscript for details. **(B)** HeLa cells were transfected with eGFP-PI4KIIα and stained for endogenous Itch. Inset values represent Pearson's correlation of Itch and trans-Golgi network marker TGN46 (mean ±SD). eGFP-PI4KIIα overexpression results in increased localization of Itch to perinuclear membranes. Scale bar, 12 µm. **(C)** Confocal images of HEK293 cells overexpressing HA-Dvl2 and mRFP-PI4KIIα stained with antibodies against HA (Dvl2) and endogenous Itch. Scale bar, 10 µm. **(D)** Analysis of mean GFP fluorescence per cell. Cells express eGFP-PI4KIIα WT, SF and D308A at equal levels. Supplementary to figure 4C. **(E)** Representative example of immublot used for quantification in fig. 4G. **(F)** HeLa cells were silenced with the indicated siRNAs and allowed to internalize Alexa488-labeled-transferrin for 10 min (mean±SEM). Transferrin uptake is not affected by PI4KIIα silencing.

Function	Accession	Ratio unmod/modified	SD(geo)	#peptides[a]	Protein ID
DUB / endosomal sorting	IPI00000728	**0.07**	1.32	18	USP15 Isoform 1 of Ubiquitin carboxyl-terminal hydrolase 15
	IPI00023191	**0.11**	1.03	2	TOM1 cDNA FLJ54710, highly similar to Target of Myb protein 1
	IPI00021048	**0.16**	1.96	6	MYOF Isoform 1 of Myoferlin
actin dynamics	IPI00328571	**0.74**	1.56	9	WASH3P Actin nucleation promoting factor
	IPI00029175	**1.09**	1.04	32	KIAA0196 WASH complex subunit strumpellin
	IPI00792422	**1.30**	1.16	12	FAM21A Isoform 1 of WASH complex subunit FAM21A
endocytosis	IPI00045219	**3.02**	2.01	6	SNX18 Isoform 1 of Sorting nexin-18
	IPI00019169	**6.93**	1.20	5	SH3GL1 cDNA, Homo sapiens SH3-domain GRB2-like 1 (SH3GL1)
	IPI00019171	**7.06**	1.54	3	SH3GL2 Endophilin-A1
	IPI00001883	**7.22**	1.15	4	SNX9 Sorting nexin-9

Supplementary Table 1: Quantitative proteomic analysis of proteins associated with unmodified (heavy) vs. ubiquitin-modified (light) GST-PI4KIIα from HeLa cell extracts. Proteins preferentially binding to ubiquitinated PI4KIIα display a low heavy/ light isotope ratio and are highlighted in green. [a]Number of identified peptides used for relative quantification

Figure S1

A



B

ITCH_HUMAN (100%), 102.805,1 Da
sp|Q96J02|ITCH_HUMAN E3 ubiquitin-protein ligase Itchy homolog
10 unique peptides, 10 unique spectra, 11 total spectra, 124/903 amino acids (14% coverage)

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MSDSGSQLGS MGSLTMKSQL QITVISAKLK ENKKNWFGPS
PYVEVTVDGQ SKKTEKCNNT NSPKWKQPLT VIVTPVSKLH
FRVWSHQTLK SDVLLGTAAL DIYETLKSNN MKLEEVVVTL
QLGGDKEPTE TIGDLSICLD GLQLESEVVT NGETTCSENG
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SHEVLNPMYC LFEYAGKDNY CLQINPASYI NPDHLKYFRF
IGRFIAMALF HGKFIDTGFS LPFYKRILNK PVGLKDLESI
DPEFYNSLIW VKENNIEECD LEMYFSVDKE ILGEIKSHDL
KPNGGNILVT EENKEEYIRM VAEWRLSRGV EEQTQAFFEG
FNEILPQQYL QYFDAKELEV LLCGMQEIDL NDWQRHAIYR
HYARTSKQIM WFWQFVKEID NEKRMRLLQF VTGTCRLPVG
GFADLMGSNG PQKFCIEKVG KENWLPRSHT CFNRLDLPPY
KSYEQLKEKL LFAIEETEGF GQE
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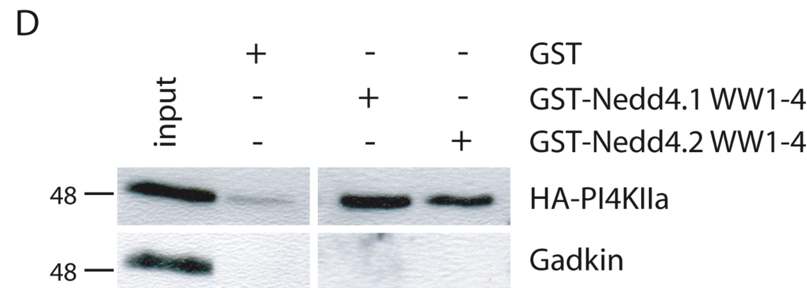
C

Protein name	number of unique peptides	% sequence coverage
sp|O9JME5|AP3B2_MOUSE AP-3 complex subunit beta-2	38	38
sp|O54774|AP3D1_MOUSE AP-3 complex subunit delta-1	24	23
sp|P62990|UBIQ_BOVIN Ubiquitin	2	33
sp|Q6ZPJ3|UBE2O_MOUSE Ubiquitin-conjugating enzyme E2 O	21	19
sp|P70398|USP9X_MOUSE Probable ubiquitin carboxyl-terminal hydrolase FAF-X	40	17
sp|Q96J02|Itch_HUMAN E3 ubiquitin-protein ligase Itchy homolog	10	14
sp|O00203|AP3B1_HUMAN AP-3 complex subunit beta-1	6	14
sp|Q62940|NEDD4_RAT E3 ubiquitin-protein ligase NEDD4	5	6
sp|Q9H0M0|WWP1_HUMAN NEDD4-like E3 ubiquitin-protein ligase WWP1	3	4

D



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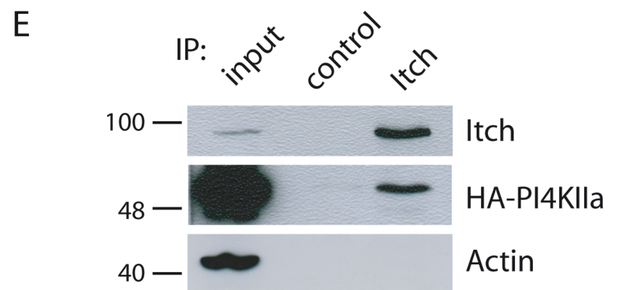

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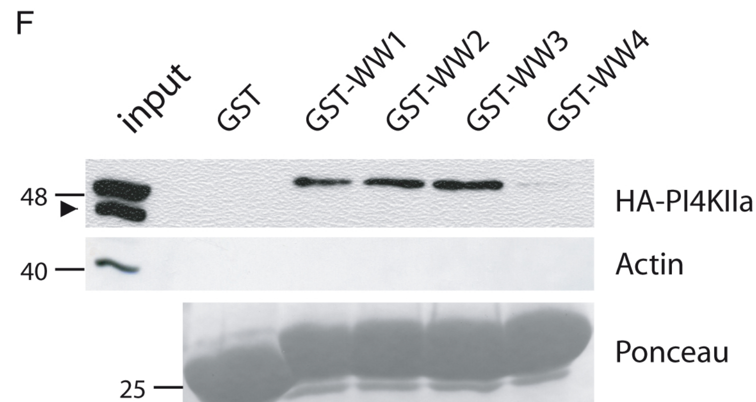

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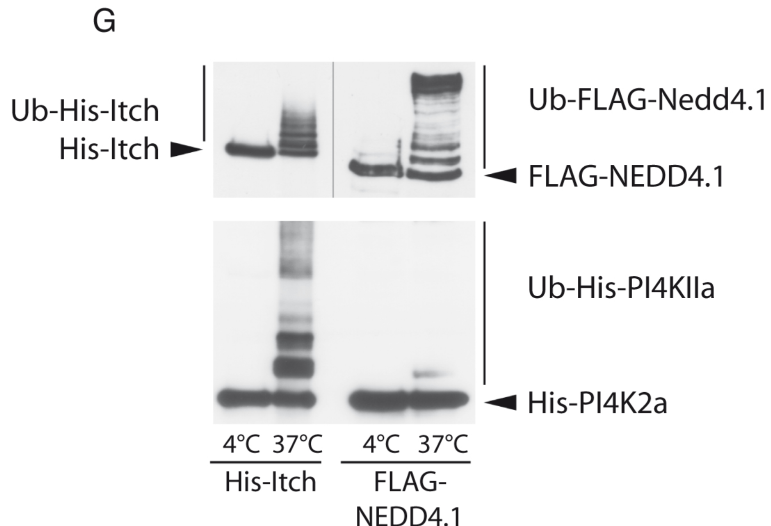
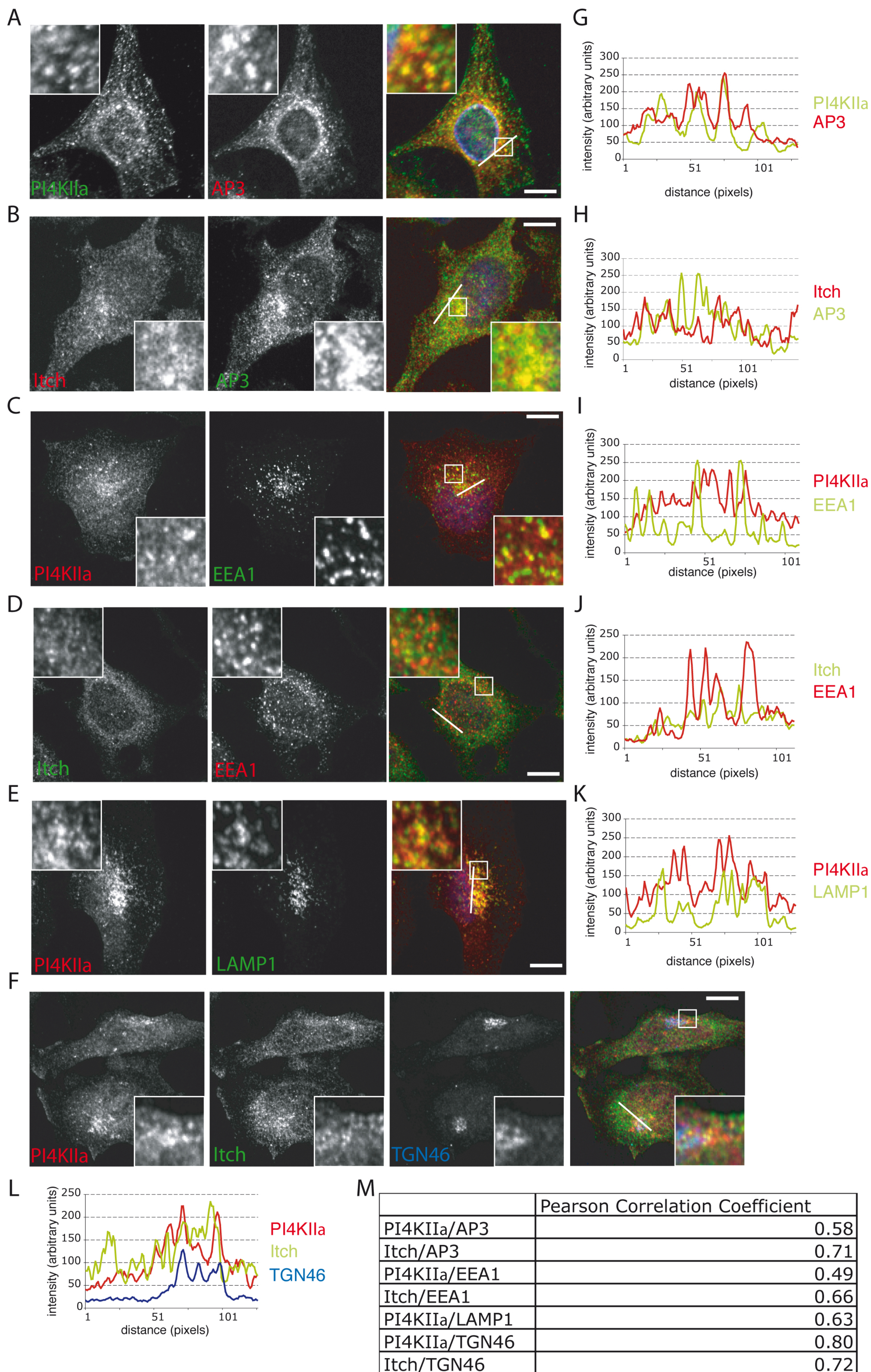
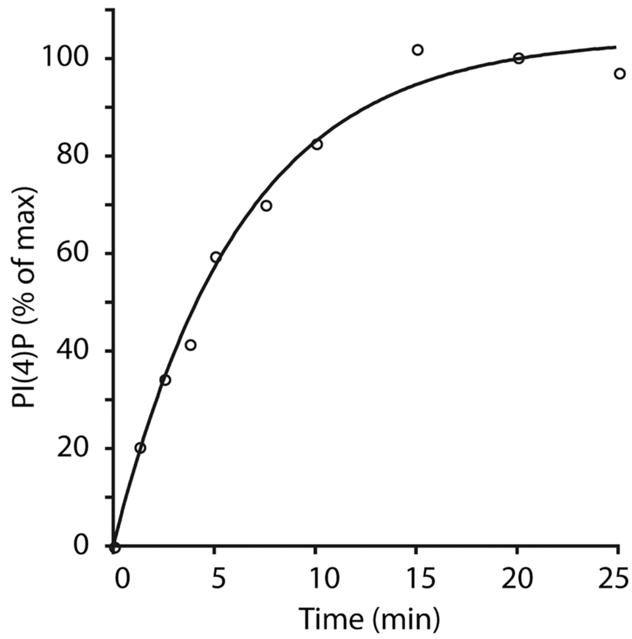


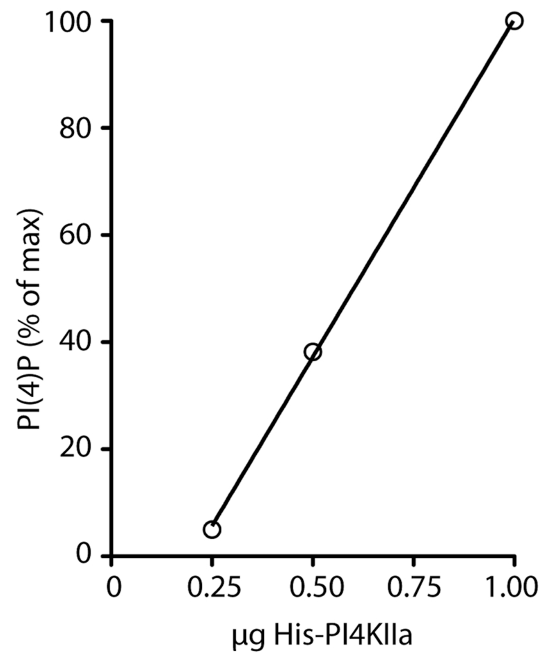
Figure S2

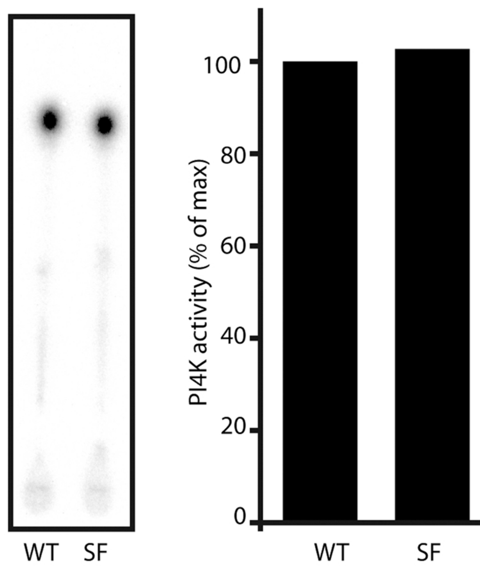


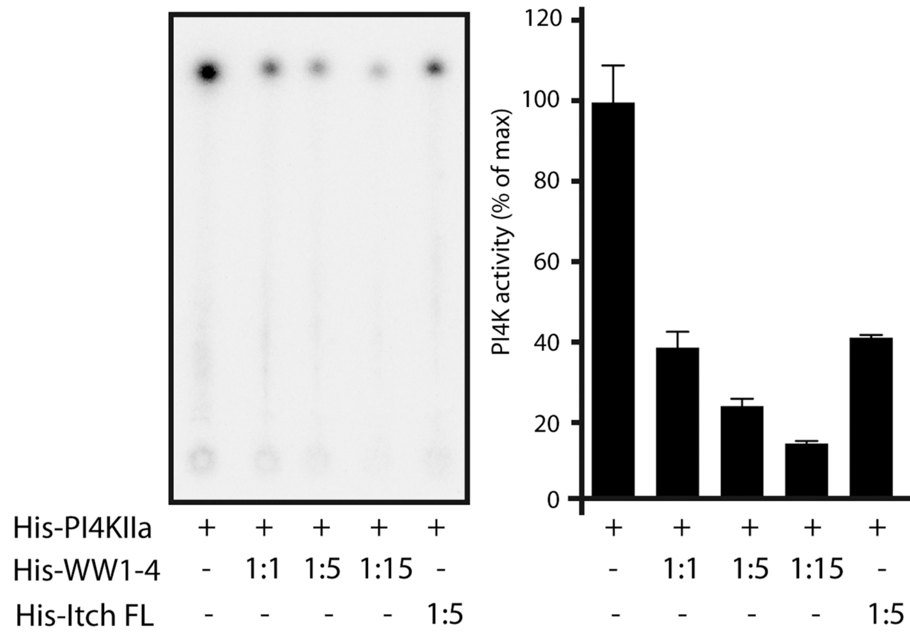
	Pearson Correlation Coefficient
PI4KIIa/AP3	0.58
Itch/AP3	0.71
PI4KIIa/EEA1	0.49
Itch/EEA1	0.66
PI4KIIa/LAMP1	0.63
PI4KIIa/TGN46	0.80
Itch/TGN46	0.72

Figure S3









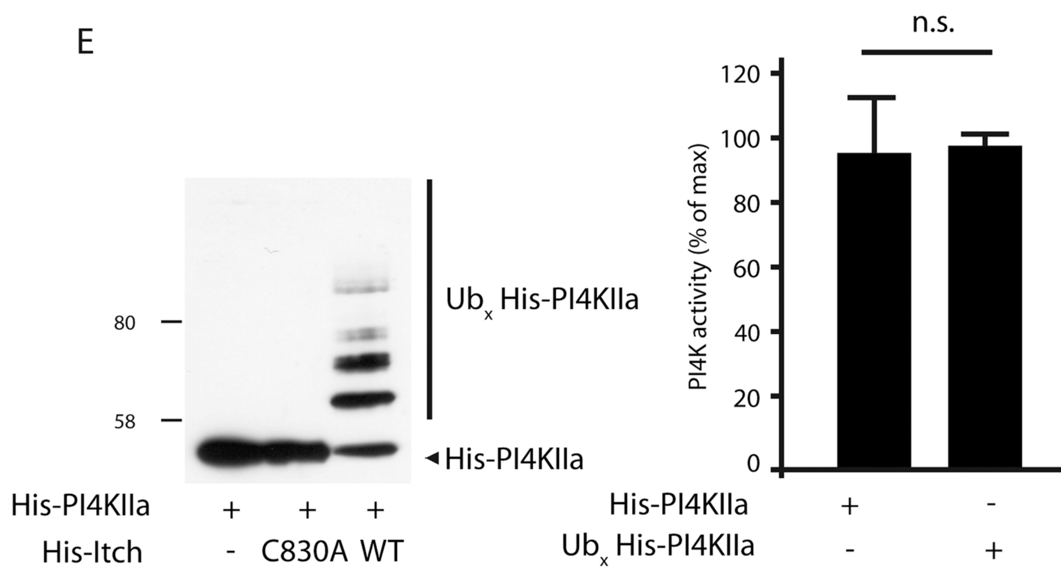
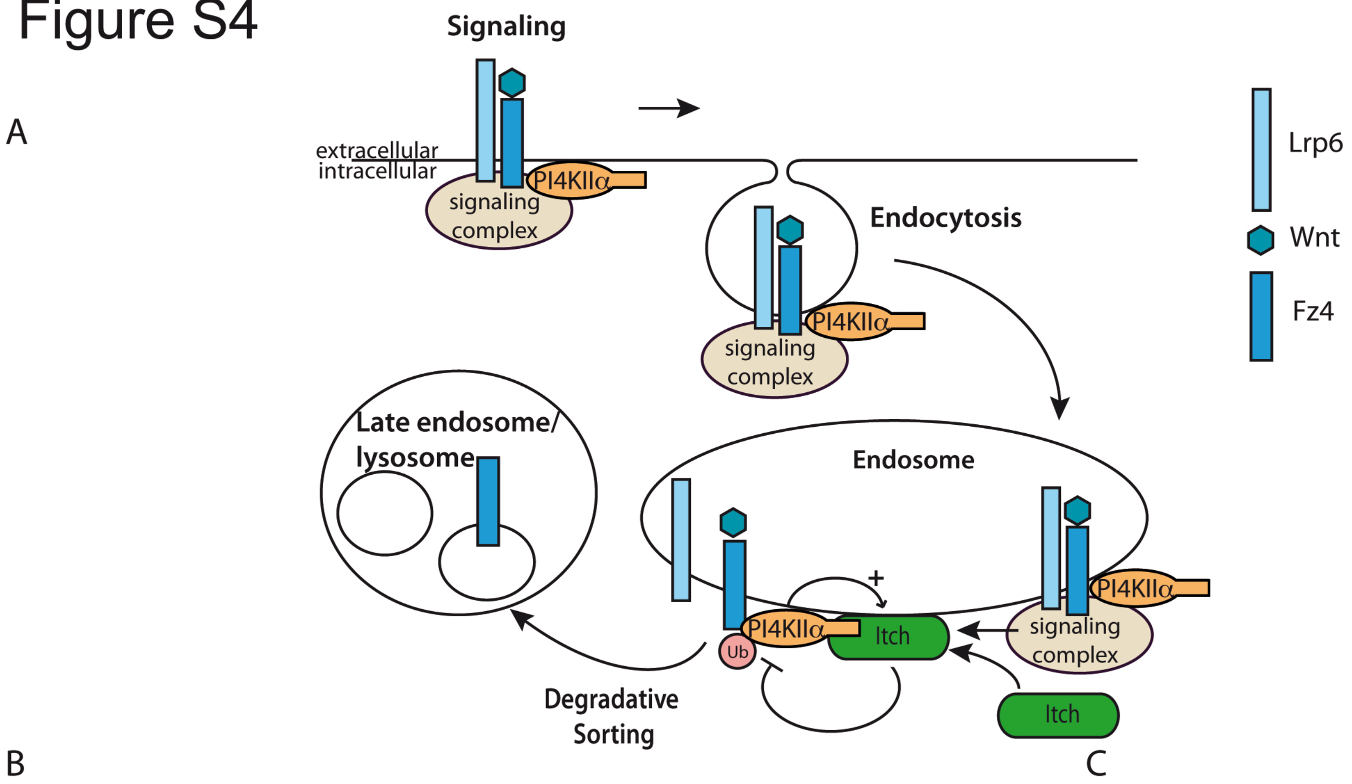
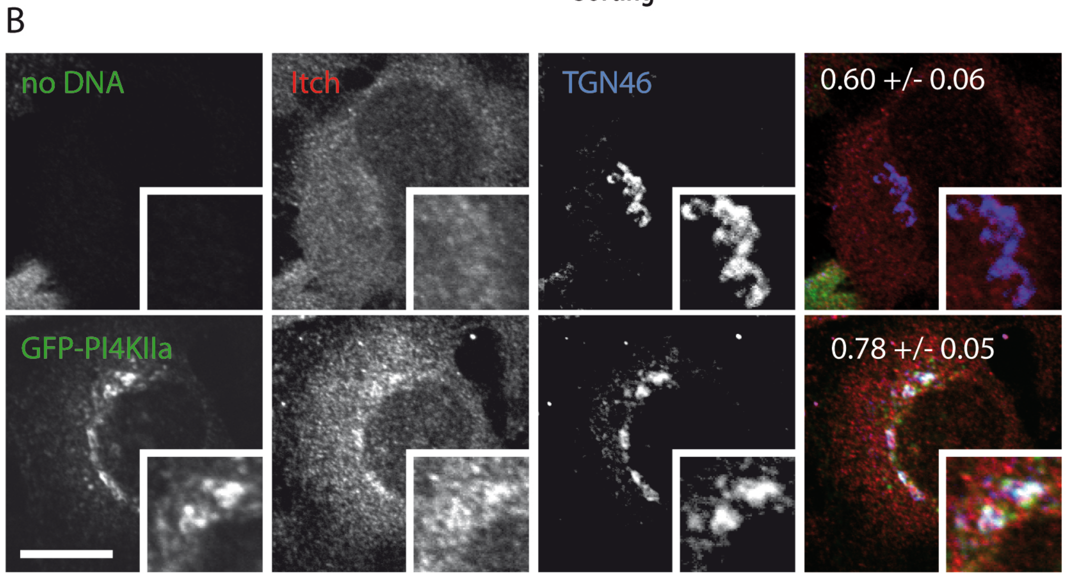


Figure S4

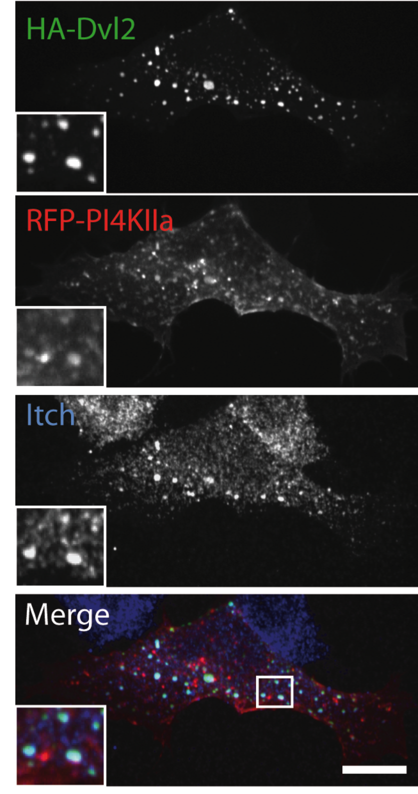
A



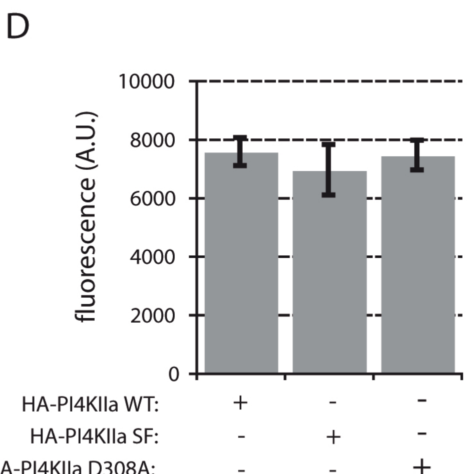
B



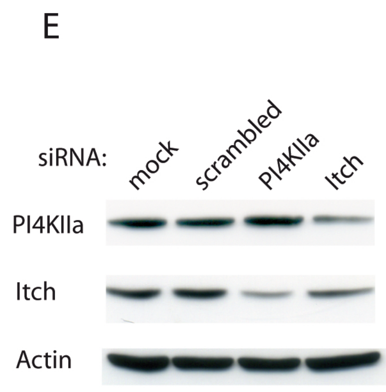
C



D



E



F