Supporting Information

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SI Experimental Procedures

Antibodies. Antibodies used in this study were as follows: goat anti-AP3B1 (Santa Cruz Biotehnology), goat anti-AP3D1 (Santa Cruz), rabbit anti-Kif3A (Abcam), mouse monoclonal anti-HA (Clone HA-7; Sigma), mouse monoclonal anti-Myc (Clone 4A6; Millipore; Upstate), goat anti-actin (Santa Cruz Biotechnology), mouse anti-HSP60 (Calbiochem), mouse anti-HIV-1 p24 [EVA365, EVA366; National Institute for Biological Standards and Control (NIBSC) Centre for AIDS Reagents], rabbit anti-glutathione-S-transferase (anti-GST; Sigma).

Expression Constructs. Unless otherwise stated all cDNAs are full-length. All of the constructs used in this study, expect NSR1, IP6K1, and mKif3A, encode human proteins with the following accession numbers: NP003655 (AP3B1); NP008985 (Kif3A); NP066267 (Ankyrin); and NP005372 (Nucleolin). The NSR1 encodes a yeast protein (CAA59817) and mKif3A encodes a mouse protein (NP032469). Plasmids for expression in yeast cells were cloned as follows: (*i*) The yeast two-hybrid bait pGBKT7-AP3B1 (576–902) was cloned into the *Bam*HI and *Pst*I sites of pGBKT7 (Clontech); (*ii*) pADH:GST vector was engineered by replacing the Gal1 promoter from pYesGST (1) with the ADH1 promoter using the *Hin*dIII and *Bam*HI cloning sites, and all derived constructs were cloned into the *Sal*I and *Not*I sites—GST-AP3B1, GST-AP3B1 (1–676), GST-AP3B1 (1–706), GST-AP3B1 (576–902), GST-AP3B1 (576–706), GST-AP3B1 (576–686), GST-AP3B1 (576–691), GST-AP3B1 (707–774), GST-AP3B1 (775–882), GST-NSR1, GST-Nucleolin, GST-Kif3A; and (*iii*) HA-Kif3A (601–702) and HA-ANK (3,864–4,082) in pACT2 (Clontech) were obtained from the YTH screen. Plasmids for expression in mammalian cells were cloned as follows: (*i*) Constructs in pCMV-GST (2) were cloned into the *Sal*I and *Not*I sites—GST-AP3B1, GST-AP3B1(577–1094), GST-Kif3A; and (*ii*) constructs in pCMV-Myc (Clontech) were cloned into the *Sal*I and *Not*I sites—Myc-AP3B1, Myc-AP3B1 (576–902), Myc-Kif3A, Myc-Kif3A (1–342) and Myc-Kif3A (354–702), Myc-mKif3A (1–701). Myc-IP_6K1 (mouse), Myc-IP_6K1K/A (mouse), Myc-IP_6K2 (human), Myc-IP_6K2K/A (human) were described (3); constructs in pEGFP-C1 (Clontech) were cloned into the *Sac*II and *Xho*I sites—EGFP-AP3B1 and EGFP-Kif3A. For expression in *E. coli* (BL21), AP3B1 (576–902) was cloned into the *Sal*I and *Not*I sites of pGex-4T-2 (GE Healthcare), and Kif3A was cloned into pHisTrcA (Invitrogen). A plasmid expressing Gag-GFP (4) was kindly provided by Wes Sundquist (University of Utah, Salt Lake City, Utah).

Yeast Strains and Yeast Two-Hybrid Screen. The following yeast strains were used in this study: *kcs1*Δ, *vip1*Δ, and wild-type background DDY1810 (indicated as WT). Nontransformed yeast strains were grown in YPD media (Bacto Yeast extract, Bacto Peptone, Dextrose; Formedium), yeast strains transformed with pADH:GST vector were grown on SD-Ura$^-$ (minimum synthetic dropout medium without uracil; Formedium), and yeast strains transformed with pACT2 vector (Clontech) were grown on SD-Leu$^-$ (SD without leucine; Formedium). Yeast transformations were performed using the lithium acetate method according to Matchmaker Gal4 Two-Hybrid System 3 (Clontech). YTH screen was performed in the yeast strain AH109 (Clontech) using pGBKT7-AP3B1 (576–902) as bait and a human fetal brain MATCHMAKER cDNA library as a prey (pACT2 based library; Clontech). The screening was performed according to the manufacturer's instructions (Clontech), and 3.5×10^6 independent clones were screened. Positive clones were retransformed with the bait into AH109 and grown on SD-LT$^-$ media (SD without leucine and tryptophan) to assess growth and in SD-LTHA$^-$ (SD without leucine, tryptophan, histidine, and adenine) to assess interaction.

RNA Interference. Twenty-one-nucleotide validated siRNA duplexes corresponding to siRNA-Kif3A (AAGACCTGATGTGGGAGTTTA), and negative control AllStars control siRNA (Qiagen) were used in the silencing experiments. siRNAs (120 μM) were transfected per well of a 24-well plate. Twenty-four hours after the initial transfection, cells were retransfected with the same amount of siRNA and subsequently split. Seventy-two hours after the initial transfection, cells were transfected with Gag-GFP. Cells and VLPs were collected 16–18 h later.

Mammalian Cell Cultures and Transfections. HeLa and MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin/streptomycin. HeLa cells were transfected with Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. Transfected HeLa cells were harvested 24 h later, and the proteins extracted accordingly. MEF cells were transfected with FuGENE HD (Roche) according to manufacturer's instructions. Forty-eight hours after transfection, cells were harvested, and the proteins extracted accordingly.

Purification of GST-AP3B1 from *E. coli*. For GST purification from *E. coli*, GST-AP3B1 (576–902) was transformed into the strain BL21. Cell cultures were induced for 4 h at 37 °C with 1 M IPTG to a final concentration of 1 mM. Cells were pelleted by centrifugation at 5,000 rpm for 15 min at 4 °C, washed, and resuspended in 12 mL ice-cold GST lysis buffer [20 mM HEPES, pH 6.8,100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% CHAPS, 5 mM DTT, and 1/500 protease inhibitor mixture (PIC; Sigma)]. The cells were lysed by sonication and clarified by centrifugation at 10,000 rpm for 20 min at 4 °C. The supernatant was incubated with 1 mL 50% glutathione beads slurry (pre-equilibrated in GST lysis buffer) and 1% Triton X-100 for 2 h at 4 °C. Beads were washed three times in GST wash buffer (50 mM Tris·HCl, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100) and equilibrated twice in GST lysis buffer.

Protein Extraction, Immunoprecipitations, and GST-Pull Downs. For the phosphorylation assays with yeast protein extracts, proteins were extracted in IP_7 phosphorylation buffer (20 mM HEPES, pH 6.8, 5 mM DTT, 1 mM EGTA, 1 mM EDTA, 0.1% CHAPS, 200 μg/mL PMSF, and 1/500 PIC). For yeast protein analyses or GST pull-down experiments with yeast proteins extracts, proteins were extracted in the following extraction buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 5 mM EDTA, 5% glycerol, 5 mM DTT, 1/500 PIC). In all cases, yeast protein were extracted through vortexing with glass beads. Cleared cell homogenates were either immediately boiled in sample buffer or normalized and used for pull-down experiments. During the experiments in which gel mobility shift was being analyzed, it was crucial that the protein samples were rapidly processed, since the shift associated with IP_7-mediated pyrophosphorylation is unstable. For GST pull-downs from yeast extracts, glutathione beads were incubated with protein extracts for 2 h at 4 °C and washed three times in the lysis buffer containing 1% Noniodet P-40. The beads were resuspended in 2× LDS sample buffer and boiled. For all of the

assays with mammalian protein extracts, except the immunoprecipitations, proteins were extracted in hypotonic buffer (10 mM Tris·HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1/500 PIC) for 10 min on ice, after which NaCl was added to a final concentration of 150 mM, and the cells were incubated on ice for a further 5 min. For AP3B1 immunoprecipitations from MEF cells, proteins were extracted in nondenaturing lysis buffer (20 mM Tris·HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 1/500 PIC). Protein extract (400 μg) was precleared with protein G-agarose (Sigma). Cleared extracts were incubated with 2 μg anti-AP3B1 antibody overnight at 4 °C, after which protein G-agarose (Sigma) was added and incubated for 4 h further. The beads were washed in lysis buffer, resuspended in 2× LDS sample buffer, and boiled.

Relative Protein Quantification. Quantification of protein bands intensities was performed with Quantity One program (version 4.6.5; Bio-Rad Laboratories) on scanned X-ray films (GS-800 calibrated densitometer scanner; Bio-Rad Laboratories).

SAX-HPLC Inositol Polyphosphates Analysis. Analysis of inositol polyphosphates from yeast or mammalian cells was performed as described in ref. 5.

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A

HsAP3B1

1
MSSNSFPYNE QSGGGEATEL GQEATSTISP SGAFGLFSSD LKKNEDLKQM LESNKDSAKL
DAMKRIVGMI AKGKNASELF PAVVKNVASK NIEIKKLVYV YLVRYAEEQQ DLALLSISTF
QRALKDPNQL IRASALRVLS SIRVPIIVPI MMLAIKEASA DLSPYVRKNA AHAIQKLYSL
DPEQKEMLIE VIEKLLKDKS TLVAGSVVMA FEEVCPDRID LIHKNYRKLC NLLVDVEEWG
QVVIIHMLTR YARTQFVSPW KEGDELEDNG KNFYESDDDQ KEKTDKKKKP YTMDPDHRLL
IRNTKPLLQS RNAAVVMAVA QLYWHISPKS EAGIISKSLV RLLRSNREVQ YIVLQNIATM
SIQRKGMFEP YLKSFYVRST DPTMIKTLKL EILTNLANEA NISTLLREFQ TYVKSQDKQF
AAATIQTIGR CATNILEVTD TCLNGLVCLL SNRDEIVVAE SVVVIKKLLQ MQPAQHGEII
KHMAKLLDSI TVPVARASIL WLIGENCERV PKIAPDVLRK MAKSFTSEDD LVKLQILNLG
576
AKLYLTNSKQ TKLLTQYILN LGKYDQNYDI RDRTRFIRQL IVPNVKSGAL SKYAKKIFLA
QKPAPLLESP FKDRDHFQLG TLSHTLNIKA TGYLELSNWP EVAPDPSVRN VEVIELAKEW
676 686 691 698 706
TPAGKAKQEN SAKKFYSESE EEEDSSDSSS DSESESGSES GEQGESGEEG DSNEDSSEDS
774
SSEQDSESGR ESGLENKRTA KRNSKAKGKS DSEDGEKENE KSKTSDSSND ESSSIEDSSS
II
DSESESEPES ESESRRVTKE KEKKTKQDRT PLTKDVSLLD LDDFNPVSTP VALPTPALSP
III
882
SLMADLEGLH LSTSSSVISV STPAFVPTKT HVLLHRMSGK GLAAHYFFPR QPCIFGDKMV
902
SIQITLNNTT DRKIENIHIG EKKLPIGMKM HVFNPIDSLE PEGSITVSMG IDFCDSTQTA
SFQLCTKDDC FNVNIQPPVG ELLLPVAMSE KDFKKEQGVL TGMNETSAVI IAAPQNFTPS
VIFQKVVNVA NVGAVPSGQD NIHRFAAKTV HSGSLMLVTV ELKEGSTAQL IINTEKTVIG
SVLLRELKPV LSQG 1094

Trunk

Hinge

Ear

B

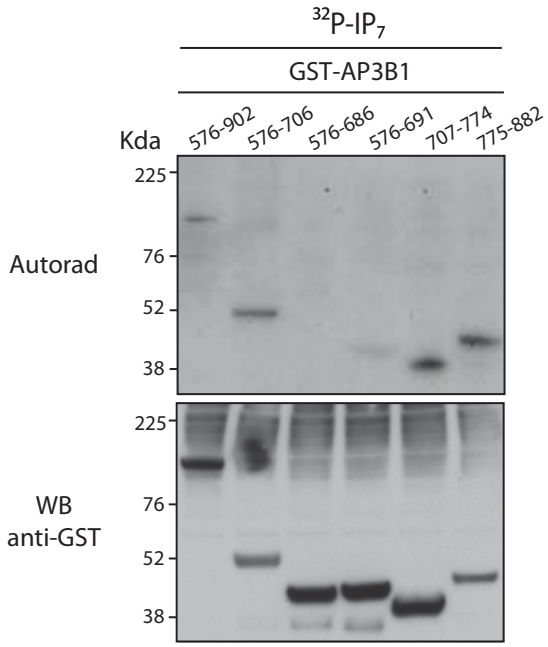

Fig. S1. Protein sequence of human AP3B1. AP3B1 is composed of three main domains: (*A*) The N-terminal head domain (in red), the hinge (in green), and the C-terminal ear domain (in black). The region used as bait for the yeast two-hybrid screening (576- 902; represented in bold) contains the three acidic serine-rich regions that correspond to the putative target of IP_7 phosphorylation (regions I–III). (*B*) AP3B1 acidic regions are targets of IP_7-mediated pyrophosphorylation. Protein extracts of /kcs1/D yeast expressing different GST-AP3B1 derivatives were treated as in Fig. 1*B*.

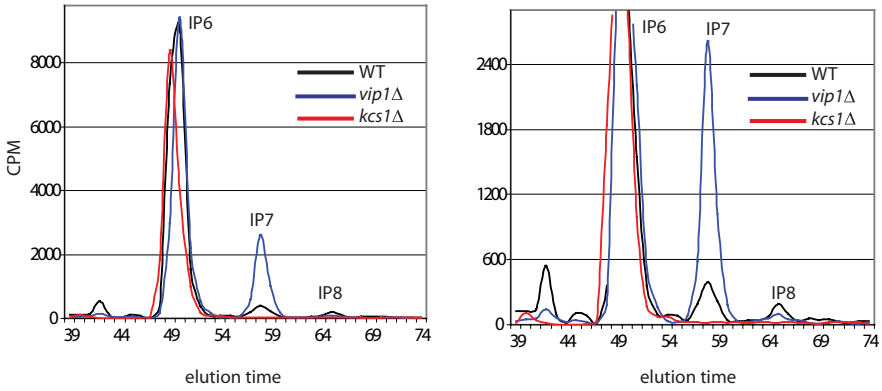

Fig. S2. Yeast *vip1*Δ single mutant accumulate IP_7. SAX-HPLC analyses of inositol phosphates in [^{3}H]inositol-labeled yeast (5) wild-type (WT), the mutants *kcs1*Δ (6, 7), and VIP1 (8) deleted strain *vip1*Δ.

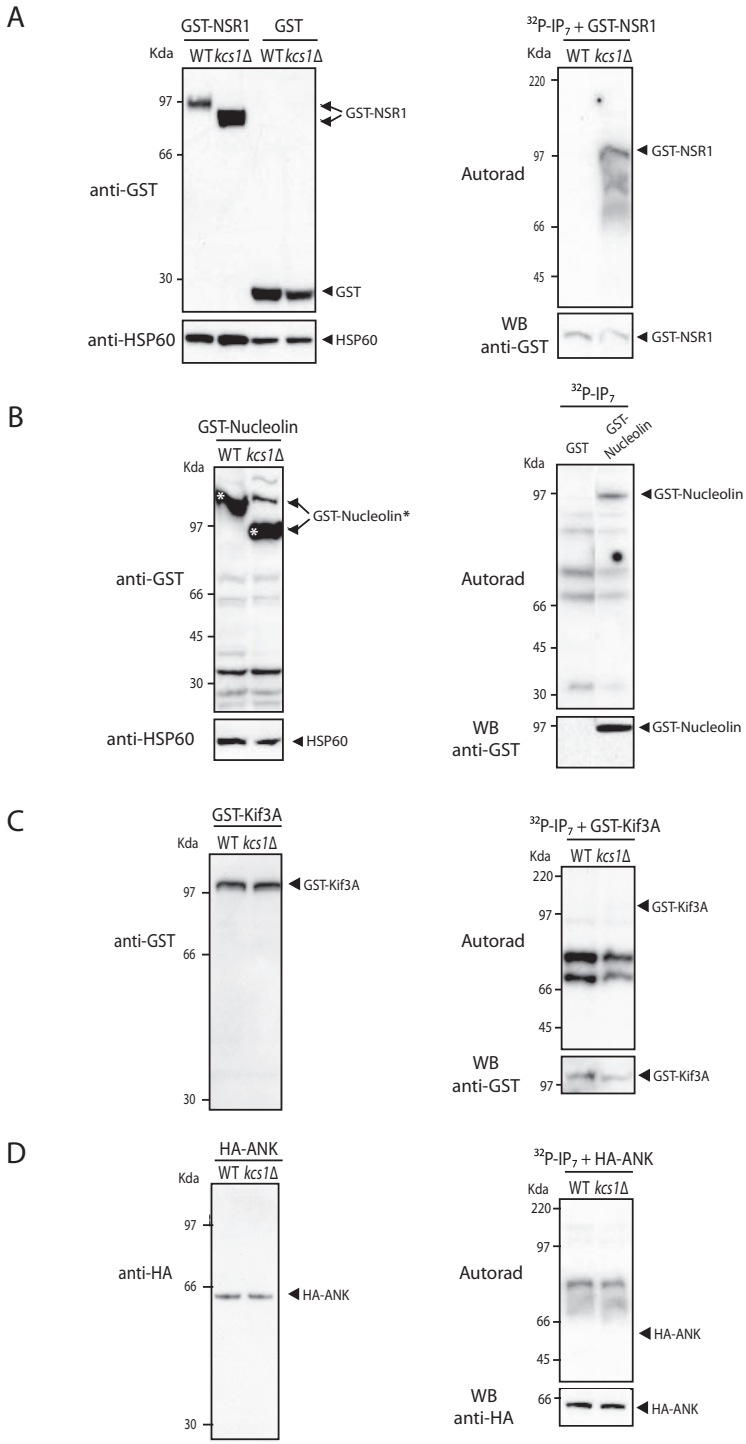

Fig. S3. Gel mobility shift of known targets of IP_7-mediated pyrophosphorylation. (*A*) Protein from WT and *kcs1*Δ yeast strains expressing: GST-NSR1 or empty GST vector control, were quickly extracted, resolved by NuPAGE (Invitrogen) and immunoblotted with anti-GST antibody (*Left*). IP_7 pyrophosphorylation assays were performed as described in the *Materials and Methods* section (*Right*). The level of in vitro pyrophosphorylation of GST-NSR1 is undetectable in WT cell due to endogenous pyrophosphorylation (see main text Fig. 1*D*). (*B*) Yeast expressing GST-Nucleolin was treated as in *A*. (*C*) Yeast expressing GST-Kif3A was treated as in *A*, the *Left* show that Kif3A is not pyrophosphorylated by IP_7. (*D*) Yeast expressing human ankyrin [HA-ANK (3864–4082)] was treated as in *A*, the *Left* show that ankyrin is not pyrophosphorylated by IP_7.

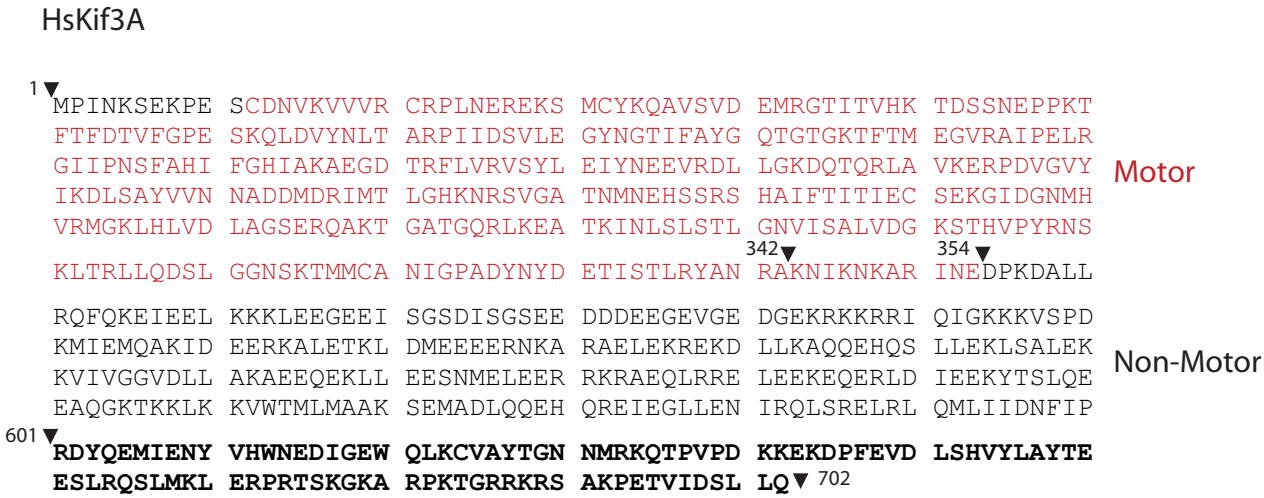

Fig. S4. Protein sequence of human Kif3A. Kif3A contains a highly conserved motor domain (in red) that binds to microtubules and hydrolyzes ATP and a stalk domain that interacts with other subunits of the kinesin-2 complex and with cargo molecules (in black). The amino acids encoded by the clone identified in the yeast two-hybrid screening with AP3B1 (576–902) as bait are represented in bold.

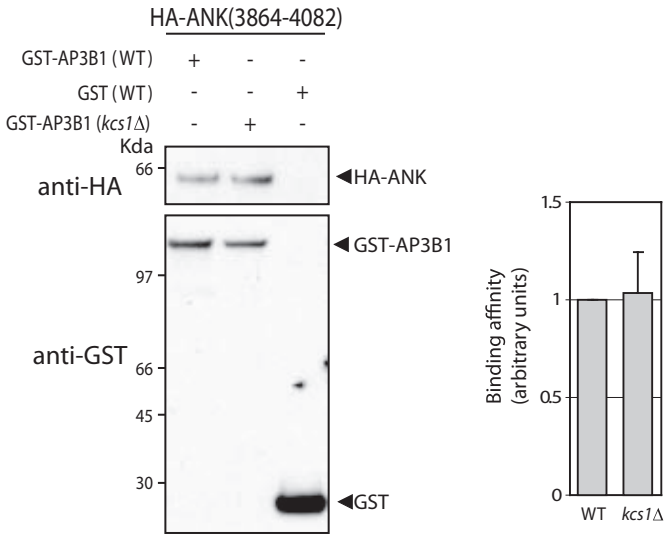

Fig. S5. AP3B1 interaction with ankyrin is not affected by IP_7 pyrophosphorylation of AP3B1. GST-pull downs (*Left*) and respective quantification (*Right*). Protein from WT yeast expressing GST-AP3B1 or GST vector control, or from *kcs1*Δ yeast expressing GST-AP3B1 were incubated with protein extracts of *kcs1*Δ yeast expressing HA-ANK (3,864–4,082). Protein extracts were subjected to pull-down with glutathione beads. Interactions were detected by immunoblotting with anti-HA and anti-GST antibodies. Quantification was done by taking the ratio between the bands intensities of the HA-ANK (3,864–4,082) and the GST-AP3B1 and normalizing *kcs1*Δ expressed GST-AP3B1 against WT expressed protein. Data represent means ± standard deviation of the mean from three independent experiments.

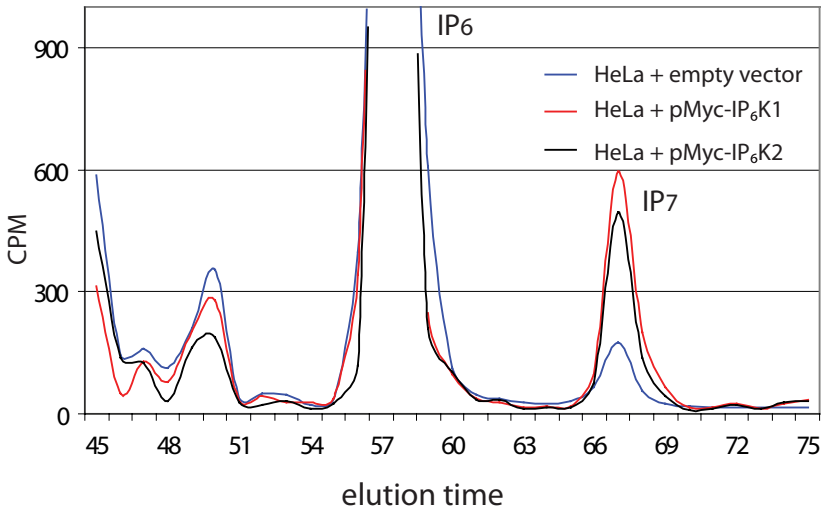

Fig. S6. SAX-HPLC analysis of IP_6K1 and two overexpression in HeLa cells. Cells were labeled with [^{3}H]inositol for 4 days (5) before transfection with empty vector, Myc- IP_6K1, or Myc-IP_6K2. Acidic extraction of soluble inositol polyphosphate was performed 48 h posttransfection, and inositol polyphosphates were analyzed by SAX-HPLC as described in ref. 5.