Supporting online materials

MATERIALS AND METHODS

Cloning of Drosophila melanogaster and human Vps34

We amplified, via polymerase chain reaction (PCR), residues 258-949 of *Drosophila melanogaster* from a cDNA library generously supplied by Dr. Marc Fiedler. The DmVps34 gene was cloned into the bacterial expression plasmid pOPTH(tev) between the NdeI and BamHI sites. The vector expresses an N-terminal His_6 tag that is cleavable with TEV protease, leaving the additional residues GSHM at the N-terminus of the DmVps34 HELCAT (helical and catalytic domains) construct. Sequencing of the plasmid revealed a G455A difference with respect to the database. This residue is highly variable among orthologues and may represent a polymorphism of the strain that we used for PCR. Full-length HsVps34, HsVps34-HELCAT (residues 257-887), HsVps34-HELCAT-ΔC18 (lacking C-terminal 18 residues) and HsVp34-ΔC10 (lacking C-terminal 10 residues) were all cloned into the pOPTH(tev) vector. Point mutations in the HsVps34 HELCAT were generated using the Quick-Change protocol (Stratagene).

Expression and purification of Vps34

The DmVps34 constructs were transformed into *Escherichia coli* C41 (DE3) RIPL cells, which were cultured in 2xTY (0.1 mg/ml ampicillin) at 37°C to OD_{600} 0.8-1.0 and induced with 50 μM IPTG at 12°C for approximately 14 hours. Cell pellets were resuspended in 10 mM Tris-HCl pH 8.0 (4°C), 50 mM KCl pH 8.0 (4°C), 200 mM NaCl, 1 Roche Complete EDTA-free Protease Inhibitor Cocktail Tablet and 0.1 mg/ml DNaseI, sonicated on ice for

3 minutes, then additionally lysed by French Press. Lysates were subjected to ultracentrifugation at 35,000 rpm for 40 minutes at 4°C. The supernatant was filtered through a 0.2 μm filter before being passed over a Ni-NTA 5 ml Fast Flow column (GE Healthcare) and eluted with an imidazole concentration gradient. The N-terminal His_6 tag was cleaved with TEV protease overnight at 4°C and the protein was further purified on a heparin affinity column (GE Healthcare) and by gel filtration on a Superdex-200 column equilibrated in 20 mM Tris-HCl pH 7.5 (4°C), 100 mM NaCl and 2 mM DTT. After gel filtration, $NaNO_3$ was added to the pooled fractions to a final concentration of 100 mM to prevent precipitation during concentration. The protein was concentrated in a 30 kDa MWCO Amicon Ultra15 Concentrator to 4.0-4.5 mg/ml. All HsVps34 constructs were expressed and purified using the same protocol as DmVps34 HELCAT, except cells were induced with 0.3 mM IPTG. The HsVps34 constructs used for enzymatic assays were concentrated without the addition of $NaNO_3$.

Crystallisation of DmVps34 HELCAT

The protein was screened in over 1700 crystallisation conditions using the LMB Innovadyne robotic crystallisation setup (1), using 100 nl of protein solution and 100 nl of the reservoir solution in 96-well plates. Initial crystals of DmVps34 were obtained with the Molecular Dimensions MemStart screen reagent 10, containing 0.1 M ammonium sulphate, 0.5 M K_2HPO_4 and 0.5 M Na_2HPO_4 (titrated to pH 7.5 with orthophosphoric acid). The crystals were optimised in grids varying the concentration of all the reagents, adjusting pH and screening cryogenic protectants, detergents and other additives. For data collection,

crystals were produced in 24-well Chryschem plates with 0.5 ml of reservoir solution, containing 0.88 M ammonium sulphate, 0.1 M K_2HPO_4 and 0.1 M Na_2HPO_4 (pH 7.5). DmVps34 protein was first mixed with Anapoe-58/Brij-58 (Hampton Research, 0.05% final concentration). Next, 1 μl of protein solution was mixed with 1 μl of reservoir in sitting drops. Crystals grew after 1-2 days and reached their maximum size of 100x50x20 μm^3, after about 10 days. Crystals were cryo-protected by equilibrating the drop with 0.7 M ammonium sulphate, 0.1 M K_2HPO_4 and 0.1 M $Na_2H\ PO_4$ (pH 7.5), 50 mM L-arginine pH 11.4 and 27% ethylene glycol. Crystals were mounted in nylon loops and flash cooled in liquid nitrogen.

Vps34 crystal complexes with inhibitors

DmVps34 HELCAT crystals were obtained as described previously and the drops equilibrated with mother liquor containing either 500 μM (for PI-103, PIK-90 and PIK-93) or 1 mM [for 3-methyladenine (3-MA)] of inhibitor. Inhibitor stocks were prepared in dimethyl sulfoxide (DMSO). Crystals were incubated in the inhibitor-containing solution for 24 hours before freezing.

Data collection, model building and refinement

Diffraction data for the apo-enzyme DmVps34 HELCAT were collected at station ID14EH4 of the European Synchrotron Radiation Facility (ESRF). Images were processed with Mosflm (2) and scaled with SCALA (3). Data collection statistics are summarised in Table S1. The structure was determined using molecular replacement with the programme

BALBES and p110γ (PDB ID 1E8X) (4). Model building was performed using Coot (5) alternating with refinement in REFMAC5 (6).

Modelling and structure analysis

The program Modeller (7) was used to build a model of the putative closed conformation of Vps34. For this model, the C-terminal helix was rotated to place it in contact with the surface of the monomer, rather than reaching to the other molecule in the asymmetric unit. For illustrations that include the C2 domain of Vps34, the HELCAT structure was simply superimposed on the helical domain/catalytic domain structure of p110γ using the BRUTE option of Lsqman (RMSD of 1.7 Å for 330 residues) and the C2 domain of the p110γ is illustrated after the superposition. In order to calculate the volume of the ATP-binding pocket, the CASTp server (8) was used to define an initial ATP-binding pocket from the p110γ /ATP complex (PDB ID 1E8X). Residues in this initially defined pocket that were remote from the ATP were removed from pocket definition and the volume was recalculated for the apo-enzyme. After superimposing Vps34 on the p110γ catalytic domain, a structure containing only the Vps34 residues structurally analogous to the p110γ /ATP-binding pocket was supplied to the CASTp server, in order to calculate the Vps34 apo-enzyme pocket volume.

Multi-angle light scattering

Purified HsVps34 constructs were subjected to size exclusion chromatography/multi-angle laser light scattering (SEC/MALLS) using an ÄKTA FPLC Chromatographic system (GE Healthcare) connected to a Dawn Heleos II 18-angle light scattering detector (Wyatt)

combined with an Optilab rEX differential refractometer (Wyatt). Protein samples (100 μl) were loaded onto a Superdex-200 HR10/30 gel filtration column (GE Healthcare) at a concentration of approximately 2.5 mg/ml and run at 0.5 ml/min in a buffer consisting of 20 mM Tris pH 7.5, 100 mM NaCl, 2 mM DTT and 0.01% NaN_3. Data were processed using Astra V software.

Lipid kinase activity assay

Lipid kinase activity of human Vps34 full-length, HELCAT and its mutants was determined using an Adapta Kinase Assay (Invitrogen) according to manufacturer's protocol. Briefly, kinase reactions were performed in a 10 μl volume in 384-well NBS black plates (Corning, USA), containing 10 nM enzyme in 1x kinase reaction buffer (50 mM HEPES pH 7.5, 1 mM EGTA, 0.1% CHAPS, 2 mM $MnCl_2$ and 2 mM DTT). The reaction was initiated by the addition of 10 μM ATP/100 μM PtdIns:PS substrate (PV5122, Invitrogen) solution and carried out for 1 hour. The reaction for the intrinsic ATPase activity of the enzyme included the same components except for the substrate. Both reactions were stopped by addition of 5 μl of the stop/detection solution (10 mM EDTA, 2 nM Adapta Eu-antiADP antibody, 3 nM AlexaFluor647-ADP tracer) and allowed to equilibrate at room temperature for 1 hour before reading. Time-resolved fluorescence measurements were performed using a PHERAStar HTS microplate reader (BMG Labtech, Aylesbury, UK) using a 340 nm excitation filter with both 620 nm and a 665 nm emission filters to measure Europium and Alexa Fluor® 647 TR-FRET emissions, respectively. In addition, a series of ATP/ADP mixtures ranging from 100% to 0.01% ADP (ADP/ATP standard curve) were prepared to convert the emission ratio signal to percentage of

hydrolysis. The ADP/ATP data were fitted in Kaleidagraph (Synergy Software, Reading, USA) using a three-parameter exponential decay model.

FRET assays for membrane binding

PI3P-containing liposomes were prepared with 10% w/v Dansyl-PS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine-N-(5-dimethylamino-1-naphthalenesulfonyl) (ammonium salt)) (Avanti Polar Lipids, 810225C), 10% w/v PI3P 1,2-dipalmitoyl (Cayman Chemical, 64921) and 80% w/v bovine brain extract type I Folch fraction 1 (Sigma-Aldrich, B1502). PI-containing vesicles were composed of 80% w/v porcine brain PS (Avanti Polar Lipids, 840032C), 10% w/v bovine liver PI (Avanti Polar Lipids, 840042C) and 10% w/v Dansyl-PS. Phospholipids in 100% chloroform were dried with nitrogen gas, then desiccated under vacuum for 15 min. Lipids were resupsended in 10 mM Tris pH 7.5 (room temperature), 50 mM NaCl, 2 mM EGTA, at a concentration of 1 mg/ml, freeze-thawed five times and then extruded through a 0.1 μm filter. The lipids were then diluted in FRET reaction buffer (20 mM Tris pH 7.5 (room temperature), 100 mM NaCl, 1 mM 2-mercaptoethanol, 0.5 mM $MgCl_2$) to a concentration of 25 μg/ml. A 7.5 μl aliquot of a protein, diluted in FRET reaction buffer to 16 μM, was mixed with 7.5 μl of liposomes (25 μg/ml)(0.19 μg) in a total volume of 15 μl (final protein concentration 8 μM, final lipid concentration 12.5 μg/ml). Reactions were carried out for 30 minutes at room temperature before reading with a PHERAStar HTS microplate reader (BMG Labtech, Aylesbury, UK) using a 280 nm excitation filter with 350 nm and 520 nm emission filters to measure Trp and Dansyl-PS FRET emissions, respectively. The FRET signal shown on the Y-axis is $(I-I_0)/I_0$, where I is the intensity at 520 nm, and was corrected by subtracting the fluorescence intensity for a

solution with protein only but without lipids. I_0 value is the fluorescence intensity of a solution containing lipids without protein at 520 nm, corrected by subtracting a solution containing only the reaction buffer.

Sedimentation assays for membrane binding

For sedimentation assays, liposomes composed of 80% porcine brain PS (Avanti Polar Lipids), 10% bovine liver PI (Avanti Polar Lipids) and 10% Dansyl-PS were made, as described above. Sedimentation assays contained 0.5 mg/ml liposomes and 8 uM proteins in 20 mM Tris 7.5, 100 mM NaCl, 0.5 mM $MgCl_2$ and 1 mM β-mercaptoethanol in a total volume of 100 µl. After 30 min incubation at room temperature, reactions were sedimented at 128,000 x g at 4°C for 30 min. The pellets and supernatants were resuspended in equal volume of buffer and analysed by SDS-PAGE. The intensities of bands in the SDS-PAGE gels were quantified using GeneTools 3.08 (SynGene, Cambridge, UK) programme.

Yeast plasmids and Vps34 expression in vivo

The *S. cerevisiae vps34Δ* deletion strain in the genetic background of the BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) strain was obtained from the EUROSCARF consortium. The wild-type *VPS34* ORF was PCR-amplified from *S. cerevisiae* genomic DNA (ATCC 9763D) using primers with engineered 5' BamHI site and 3' NheI site for cloning between the BamHI and NheI sites of the expression vector pRB415A-FLAG. The pRB series of CEN plasmids has been described previously (9, 10). Briefly, the pRB415A-FLAG plasmid variant (a kind gift from Scott Emr, Cornell University, Ithaca) encodes two tandem copies

of a FLAG tag (cloned between the XbaI and BamHI sites) and expresses the proteins under the control of an *ADH1* promoter. Cloning was done using In-Fusion protocol (Clontech).

The *vps34*Δ null yeast was transformed with plasmids encoding wild-type *S. cerevisiae* Vps34p (ScVps34p-WT, plasmid pSM126), C-terminally truncated ScVps34p-ΔC10 (plasmid pSM127), point-mutant H867A in the C-terminal helix of Vps34p (ScVps34p-H867A, plasmid pSM128), catalytic loop kinase-dead ScVps34p-D731N mutant (plasmid pSM134) or empty vector (pRB415AF). Cells were grown overnight in SC media without leucine, diluted to OD_{600}=0.5 and 2.5 μl aliquots of 10-fold serial dilutions were spotted onto selective plates and grown at 30 °C, 37 °C or 39 °C for three days.

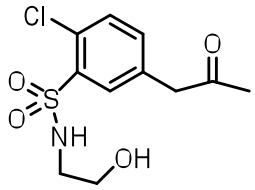
PI3K inhibitor assays

Assays of p110α, p110β and p110γ were performed with L-α-phosphatidylinositol (Avanti) as described previously (11). Inhibitors were prepared in 10% DMSO as 5x stocks and assayed in duplicate. Assays of HsVps34 were performed as described previously (11), except that the final assay composition used was changed to 20 mM HEPES 7.5, 3 mM $MgCl_2$, 1 mg/ml PI and 44 nM HsVps34. For inhibitors with an apparent IC50 less than or equal to 22 nM, values were re-assayed using 4.4 nM HsVps34 with 3 mM $MnCl_2$.

Synthesis of PIK-93 analogs

Synthesis of PIK-93 was reported previously (11). For completeness, this is included here as products 1-3.

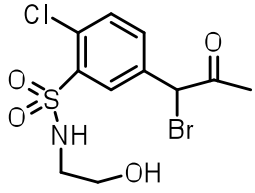
(1) 2-chloro-N-(2-hydroxyethyl)-5-(2-oxopropyl)benzenesulfonamide



1-(4-chlorophenyl)propan-2-one (1 ml, 14.8 mmol) was added dropwise to HSO_3Cl (5 ml) in an ice bath, and the reaction was then heated to 40º C for 2 hours. The reaction was stopped by transferring dropwise to 200 ml ice. The aqueous phase was then extracted 3 times with EtOAc, the combined organic phases were dried with Na_2SO_4, filtered, and then concentrated *in vacuo* to give a brown oil. This oil was dissolved in THF (10 ml), ethanolamine was added (~1.5 ml), and the reaction was allowed to stir overnight at room temperature. The following day the reaction was concentrated *in vacuo*, water was added, and the aqueous phase was extracted three times with EtOAc. The combined organic phase was concentrated, and the product purified by silica gel flash chromatography using a gradient of 2% - 10% MeOH in CH_2Cl_2. The product containing fractions from this column were combined, solvent removed *in vacuo,* and the product was subjected to a second purification by reverse phase HPLC using a MeCN/H_2O/0.1% TFA solvent system. Product containing fractions were lyophilized to yield 414 mg (24% yield) of product.

LC-ESI-MS: [MH]+ m/z calculated for C11H15ClNO4S, 292.03; found, 292.17.

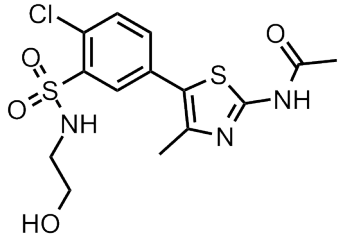
(2) 5-(1-bromo-2-oxopropyl)-2-chloro-N-(2 hydroxyethyl)benzenesulfonamide



$Ph_3PCH_2CH_2COOH\ Br_3$ (900 mg, 1.5 mmol) was dissolved in THF (15 ml) (Armstrong et al., 1975). Product 7 (414 mg, 1.42 mmol) was dissolved in THF (10 ml) and was added dropwise to the first solution at room temperature. The reaction was allowed to proceed one hour at room temperature, at which point the solvent was removed *in vacuo* and the product purified by silica gel flash chromatography to yield 377 mg (72% yield) of product.

LC-ESI-MS: [MH]+ m/z calculated for C11H14BrClNO4S, 369.94; found, 369.83.

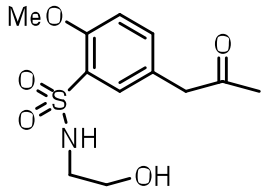
(3) PIK-93 (N-(5-(4-chloro-3-(N-(2-hydroxyethyl)sulfamoyl)phenyl)-4-methylthiazol-2-yl)acetamide)



Product 8 (377 mg, 1.02 mmol) was dissolved in ethanol (6 ml), and N-acetylthiourea (130 mg, 1.1 mmol) was added at room temperature. The reaction was heated to reflux for 30 minutes, then cooled to room temperature, and the product purified by silica gel flash chromatography using a gradient of 0% - 10% MeOH in CH_2Cl_2 to yield 167 mg (42% yield) of PIK-93.

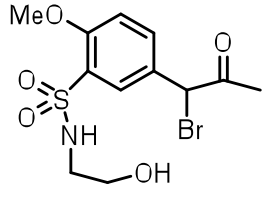
LC-ESI-MS: [MH]+ m/z calculated for C14H17ClN3O4S2, 390.03; found, 390.14.

(4) N-(2-hydroxyethyl)-2-methoxy-5-(2-oxopropyl)benzenesulfonamide



1-(4-methoxyphenyl)propan-2-one (2 ml, 13 mmol) was added dropwise to 10 ml HSO_3Cl cooled on a dry ice ethanol/ethylene glycol (1:9) bath over 5 minutes. The reaction was allowed to stir for 30 minutes, warmed to room temperature and allowed to stir for 2 hours. The reaction was then cooled on ice and quenched by dropwise addition to 200 ml ice/water with stirring. Once the ice had melted, the reaction was extracted 3x with ethyl acetate. The combined organic layers were dried with brine and sodium sulfate and reduced *in vacuo*. The residue was dissolved in 10 ml dry THF and added dropwise to a solution of 1.5 ml ethanolamine in 10 ml THF. The reaction was allowed to stir overnight and then reduced *in vacuo*. 50 ml water was added, and the aqueous layer was extracted 3x with ethyl acetate. The combined ethyl acetate was dried with brine and sodium sulfate, reduced *in vacuo*, and purified by flash chromatography in 0-10% methanol in dichloromethane. Yield 0.8 g (21%). LC-ESIMS [MH]+ m/z calculated for C12H17NO5S, 288.08; found 287.94.

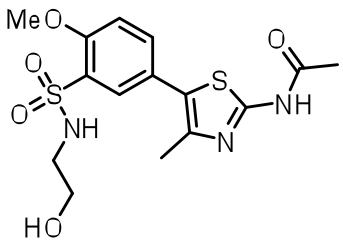
(5) 5-(1-bromo-2-oxopropyl)-N-(2-hydroxyethyl)-2-methoxybenzenesulfonamide



Synthesis was performed as in (2), substituting (4) for (1). Compound was purified by silica flash chromatography in ethyl acetate. Yield 0.82 g (80%).

LC-ESI-MS: [MH]+ m/z calculated for C12H16BrNO5S, 365.99; found 365.79.

(7) PT-21 (N-(5-(3-(N-(2-hydroxyethyl)sulfamoyl)-4-methoxyphenyl)-4-methylthiazol-2-yl)acetamide)

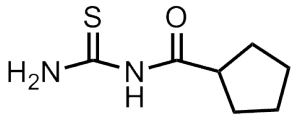


Product (5) (100 mg, 0.28 mmol) was dissolved in ethanol (2 ml), and N-acetylthiourea (35 mg, 0.3 mmol) was added. The reaction was heated to reflux for 1 hour, reduced *in vacuo*, and the residue was purified by flash chromatography in a gradient of 0-10% methanol in dichloromethane. Yield 57 mg (53%).

1H NMR (400 MHz, DMSO) δ 7.72 (d, J = 2.3, 1H), 7.67 (dd, J = 8.5, 2.2, 1H), 7.30 (d, J = 8.7, 1H), 7.18 (s, 1H), 4.64 (s, 1H), 3.93 (s, 3H), 3.35 (dd, J = 12.3, 6.3, 2H), 2.85 (dd, J = 12.5, 6.3, 2H), 2.49 (m, DMSO), 2.31 (s, 3H), 2.13 (s, 3H).

LC-ESI-MS: [MH]+ m/z calculated for C15H19N3O5S2, 386.08; found 385.94.

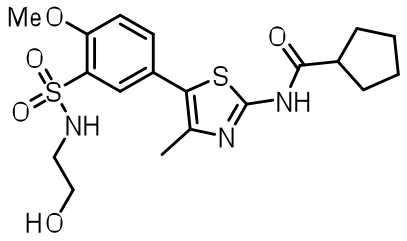
(8) N-carbamothioylcyclopentanecarboxamide



Cyclopentanecarboxylic acid (1.48 g, 13 mmol) was treated with oxalyl chloride (1.65 g, 32.5 mmol) by stirring in a dry, argon-purged flask at room temperature for 30 minutes and then refluxing at 72 degrees for 1 hour. Dry toluene (26 ml) and ethanol-recrystallized thiourea (5 g, 65.7 mmol) was added. The reaction was heated to reflux and allowed to stir for 18 hours. Toluene was then removed *in vacuo* and the reaction was diluted in 200 ml ethyl acetate and filtered. The organic layer was washed 2x with water and dried with brine and sodium sulfate before reducing *in vacuo*. The compound was adsorbed onto silica and purified by silica flash chromatography using 25% ethyl acetate in hexanes. Yield 1.28 g (57%).

LC-ESI-MS: [MH]+ m/z calculated for C7H12N2OS, 173.07; found 172.92.

(9) PT210 (N-(5-(3-(N-(2-hydroxyethyl)sulfamoyl)-4-methoxyphenyl)-4-methylthiazol-2-yl)cyclopentanecarboxamide)

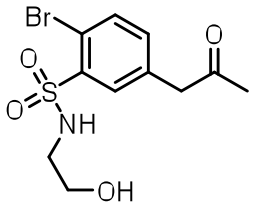


Product (5) (100 mg, 0.274 mmol) was dissolved in ethanol (2 ml), and (8) (47 mg, 0.300 mmol) was added. The reaction was heated to reflux for 1 hour, reduced *in vacuo*, and the residue was purified by flash chromatography in a gradient of 0-10% methanol in dichloromethane. Yield 90 mg (75%).

1H NMR (400 MHz, DMSO) δ 7.72 (s, 1H), 7.65 (m, 1H), 7.30 (d, J = 8.6, 1H), 7.18 (m, 1H), 3.89 (m, 3H), 3.7 (broad, H2O), 3.36 (m, 2H), 2.85 (m, 2H), 2.49 (m, DMSO), 2.31 (s, 1H), 1.86 (m, 2H), 1.67 (m, 4H), 1.5-2.9 (m, broad, 6H).

LC-ESI-MS: [MH]+ m/z calculated for C19H25N3O5S2, 440.12; found 440.4.

(10) 2-bromo-N-(2-hydroxyethyl)-5-(2-oxopropyl)benzenesulfonamide

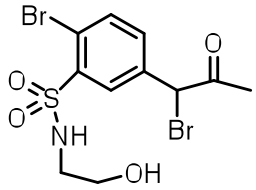


1-(4-bromophenyl)propan-2-one (2 ml, 13 mmol) was added dropwise to 10 ml HSO_3Cl cooled on a dry ice ethanol/ethylene glycol (1:9) bath over 5 minutes. The reaction was allowed to stir for 30 minutes, warmed to room temperature, and heated at 40 degrees for 2 hours. The reaction was cooled on ice and quenched by dropwise addition to 200 ml ice/water with stirring. Once the ice had melted, the reaction was extracted 3x with ethyl acetate. The combined organic layers were dried with brine and sodium sulfate and reduced *in vacuo*. The residue was dissolved in 10 ml dry THF and added dropwise to a solution of 1.5 ml ethanolamine in 10 ml THF. The reaction was allowed to stir overnight and then reduced *in vacuo*. 50 ml water was added, and the aqueous layer was extracted 3x with ethyl acetate. The combined ethyl acetate was dried with brine and sodium sulfate, reduced *in vacuo*, and purified by flash chromatography in 0-10% methanol in dichloromethane. The product containing fractions from this column were combined,

solvent removed *in vacuo*, and the product was subjected to a second purification by reverse phase HPLC using a MeCN/H2O/0.1% TFA solvent system. Yield 0.248 g (5.6%).

LC-ESI-MS: [MH]+ m/z calculated for C11H14BrNO4S, 335.98; found 335.8

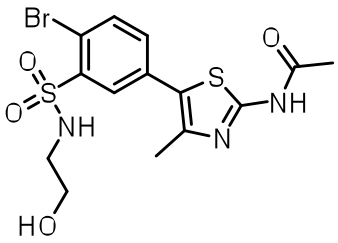
(11) 2-bromo-5-(1-bromo-2-oxopropyl)-N-(2-hydroxyethyl)benzenesulfonamide



Product (10) (0.248 g, 0.737 mmol) in 10 ml THF was added dropwise to Ph3PCH2CH2COOH Br3 (0.442 g, 0.737 mmol) in 10 ml THF. The reaction was allowed to stir for 1 hour at room temperature. Ethyl acetate (~5 ml) was added, and the reaction was filtered and reduced *in vacuo*. The product was used without further purification. Yield 0.25 g (82%).

(12) 3-92A (N-(5-(4-bromo-3-(N-(2-hydroxyethyl)sulfamoyl)phenyl)

-4-methylthiazol-2-yl)acetamide)

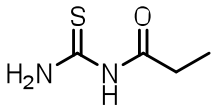


Product (11) (50 mg, 0.120 mmol) was dissolved in 5 ml dry ethanol and N-acetylthiourea (15.6 mg, 0.132 mmol) was added. The reaction was refluxed for 1 hr and the ethanol was removed *in vacuo*. The residue was resuspended in 1 ml DMSO and purified by reverse phase HPLC using a MeCN/H2O/0.1% TFA solvent system. Yield 15 mg (29%).

1H NMR (400 MHz, DMSO) 7.97 (d, J = 2.3, 1H), 7.88 (d, J = 8.3, 2H), 7.59 (d, J = 8.3, 1H), 3.95 (broad), 3.39 (t, J = 6.4, 2H), 2.95 (d, J = 6.0, 2H), 2.49 (m, DMSO), 2.36 (s, 3H), 2.15 (s, 3H).

LC-ESI-MS: [MH]+ m/z calculated for C14H16BrN3O4S2, 433.98; found 433.79.

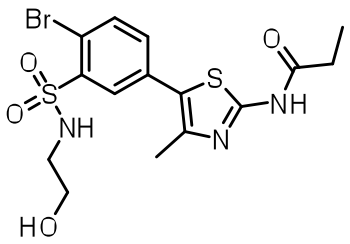
(13) N-carbamothioylpropionamide



To ethanol-recrystallized thiourea (1.0 g, 13.1 mmol) in 13 ml dry toluene was added 1.14 ml (13.1 mmol) propionyl chloride. The reaction was heated to reflux for 16 hours, and then toluene was removed *in vacuo*. The product was recrystallized from ethanol to yield 0.8 g product (46%).

LC-ESI-MS: [MH]+ m/z calculated for C4H8N2OS, 133.04; found 132.89.

(14) 3-92B (N-(5-(4-bromo-3-(N-(2-hydroxyethyl)sulfamoyl)phenyl)-4-methylthiazol-2-yl)propionamide)

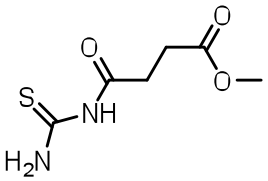


Product (11) (50 mg, 0.120 mmol) was dissolved in 5 ml dry ethanol and product (13) (17.5 mg, 0.132 mmol) was added. The reaction was refluxed for 1 hr and the ethanol was removed *in vacuo*. The residue was resuspended in 1 ml DMSO and purified by reverse phase HPLC using a MeCN/H2O/0.1% TFA solvent system. Yield 10 mg (19%).

1H NMR (400 MHz, DMSO) δ 7.97 (s, 1H), 7.88 (d, J = 8.3, 1H), 7.59 (d, J = 8.3, 1H), 4.19 (broad), 3.39 (t, J = 6.4, 2H), 2.95 (m, 2H), 2.49 (m, DMSO), 2.44 (d, J = 7.5, 2H), 2.36 (s, 3H), 1.09 (m, 3H).

LC-ESI-MS: [MH]+ m/z calculated for C15H18BrN3O4S2, 447.99; found 447.81.

(15) Methyl 4-oxo-4-thioureidobutanoate

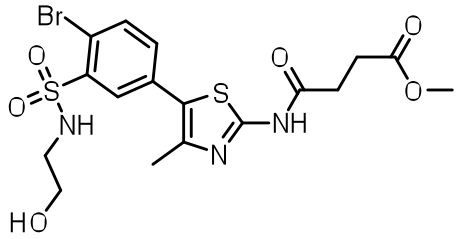


Methyl 4-chloro-4-oxobutanoate (2.93 ml, 23.9 mmol) was added dropwise to thiourea (2.0 g, 26.3 mmol) in 30 ml dry toluene. The reaction was heated to reflux for 18 hours, then toluene was removed *in vacuo*. The residue was dissolved in 200 ml ethyl acetate, washed 3x with brine, dried with sodium sulfate, and reduced *in vacuo*. The dry product was

adsorbed onto silica and purified by flash chromatography in 50% ethyl acetate in hexanes. Yield 1.0 g (22%).

LC-ESI-MS: [MH]+ m/z calculated for C6H10N2O3S, 191.04; found 191.3.

(16) 3-92D (Methyl 4-(5-(4-bromo-3-(N-(2-hydroxyethyl)sulfamoyl)phenyl)-4-methylthiazol-2-ylamino)-4-oxobutanoate)

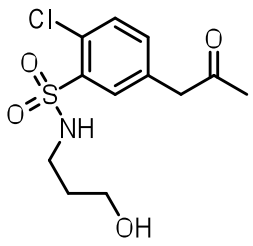


Product (11) (50 mg, 0.120 mmol) was dissolved in 5 ml dry ethanol and product (15) (25.2 mg, 0.132 mmol) was added. The reaction was refluxed for 1 hr and the ethanol was removed *in vacuo*. The residue was resuspended in 1 ml DMSO and purified by reverse phase HPLC using a MeCN/H2O/0.1% TFA solvent system. Yield 11 mg (18%).

1H NMR (400 MHz, DMSO) δ 7.97 (s, 1H), 7.88 (m, 1H), 7.59 (m, 1H), 3.7 (broad), 3.59 (d, J = 2.6, 3H), 3.39 (m, 2H), 2.95 (m, 2H), 2.72 (m, 2H), 2.64 (m,2H), 2.49 (m, DMSO), 2.37 (s, 3H).

LC-ESI-MS: [MH]+ m/z calculated for C17H20BrN3O6S2, 505.99; found 505.77.

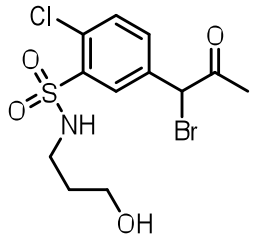
(17) 2-chloro-N-(3-hydroxypropyl)-5-(2-oxopropyl)benzenesulfonamide



1-(4-chlorophenyl)propan-2-one (1 ml) was added dropwise to 5 ml HSO_3Cl cooled on a dry ice ethanol/ethylene glycol (1:9) bath over 5 minutes. The reaction was allowed to stir for 30 minutes, warmed to room temperature and heated to 40 degrees Celsius for 2 hours. The reaction was then cooled on ice and quenched by dropwise addition to 200 ml ice/water with stirring. Once the ice had melted, the reaction was extracted 3x with ethyl acetate. The combined organic layers were dried with brine and sodium sulfate and reduced *in vacuo*. The residue was dissolved in 10 ml dry THF and added dropwise to a solution of 2.0 ml 3-aminopropan-1-ol in 10 ml THF. The reaction was allowed to stir overnight and then reduced *in vacuo*. 50 ml water was added, and the aqueous layer was extracted 3x with ethyl acetate. The combined ethyl acetate was dried with brine and sodium sulfate, reduced *in vacuo*, and purified by flash chromatography in 0-10% methanol in dichloromethane. The product containing fractions from this column were combined, solvent removed *in vacuo*, and the product was subjected to a second purification by reverse phase HPLC using a MeCN/H2O/0.1% TFA solvent system. Yield 83 mg.

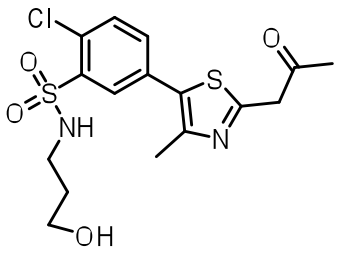
LC-ESI-MS: [MH]+ m/z calculated for C12H16ClNO4S, 306.05; found 306.3.

(18) 5-(1-bromo-2-oxopropyl)-2-chloro-N-(3-hydroxypropyl)benzenesulfonamide



Product (17) (83 mg, 0.236 mmol) in 10 ml THF was added dropwise to Ph3PCH2CH2COOH Br3 (0.142 g, 0.236 mmol) in 10 ml THF. The reaction was allowed to stir for 1 hour at room temperature. Ethyl acetate (~5 ml) was added, and the reaction was filtered and reduced *in vacuo*. The product was used without further purification. Yield 92 mg (100%).

(19) 3-94B (N-(5-(4-chloro-3-(N-(3-hydroxypropyl)sulfamoyl)phenyl)-4-methylthiazol-2-yl)acetamide)

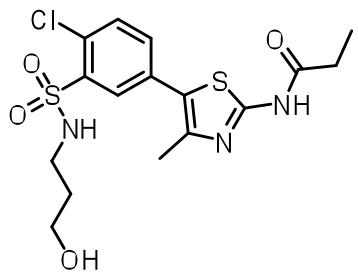


Product (18) (46 mg, 0.120 mmol) was dissolved in 5 ml dry ethanol and N-acetylthiourea (15.6 mg, 0.132 mmol) was added. The reaction was refluxed for 1 hr and the ethanol was removed *in vacuo*. The residue was purified by reverse phase HPLC using a MeCN/H2O/0.1% TFA solvent system. Yield 8 mg (16%).

1H NMR (400 MHz, DMSO) δ 7.93 (m, 1H), 7.90 (m, 1H), 7.70 (s, 1H), 4.25 (broad), 3.35 (m, 2H), 2.93 (m, 2H), 2.49 (m, DMSO), 2.36 (d, J = 1.9, 3H), 2.15 (d, J = 1.9, 3H), 1.54 (m, 2H).

LC-ESI-MS: [MH]+ m/z calculated for C15H18ClN3O4S2, 404.04; found 403.88.

(20) 3-94C (N-(5-(4-chloro-3-(N-(3-hydroxypropyl)sulfamoyl)phenyl)-4-methylthiazol-2-yl)propionamide)



Product (18) (46 mg, 0.120 mmol) was dissolved in 5 ml dry ethanol and product (13) (17.4 mg, 0.132 mmol) was added. The reaction was refluxed for 1 hr and the ethanol was removed *in vacuo*. The residue was purified by reverse phase HPLC using a MeCN/H2O/0.1% TFA solvent system. Yield 10 mg (20%).

1H NMR (400 MHz, DMSO) δ 7.94 (m, 1H), 7.90 (m, 1H), 7.71 (s, 1H), 4.08 (broad), 3.35 (m, 2H), 2.93 (m, 2H), 2.49 (m, DMSO), 2.43 (m, 2H), 2.36 (s, 3H), 1.54 (m, 2H), 1.09 (t, J = 7.5, 3H).

LC-ESI-MS: [MH]+ m/z calculated for C16H20ClN3O4S2, 418.06; found 417.90.

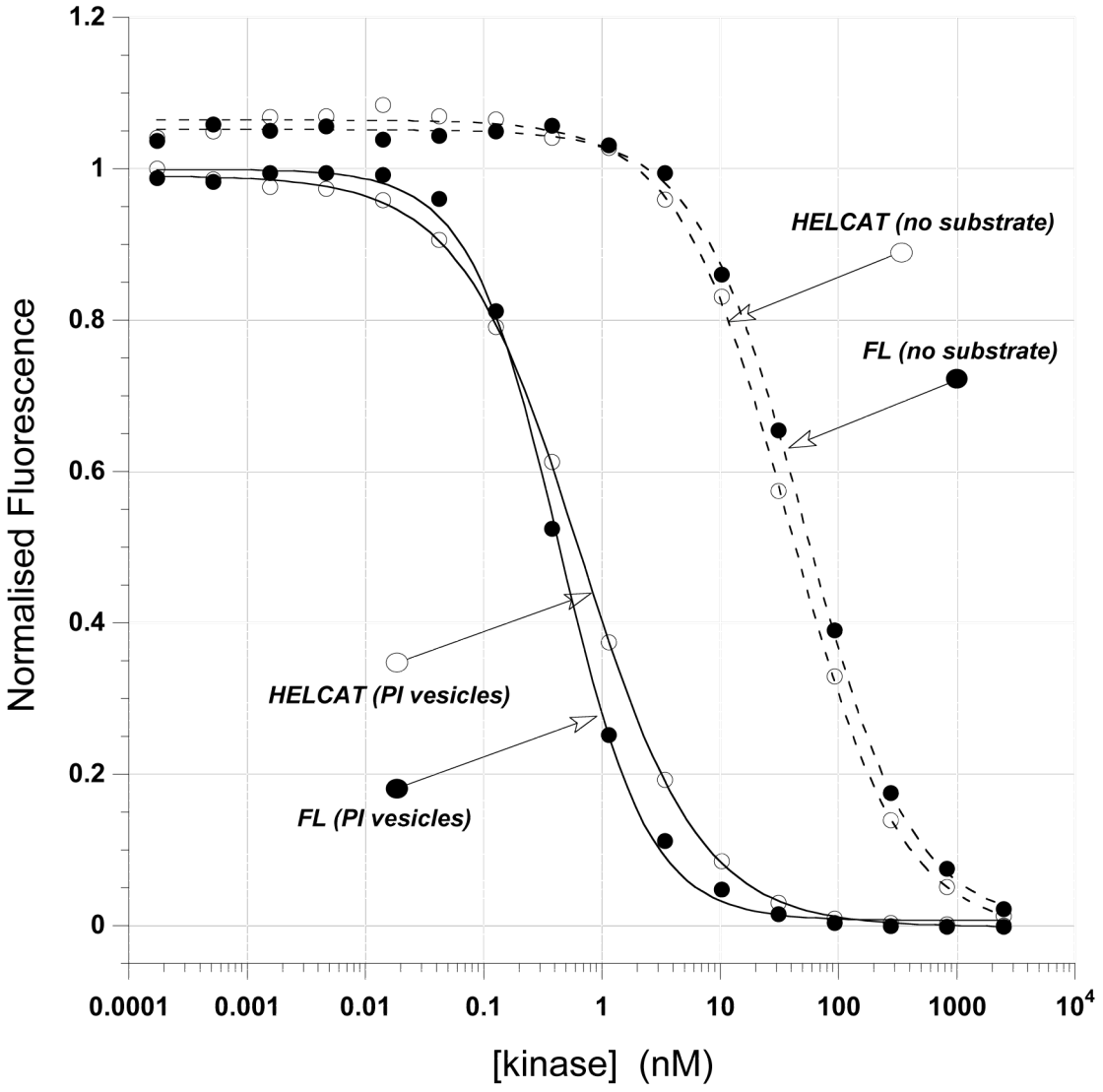


Fig. S1. Enzymatic activity of the human Vps34 HELCAT construct (open circles) and the full-length, wild-type human Vps34 (closed circles). The fluorescence emission 655 nm/620 nm ratio reflects the assay window with the maximum corresponding to no conversion of ATP to ADP and the minimum corresponding to 100% conversion. This ratio was normalised relative to the ratio for HELCAT with PI vesicles and is plotted on the Y-

axis. Activity was determined in the presence of 10 μM ATP either with PtdIns:PS substrate (PV5122, Invitrogen) or in the absence of vesicles.

Fig. S2. Sequence alignment of HsVps34 and DmVps34 helical and kinase domains with three class I PI3K isotypes. The alignments were adjusted to reflect the structural superpositions. The blue squares denote all inhibitor-contacting residues as determined by the programme CCP4i Contacts (interatomic distance ≤4.0Å). The sequence is shaded by

domain color as in Fig. 1A. Completely conserved regions among the species are colored bright red and enclosed in boxes predominately conserved regions are colored yellow and enclosed in boxes.

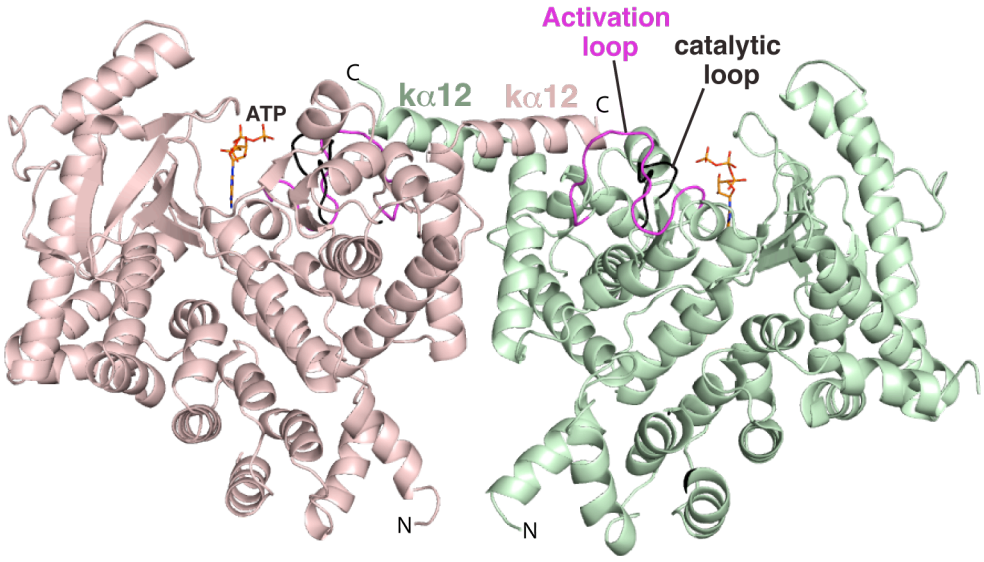


Fig. S3. The Vps34 dimer present in the asymmetric unit of the crystals. The two-fold axis of the dimer is in the plane of the page and runs perpendicular to the putative membrane interface. The C-terminal helix from one molecule fits onto the surface of its dyad-related partner. Two ATPs are shown modelled in stick representation based on the structure of p110γ to act as reference points for visualising the Vps34 active sites.

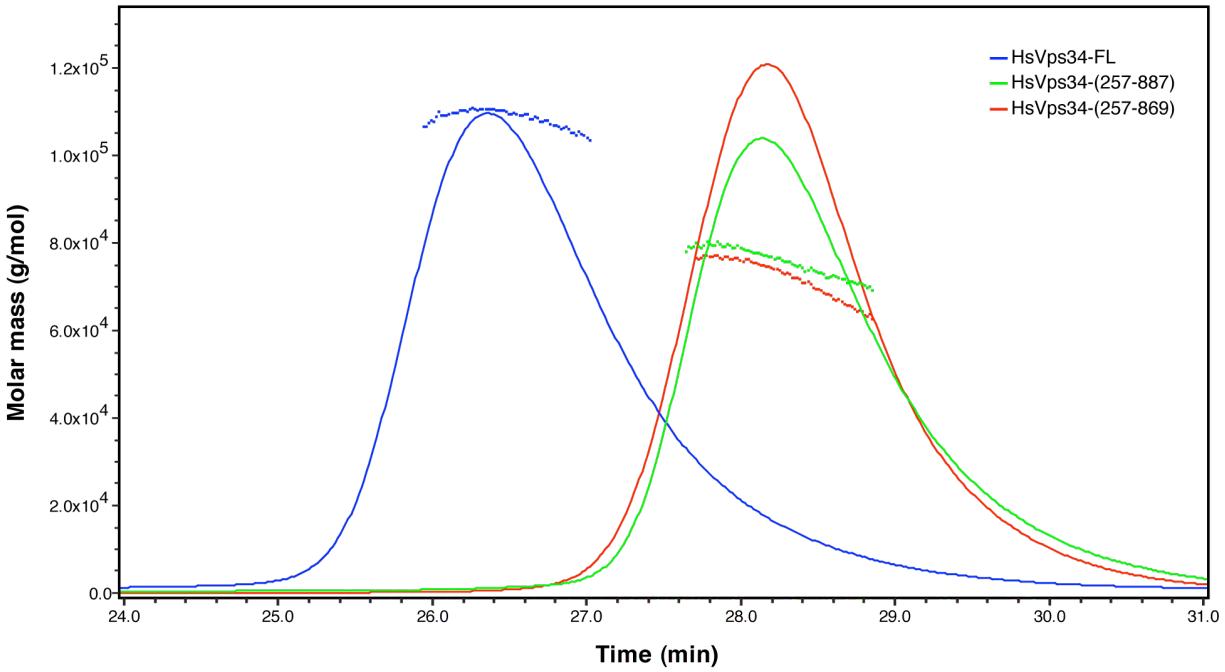
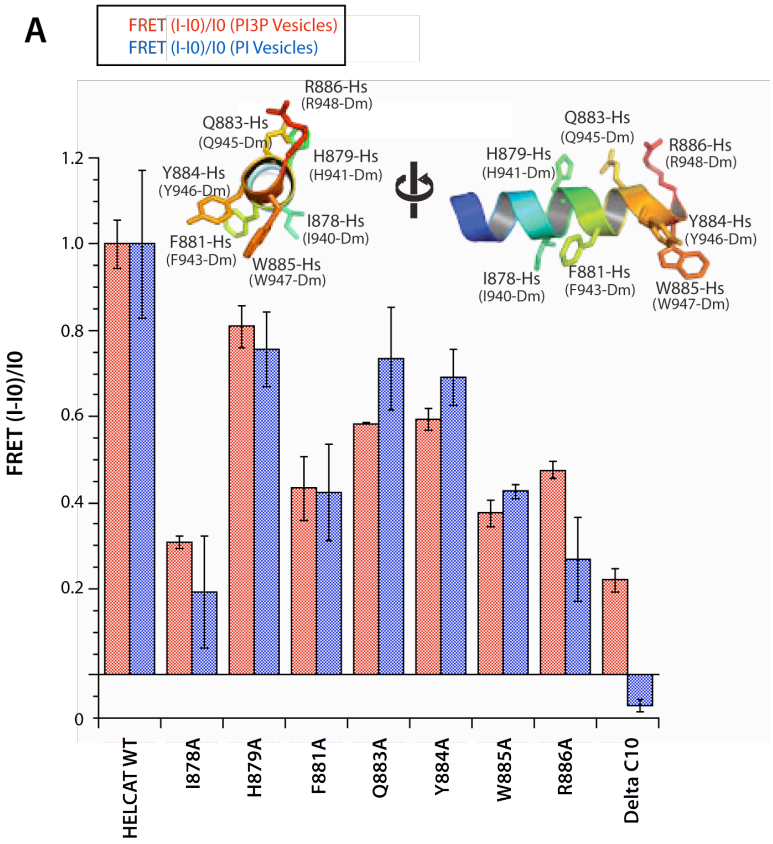


Fig. S4. Multi-angle light scattering of HsVps34 full-length (blue), HELCAT HsVps34 (green) and a C-terminal truncation variant of the HsVps34 HELCAT (red). The gel-filtration elution profiles (OD280) are represented by solid lines. The weight-averaged molecular weights determined across the peaks using the Astra V software are shown as points. The expected masses for the full-length, HELCAT and HELCAT C-terminal truncation enzymes were 102 kDa, 73 kDa and 70 kDa, respectively. The molecular masses calculated from scattering were 110 kDa, 75 kDa and 71 kDa, respectively.


A
FRET (I-I0)/I0 (PI3P Vesicles)
FRET (I-I0)/I0 (PI Vesicles)
R886-Hs
(R948-Dm)
Q883-Hs
(Q945-Dm)
H879-Hs
(H941-Dm)
Y884-Hs
(Y946-Dm)
I878-Hs
(I940-Dm)
F881-Hs
(F943-Dm)
W885-Hs
(W947-Dm)
FRET (I-I0)/I0
1.2
1.0
0.8
0.6
0.4
0.2
0
HELCAT WT
I878A
H879A
F881A
Q883A
Y884A
W885A
R886A
Delta C10

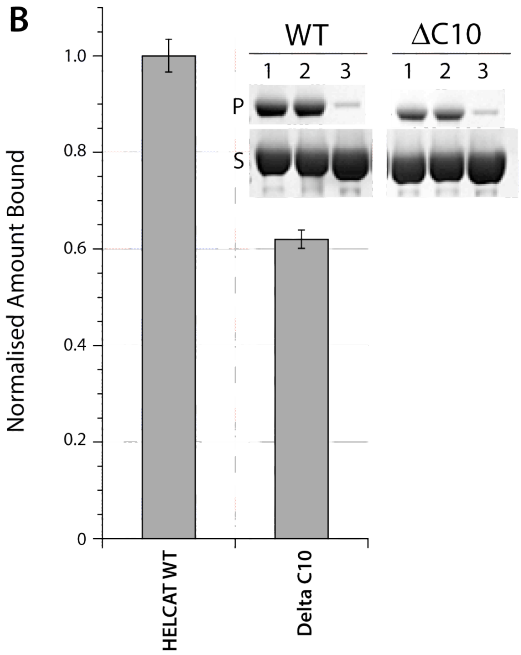

B
Normalised Amount Bound
1.0
0.8
0.6
0.4
0.2
0
WT
ΔC10
1 2 3
1 2 3
P
S
HELCAT WT
Delta C10

Fig. S5. The C-terminus can regulate membrane binding. (**A**) A plot of FRET intensity $(I-I_0)/I_0$ for human Vps34 HELCAT WT and C-terminal mutants. Point mutations and the ΔC10 truncation reduced binding to PtdIns- and PtdIns3P-containing vesicles. The C-terminal helix (kα12) is shown as a rainbow-coloured cartoon with the three most important residues (I878, F881 and W885 HsVps34) for membrane interaction orientated downwards. Error bars represent standard deviation for duplicate or triplicate assays with PtdIns- and PtdIns3P-containing vesicles, respectively. (**B**) Liposome sedimentation assays show the C-terminal helix is important for membrane binding. The bands labelled "P" and "S" on the inset coomassie-stained SDS-PAGE gel represent the pellet and the supernatant, respectively. The histogram on the left is a quantification of the pellet bands normalised relative to the wildtype HELCAT. Lanes 1 and 2 are two replicates of protein incubated with PI-containing vesicles. Lane 3 is a sample of the protein with no vesicles. Intensities of the bands were quantified using GeneTools 3.08 (SynGene, Cambridge, UK) programme. The intensity of the lane 3 pellet was subtracted from the intensities of lane 1 and 2. Error bars show the standard deviation of the mean of the two assays.

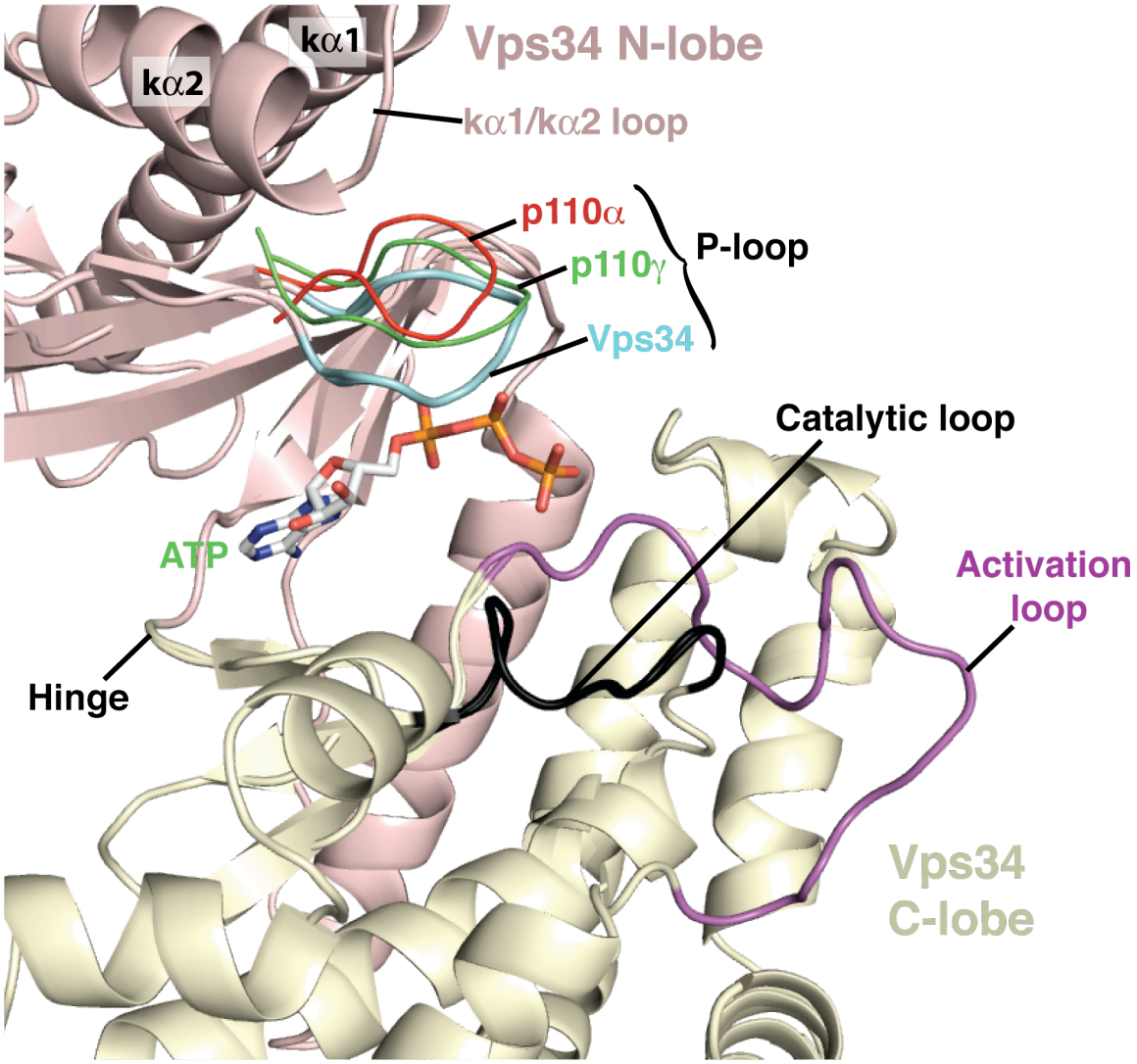


Fig. S6. A comparison of some key features of the ATP-binding pockets of Vps34 with p110γ (PDB ID 1E8X) and p110α (PDB ID 2RD0). The N-lobe of the DmVps34 catalytic domain (residues 592-747) was superimposed onto the N-lobes of p110γ and p110α using MUSTANG (12) **(A)** The P-loop of Vps34 (cyan) curls inward toward the ATP-binding pocket when compared with the other isotypes so that the ATP-binding pocket of Vps34 is more closed than the pocket of p110γ (green) or p110α (red).

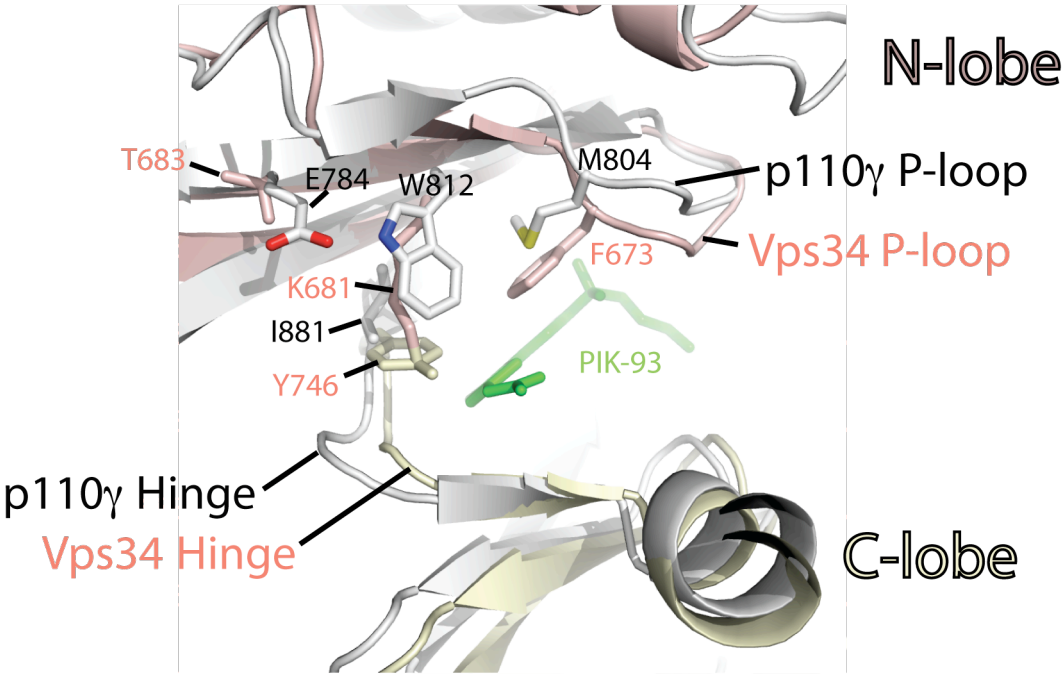
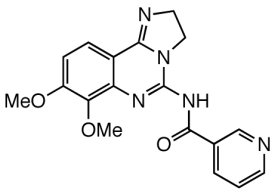
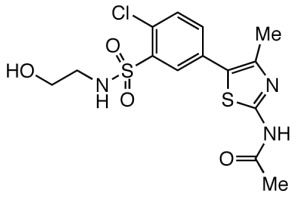


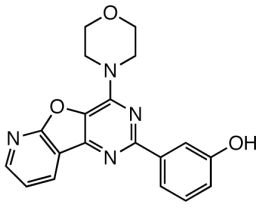
Fig. S7. A comparison of the structural elements controlling the allosteric pocket of the class I enzymes with Vps34. The presence of the rotationally constrained Phe673/Y746-Dm pair in Vps34 results in a less flexible pocket, in which one corner of the adenine-binding pocket is closed off. The superposition of the p110γ catalytic domain onto the Vps34 catalytic domain (residues 592-949) was performed using the BRUTE option of LSQMAN.



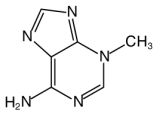
PIK-90	IC50 (nM)
HsVps34	830 *
p110α	11
p110β	350
p110δ	58
p110γ	18



PIK-93	IC50 (nM)
HsVps34	36
p110α	39
p110β	590
p110δ	120
p110γ	4

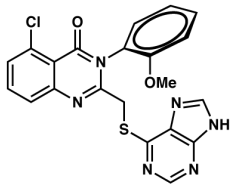


PI-103	IC50 (nM)
HsVps34	2,300 *
p110α	8
p110β	88
p110δ	48
p110γ	150



3-MA	IC50 (nM)
HsVps34	25,713
p110α	145,600
p110β	225,900
p110δ	-
p110γ	60,671

Propeller-shaped compound



PIK-39	IC50 (nM)
HsVps34	>100,000 *
p110α	>200,000
p110β	11,000
p110δ	180
p110γ	17,000

Fig. S8. Structures of the inhibitors PIK-90, PIK-93, PI-103, 3-MA and the propeller-shaped PIK-39. The IC50 values for PIK-93 and 3-MA were obtained from assays containing $MgCl_2$. *The IC50 values for Vps34 with PIK-90, PI-103 and PIK-39 were derived from enzymatic assays containing the co-factor $MnCl_2$.

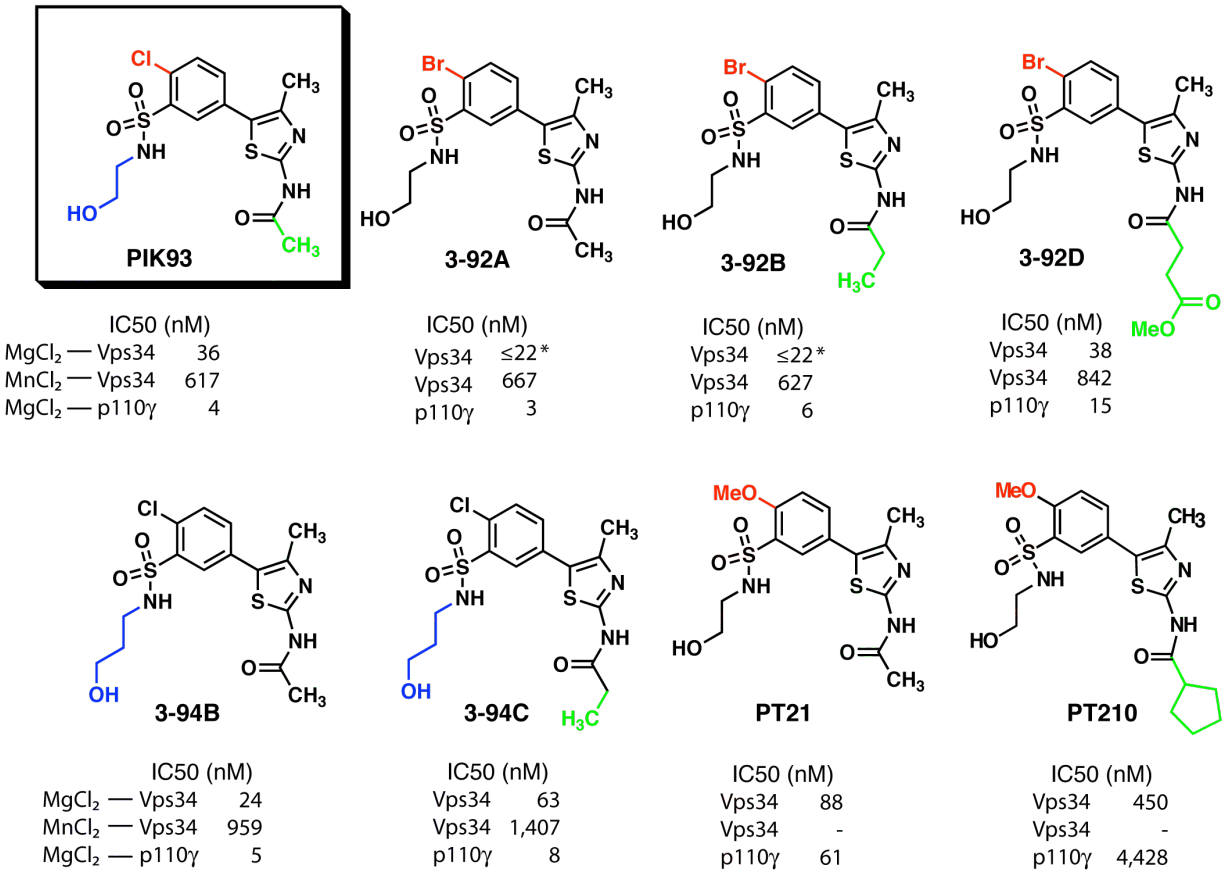


Fig. S9. Structures of the PIK-93 analogues 3-92A, 3-92B, 3-92D, 3-94B, 3-94C, PT21 and PT210. IC50 values are shown for lipid kinase assays containing $MgCl_2$ or $MnCl_2$ (where applicable). *The use of Mg^{2+} precluded accurate measurement of IC50 values below 22 nM. Thus, the IC50 values for high affinity compounds were also measured with Mn^{2+}. IC50 values measured with Mn^{2+} are approximately 10-20-fold higher than those measured with the physiologically more abundant divalent metal Mg^{2+}, because the enzyme is more active with the Mn^{2+} co-factor.

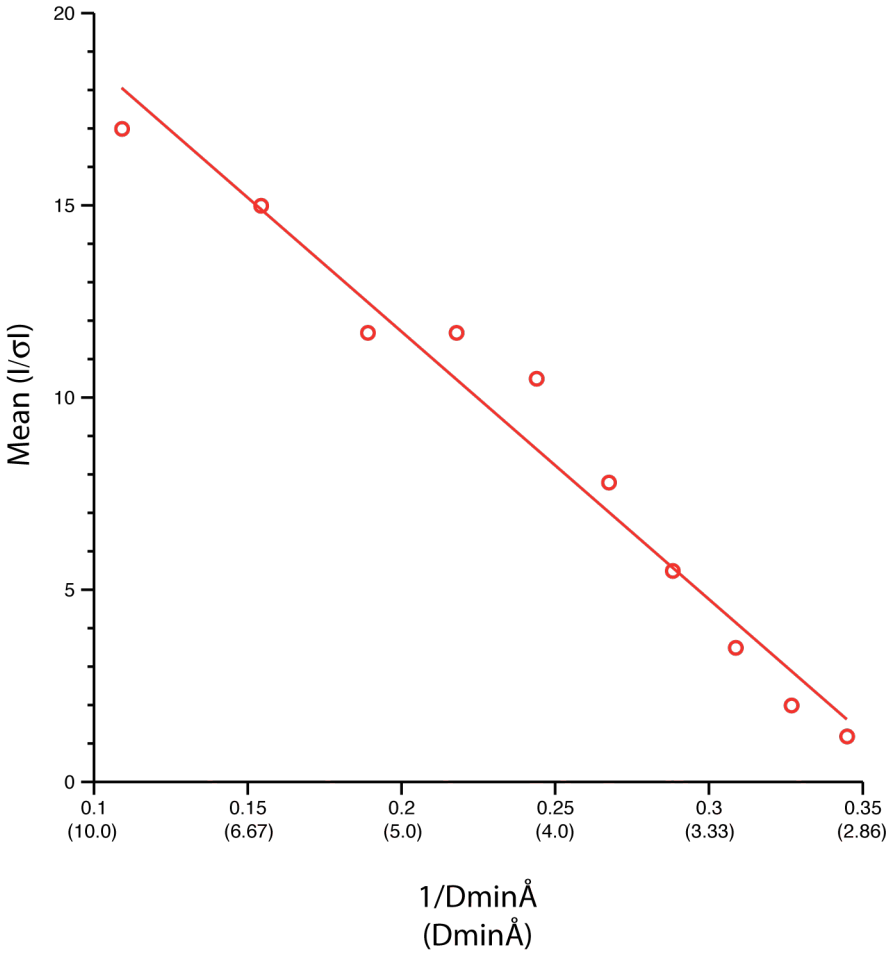


Fig. S10. Graph of resolution versus mean I/σ (SCALA) for the *Drosophila* Vps34 apo-enzyme dataset. Resolution is 3.06 Å at a mean (I /σI) of 2.0.

Table S1. X-ray diffraction data collection and refinement statistics *

	Apo	PIK-90	PIK-93	PI-103	3-MA
Data collection					
ESRF Beamline	ID14-4	ID23-1	ID23-1	ID29	ID23-1
Space group	I $2_12_12_1$	I $2_12_12_1$	I $2_12_12_1$	I $2_12_12_1$	I $2_12_12_1$
Cell dimensions					
a, *b*, *c* (Å)	110.6, 154.8, 243.1	111.5, 156.2, 244.2	110.0, 156.3, 242.9	111.4, 155.7, 244.0	112.1, 155.1, 244.5
Resolution (Å)	2.9†	3.4	3.5	3.5	3.3
R_{merge}	0.10 (0.88)	0.11 (0.56)	0.11 (0.45)	0.10 (0.54)	0.11 (0.69)
$I/\sigma I$	7.1 (1.2)	8.6 (2.6)	9.6 (4.6)	8.8 (2.4)	7.3 (1.9)
Completeness (%)	95.8 (84.5)	98.3 (98.7)	99.7 (99.9)	99.7 (100)	98.2 (99.7)
Redundancy	3.4 (2.9)	3.5 (3.6)	3.5 (3.6)	3.6 (3.6)	3.6 (3.7)
Refinement					
Resolution (Å)	72.4-2.9	72.8-3.4	61.6-3.5	61.7-3.5	61.1-3.3
No. reflections	44559	29052	26700	27069	31662
R_{work} / R_{free}	0.24/0.28	0.22/0.28	0.23/0.28	0.22/0.29	0.24/0.29
No. atoms					
Protein	8894	8932	8949	9003	8883
Ligand/ion	0	52	48	52/15	22
Water	31	0	0	0	0
B-factors					
Protein	40.2	68.8	64.5	74.9	87.7
Ligand/ion	-	81.1	101.0	128.2/178.4	89.7
Water	33.6	-	-	-	-
R.m.s. deviations					
Bond lengths (Å)	0.011	0.014	0.012	0.014	0.011
Bond angles (°)	1.70	1.82	1.65	1.82	1.67

* Each dataset was collected from a single crystal. Values in parentheses are for the highest resolution shell.
† At a mean ($I/\sigma I$) of 2.0, resolution is 3.06Å (Fig. S10).

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