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PIP4K β interacts with and modulates nuclear localisation of the high activity PtdIns5P-4-kinase isoform, PIP4K α

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Abstract.

The β -isoform of phosphatidylinositol-5-phosphate (PtdIns5P)-4-kinase (PIP4K) regulates the levels of nuclear PtdIns5P, which in turn modulates the acetylation of the tumour suppressor p53. The crystal structure of PIP4K β demonstrated that it can form a homodimer with the two subunits arranged in opposite orientations. Using mass spectrometry, isoform specific antibodies to PIP4Ks, RNAi suppression and overexpression studies, we show that PIP4K β interacts *in vitro* and *in vivo* with the PIP4K α isoform. As the two isoforms phosphorylate the same substrate to generate the same product, the interaction could be considered to be functionally redundant. However, contrary to expectation, we find that PIP4K β has 2000 times less activity towards PtdIns5P, compared to PIP4K α and that the majority of PIP4K activity associated with PIP4K β comes from its interaction with PIP4K α . Furthermore, PIP4K β can modulate the nuclear localisation of PIP4K α and PIP4K α has a role in regulating PIP4K β functions. Our data present a rationale for the functional interaction between PIP4K α and PIP4K β and provide insight into how the relative levels of the two enzymes may be important in their physiological and pathological roles.

Introduction.

Combinations of phosphorylation at the 3,4, or 5, position of the inositol head group of phosphatidylinositol generates seven different phosphoinositides that form the basis of a ubiquitous membrane signalling system. An array of tightly regulated phosphoinositide kinases and phosphatases, ultimately control the subcellular profile of phosphoinositides [1]. Phosphoinositides can regulate protein localisation, ion channel function and protein enzymatic activity, which can impact on cellular processes including vesicle transport, cytoskeletal dynamics, cell proliferation and survival, gene transcription, cell polarity and migration [2].

Phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5) P_2) is central to phosphoinositide signalling being a substrate for both phospholipase C (PLC) and phosphatidylinositol-3-kinase (PtdIns-3-kinase) and itself a second messenger [3]. PtdIns(4,5) P_2 can be synthesised by two different enzyme families that are highly related.

Phosphatidylinositol-4-phosphate (PtdIns4 P)-5-kinases (PIP5Ks) phosphorylate PtdIns4 P [4] on the 5-position while phosphatidylinositol-5-phosphate (PtdIns5 P)-4-kinases (PIP4Ks) phosphorylate PtdIns5 P on the 4-position [5]. The cellular level of PtdIns4 P is much greater than PtdIns5 P and it is likely that the majority of PtdIns(4,5) P_2 , in the cell, is synthesised through PIP5K activity. In accord with this hypothesis, overexpression or suppression of different isoforms of PIP5K impinge on many of the processes known to be regulated by PtdIns(4,5) P_2 [3]. In contrast to PIP5Ks, the role of PIP4Ks is less well understood. Of the three isoforms of PIP4K [6-9], the α and β isoforms can phosphorylate PtdIns5 P and PtdIns3 P [10], while the γ isoform is likely to be inactive. PIP4K γ , however, can interact with PIP4K α and β , probably to target their activities to a particular subcellular localisation [11].

Overexpression of PIP4Ks does not lead to phenotypes that are induced by PIP5Ks, suggesting that PIP4Ks do not increase the cellular level of PtdIns(4,5) P_2 to the same extent as PIP5Ks. However, this may only reflect the relative levels of PtdIns5 P to PtdIns4 P in the cell. In megakaryocytes, for example, PIP4K α -mediated synthesis of PtdIns(4,5) P_2 is important in platelet formation [12] and in platelets it is required for secretion [13;14]. Deletion of the only PIP4K in *C.elegans* and *D.melanogaster* led to an increase in whole animal levels of PtdIns5 P (our unpublished data) and in murine erythroleukemia cells (MEL), RNAi-mediated suppression of PIP4K β increased, while the overexpression of PIP4K β decreased the levels of nuclear PtdIns5 P [15]. These data suggest that PIP4Ks, through phosphorylation, likely control the level of PtdIns5 P .

The relative expression level of PIP4K isoforms varies in mouse tissues. For example, in brain, PIP4K α and β are approximately equal while there is nearly 10 fold more PIP4K β mRNA in muscle and in the heart. In the spleen, PIP4K α expression is 3-4 fold higher than PIP4K β . PIP4K γ , on the other hand, is highly expressed in the kidney [11]. The differential expression suggests that each PIP4K may have specific functions within the target organ. At a cellular level, PIP4K α localises in the cytosol, while PIP4K γ associates with an endomembrane compartment that partially co-localises with the golgi [9;11]. PIP4K β appears to localise predominantly in the nucleus [16]. However, PIP4K β interacts with both a TNF receptor [8] and the EGF receptor [17] suggesting that it may also be required in the cytosol. Deletion of

PIP4K β in mice leads to increased insulin-induced PKB activation in muscle [18] and in cells, overexpression of kinase active but not kinase inactive PIP4K β attenuates insulin induced PKB activation [19]. Finally, overexpression of IppD, a bacterial PtdIns(4,5) P_2 -4-phosphatase that generates PtdIns5 P , also increased PKB activity [19]. Together, these data point to a role for cytosolic PIP4K β and PtdIns5 P in PKB activation, possibly through the regulation of PtdIns(3,4,5) P_3 [19] and/or PKB dephosphorylation [20].

PIP4K β is also present in the nucleus and its nuclear import is dictated by a sixteen amino acid α -helical insertion [16] that is not present in other PIP4Ks. In the nucleus, activation of the p38 stress pathway inhibits PIP4K β activity leading to an increase in PtdIns5 P . PtdIns5 P can interact with the PHD finger of inhibitor of growth protein 2 (ING2) [21], which in turn modulates the acetylation and transcriptional activity of the tumour suppressor protein p53 [15]. PHD finger containing proteins appear to be nuclear specific downstream targets of PtdIns5 P [22;23]. Through its interaction with SPOP (speckle-type POZ protein), PIP4K β can regulate the activity of the CUL3 (Cullin 3) ubiquitination complex. The CUL3 ubiquitination activity also appears to be stimulated by increased levels of PtdIns5 P [24].

The differential tissue expression and sub-cellular localisation of PIP4Ks provides a reasonable explanation for the requirement for different isoforms within a cell. However, during a search for proteins that regulate PIP4K β , we discovered that PIP4K β interacts with PIP4K α . Previous studies indicate that PIP4K α and PIP4K β phosphorylate the same substrate to generate the same product suggesting that their interaction may have no functional advantage. Here we have studied the activity of both enzymes *in vitro* and *in vivo* and together with RNAi and overexpression assays have developed a rationale for a functional interaction between PIP4K α and PIP4K β .

Materials and methods.

PIP4K plasmids, Antibodies and chemicals.

GST-PIP4K α and β , EE-PIP4K α , EE-PIP4K α KD (G131L/Y138F), Myr-PIP4K α and HA-PIP4K β were derived from human sequences. Myc-PIP4K α and Myc-PIP4K β and Myc-PIP4K β KD (D278A) were derived from rat sequences. Peptides (p5:CGVGGNLLCSYG, p6: CNLLSFPRFFGP, p19: CMATPGNLTGSSVL) were coupled to keyhole limpet hemocyanin and used to immunise New Zealand white rabbits (for specificity of the antibodies see supplementary figure 2). Antibody 3 is a rat monoclonal that was previously described [25]. The anti-PIP4K α -C-term was from Abgent (AP8041b). Anti-HA and anti-Myc are monoclonal supernatants from clone 12CA5 and 9E10 respectively. Protein G and A sepharose, glutathione sepharose and Ni sepharose were purchased from GE Healthcare. Disuccinimidyl suberate (DSS) was from Pierce chemicals. All other chemicals were of analar grade.

Generation of HEK293 cells stably expressing shRNAi.

ShRNAi constructs, generated by cloning the following sequences into pRetroSuper, were used to generate viral particles in Phoenix ecotropic cells.

RNAi-PIP4K α : 5'-ATAGTGGAATGTCATGGGA-3'

RNAi-PIP4K β : 5'-AGATCAAGGTGGACAATCA-3'

HEK293 cells, stably expressing the ecotropic receptor, were infected with retroviral particles using polybrene. Cell populations were selected using puromycin (2 μ g/ml). In general, cells were maintained in the absence of the antibiotic selection for at least 48 hours prior to the experiments.

SiRNA targeting of PIP4Ks.

Control SiRNA oligos (siGENOME Non-Targeting siRNA Pool, Dharmacon, D-001206-13-20) or those targeting PIP4K α (PIP4K2A siGENOME SMARTpool, Dharmacon) M-006778-01) or PIP4K β (PIP4K2B siGENOME siRNA D-006779-02, D-006779-04, D-006779-06) were transfected into HEK293 cells using Dharmafect 1. Forty eight hours later cell lysates were used in immunoprecipitation experiments.

PIP4K activity measurements and PtdIns5P mass measurements.

In vitro PIP4K activity was measured using liposomes with 1 nmol PtdIns5P and 10 nmol of phosphatidylserine (PtdSer) as a substrate, 20 μ M ATP, 10 μ Cl 32 P-ATP and the appropriate amount of enzyme in 100 μ l of PIPKIN buffer (50 mM Tris-HCl pH 7.4, 10 mM MgCl $_2$, 1 mM EGTA, 70 mM KCl) for 10 minutes. The reactions were terminated and extracted as previously described [26]. 32 P-PtdIns(4,5) P_2 , a measure of PIP4K activity, was separated by TLC and quantitated using a phosphoimager (Biorad). To determine how PIP4K activity varied with respect to the levels of PtdIns5P, liposomes were made with 50nmol of PtdSer and varying amounts of PtdIns5P. Phosphoimager counts were converted to μ mol of PtdIns(4,5) P_2 by quantitating standard amounts of 32 P-ATP spotted on to the TLC. V_{max} was determined at a saturating concentration of PtdIns5P and the K_m was determined graphically at 50% of the V_{max} value. PtdIns5P was measured as described [26].

Subcellular fractionation and cross-linking

MEL cell nuclei were purified as previously described [27] except that the final resuspension buffer (FRB, see below) was made with 10 mM HEPES instead of Tris

to prevent quenching of the cross-linker. Nuclei, controls or treated with the indicated concentrations of cross-linking agent (Disuccinimidyl suberate DSS) were terminated by the addition of 1 M glycine pH7.5 (20 mM). Nuclear proteins were extracted using FRB (see below) containing 1% CHAPS, 0.5 M NaCl and immunoprecipitated using the indicated antibody. Immune-complexes were collected on proteinG sepharose and were solubilised using 1x SDS-PAGE loading buffer, separated by SDS-PAGE and analysed by western blotting.

HEK293 cells were trypsinised, washed with PBS and resuspended in hypotonic swell buffer (5 mM Tris-HCl pH 7.4, 1.5 mM KCl, 2.5 mM MgCl₂) for six minutes on ice. The cells were then disrupted by passage through a 22 gauge needle. The nuclei were pelleted by centrifugation (4 minutes, 352xg, 4°C) and the supernatant was removed and membranes were collected by high speed centrifugation (14000rpm microfuge) at 4°C for 15 minutes. The supernatant (cytosol) was collected and the membranes were sonicated into FRB (membrane fraction). The nuclei were further purified by centrifugation through a sucrose cushion (10 mM Tris-HCl pH 7.4, 1 mM EGTA, 1.5 mM KCl, 5 mM MgCl₂, 460 mM sucrose) and washed with final resuspension buffer (FRB 10 mM Tris-HCl pH 7.4, 1 mM EGTA, 1.5mM KCl, 5 mM MgCl₂, 290 mM sucrose). Nuclei were resuspended in FRB or were extracted by resuspending them in Dignam C buffer (20 mM Tris pH7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT) followed by incubation on ice for 30 minutes. The insoluble fraction was pelleted by centrifugation and the supernatant was dialysed against Dignam D buffer (20 mM Tris pH7.9, 0.1 M KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT) and samples were stored at -80°C.

Sucrose density centrifugation.

1 ml each of 40%, 31.25%, 22.5%, 13.72% and 5% sucrose solutions were layered sequentially in a centrifuge tube and allowed to form a gradient overnight by standing at 4°C. The cytosol (50 µl) and nuclear fraction (50 µl) were adjusted to 5% sucrose and loaded on top of the cushions and then centrifuged for 18 hours at 45000 rpm. After centrifugation fifteen 1 ml fractions were isolated from the bottom of the centrifuge tube using a syringe and needle. The samples were mixed with SDS-loading buffer separated by SDS-PAGE and analysed by staining the gel or by western blotting.

Mass spectrometry

Gel band destaining and washing

Coomassie blue stained gel bands were destained with three twenty minute changes of 1ml 200mM ammonium bicarbonate, 40% (V/V) acetonitrile. Gel bands were the dehydrated by the addition of 500ul acetonitrile for fifteen minutes followed by rehydration in 500ul of water for a further fifteen minutes. This dehydration-rehydration procedure was performed a total of three times followed by a final dehydration in acetonitrile.

In-Gel tryptic digestion

Gel bands were rehydrated in 25ul of 50mM ammonium bicarbonate, 9% (v/v) acetonitrile, 20ng/ul sequencing grade trypsin (Sigma-Aldrich) for twenty minutes. The bands were then covered in 100ul of 50mM ammonium bicarbonate, 9% (v/v) acetonitrile and incubated at 37°C for 18hrs. Following digestion, samples were acidified by the addition on 10ul of 10% (v/v) formic acid. The digest supernatant

was then transferred to a fresh eppendorf tube and the digest was dried in a vacuum centrifuge at 40°C for 30 minutes. The dried peptides were then resuspended in 20ul of water, 0.1% trifluoroacetic acid (Sigma-Aldrich) prior to LCMS analysis.

nLCMSMS Analysis

Peptides were separated utilising a Nano-Acquity UPLC system (Waters) as detailed below. Sample was loaded onto a Waters C18 Symmetry trap column (180um ID, 5um 5cm) in water, 0.1% (v/v) acetonitrile, 0.1% (v/v) formic acid at a flow rate of 7ul/min for 5 minutes. Peptides were then separated using a Waters NanoAcquity BEH C18 column (75um ID, 1.7um, 25cm) with a gradient of 3 to 30% (v/v) of acetonitrile, 0.1% formic acid over 30 minutes at a flow rate of 400nl/min. The nLC effluent was sprayed directly into the LTQ-Orbitrap XL mass spectrometer aided by the Proxeon nano source at a voltage offset of 2.5KV. The mass spectrometer was operated in parallel data dependent mode where the MS survey scan was performed at a nominal resolution of 60, 000 (at m/z 400) resolution in the Orbitrap analyser between m/z range of 400-2000. The top 6 multiply charged precursors were selected for CID in the LTQ at a normalised collision energy of 35%. Dynamic exclusion was enabled to prevent the selection of a formally targeted ion for a total of 20 seconds.

Immunoprecipitations.

Washed cells were resuspended in lysis buffer (50 mM Tris pH 8.0, 50 mM KCl, 10 mM EDTA, 1% NP-40,) and after 15 minutes the nuclei and cell debris were removed by centrifugation (14000 rpm, microcentrifuge 4°C, 10 mins). Lysates were incubated with the appropriate antibodies overnight and immune-complexes were captured using proteinG sepharose (1 hour 4°C). Immunoprecipitates were washed three times with IP wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Tween-20), resuspended in 1xSDS-loading buffer and subjected to SDS-PAGE and transferred to nitrocellulose. After incubation with the indicated antibodies, antibody/protein interactions were detected with ECL (GE Healthcare) or super signal (Pierce chemicals).

SDS-PAGE and Western blotting

Protein extracts were quantitated using the BIORAD reagent and adjusted to 1X SDS-loading buffer. After boiling, the extracts were either treated or not with iodoacetamide to modify cysteine residues and then separated by SDS-PAGE. Proteins were transferred to nitrocellulose, blocked in PBS-T-5%fat free milk for 1 hour and then incubated with the primary antibody appropriately diluted in Magic mix (PBS-0.1% Tween 20 (PBS-T), 1% western blocking reagent (Roche), 3% BSA). Antibody dilutions used were: p19 and p6 1:10000, anti-PIP4K α -C-term 1:1000, anti-Myc 1:100, anti-HA 1:100. The blots were washed with PBS-T and then incubated with the appropriate HRP-conjugated secondary antibody diluted in Magic mix (anti-Rabbit, mouse and rat 1:10000 (GE H), Anti-rabbit-TrueBlot (eBioscience) 1:10000). Antibody-protein interactions were visualised using ECL (GE H) or super signal (Pierce). Blots were stripped by incubation at 55°C in 50ml of strip buffer (50 mM TRIS pH 7.4, 2% SDS, 50 mM β -mercaptoethanol) for 20 minutes followed by extensive washing in PBS-T. The blots were blocked again and then used as above.

Production of recombinant proteins.

Bacterial colonies transformed with the appropriate vector were diluted into 5 ml of LB medium containing ampicillin (50 µg/ml) and grown overnight. Cultures were diluted to 100 ml, grown at 37°C for 1 hour and then IPTG (100 µM final concentration) was added and the cultures were grown overnight at 30°C. Cells were collected by centrifugation (4000 rpm for 10 minutes), washed with PBS and resuspended in 5 mls of PBS. Cells were disrupted by sonication (Diagenode cell disruptor set at the high intensity setting) and Triton-X-100 was added to a final concentration of 1%. Cell debris was removed by centrifugation and the supernatant was incubated with Glutathione sepharose beads for 2 hours. After extensive washing (PBS-1% Triton-X-100), the bound proteins were eluted using 10mM reduced glutathione in 50 mM Tris pH 8, 0.3 M NaCl. Eluted proteins were quantitated using BIORAD reagent and by SDS-PAGE and coomassie blue staining and dialysed into an appropriate buffer. GST was cleaved by incubation with thrombin for 2 hours at room temperature. The uncleaved protein and the cleaved GST-portion were removed by an additional incubation with glutathione sepharose. The cleaved protein was quantitated using standardised amounts of BSA after SDS-PAGE electrophoresis and coomassie blue staining.

HEK293 cells were transfected with the appropriate plasmid encoding a HA-tagged protein using calcium phosphate. The transfected cells were washed once with cold PBS, trypsinised and lysed in lysis buffer. The HA-tagged protein was immunopurified by using the anti-HA antibody followed by protein-G sepharose. After extensive washing with IP wash buffer, the HA-tagged protein was eluted with 3XHA peptide (YPYDVPDYA) (1 mg/ml in 50 mM Tris pH 8, 0.3 M NaCl) by incubation at 30°C for 30 minutes. The eluted protein was quantitated using standardised amounts of BSA after SDS-PAGE electrophoresis and coomassie blue staining.

Reconstitution of PIP4K α /PIP4K β complexes using recombinant proteins.

Cleaved bacterially expressed PIP4K α was incubated alone or together with HA-PIP4K β in a volume of 150µl of PIPKIN buffer containing 0.1% NP40, 1 µg/ml BSA, 15 mM dithiothreitol for one hour. The complexes were collected by HA-immunoprecipitation overnight at 4°C and were separated by SDS-PAGE and analysed by western blotting.

Ubiquitination assays.

HEK293 cells were transfected using calcium phosphate. MG-132 (10 µM) was added for the final 6 hours, then the cells were washed once with PBS and lysed directly into PBS-0.2% SDS, 8 M urea and sonicated. His-tagged proteins were purified on Ni columns and separated using SDS-PAGE and analysed by western blotting.

Confocal microscopy.

HeLa cells were transfected on glass coverslips using Fugene. HT1080 cells were transduced with the constructs indicated, selected with the appropriate antibiotics and plated on coverslips. The cells were fixed using PBS-4% formaldehyde (20 minutes), permeabilised with PBS-0.1% triton-X100 (5 minutes) and blocked with PBS-3% BSA (10 minutes). Appropriate antibodies were diluted in PBS-3% BSA and incubated with the coverslips for 30 minutes at 37°C in a humidified incubator. The coverslips were washed with PBS and incubated for 30 minutes with fluorophore-conjugated secondary antibodies diluted appropriately in PBS-3%BSA. The

coverslips were mounted using Vectashield, sealed using nail varnish and analysed by confocal microscopy (Zeiss)

Results.

PIP4K β complexes.

In order to study the localisation of PIP4K β and identify proteins that interact with it, a cell line expressing HA-tagged PIP4K β (HA-PIP4K β) was generated. Cytosolic and nuclear extracts were isolated from these cells and were further fractionated using sucrose gradient centrifugation. Protein molecular weights between 67 and 230 kDa could be separated (supplementary Figure 1A and B) and the BRG1 chromatin remodelling complex (MWt>500000 kDa) eluted in fractions 12-15 (Figure 1A). Cytosolic HA-PIP4K β eluted between a molecular weight of 120 and 200, while the nuclear HA-PIP4K β eluted with a molecular weight between 120 and 150 kDa (Figure 1A), however, the monomeric form of PIP4K β (49 kDa), which should elute in fractions 4-6, was not seen. The observed molecular weight suggested that PIP4K β exists only in complexes in both the cytosol and in the nucleus.

To assess if the endogenous nuclear PIP4K β is also in complexes, isolated nuclei from MEL cells were treated with increasing concentrations of a non-hydrolysable cross-linking agent (DSS), immunoprecipitated and analysed by SDS-PAGE and immunoblotting. Increasing concentrations of the cross-linker induced the loss of the monomeric 49 kDa protein resulting in the appearance of a 97 kDa complex. Higher concentrations of DSS resulted in disappearance of the dimeric complex possibly through irreversible cross-linking of PIP4K β into the nuclear pellet or cross-linking dependent loss of the epitope recognised by the antibodies (Figure 1B). The data suggest that endogenous nuclear PIP4K β is predominantly a dimer, which is in line with previous data obtained from the crystal structure of the PIP4K β [28], although higher molecular weight complexes were also seen (Figure 1C). Extraction of nuclear proteins with CHAPS/NaCl or with RIPA buffer, before the addition of the cross-linking agent, did not prevent the formation of the 97 kDa PIP4K β dimer, demonstrating the strength and stability of the complex (Figure 1C).

PIP4K β associates with PIP4K α .

To identify potential interacting partners of PIP4K β , an immunoprecipitate of HA-PIP4K β was separated by SDS-PAGE and stained with coomassie blue. The gel was cut into 60 slices, all of which were analysed by mass spectrometry (Figure 2A). In slices 33 and 34, corresponding to a molecular weight of approximately 49 kDa, 14 peptides were identified, three of which uniquely identified PIP4K β and two identified PIP4K α (Figure 2A). The data suggested that endogenous PIP4K α interacts with HA-PIP4K β . To further investigate the interaction between PIP4K α and PIP4K β , lysates from HEK293 cells overexpressing Myc-PIP4K β were used for affinity purification using bacterially expressed and purified GST, GST-PIP4K α , GST-PIP4K β or GST-PIP4K γ (Figure 2B). GST-PIP4K α , β and γ could affinity purify Myc-PIP4K β but GST alone could not. To demonstrate that the interaction between PIP4K α and PIP4K β occurs *in vivo*, lysates from HEK293 cells, expressing EE-PIP4K α either alone or with HA-PIP4K β , were immunoprecipitated with the anti-EE or HA antibody and analysed by western blotting. Immunoprecipitation with the

anti-HA antibody affinity captured EE-PIP4K α only when both proteins were co-overexpressed. When the reciprocal experiment was carried out, HA-PIP4K β was co-immunoprecipitated by the anti-EE only when both proteins were also co-expressed (Figure 2C).

To demonstrate that the interaction between PIP4K α and β is direct, both proteins were purified, combined *in vitro* and were analysed by HA-immunoprecipitation followed by western blotting (Figure 2D). The blot also shows 10% of the PIP4K α and β input. The anti-HA antibody immunoprecipitated approximately 80% of HA-PIP4K β and when HA-PIP4K β was combined with PIP4K α , the anti-HA antibody immuno-affinity purified approximately 80-90% of the PIPK α showing that PIP4K α and PIP4K β can interact directly (Figure 2D).

To demonstrate that endogenous PIP4K α and PIP4K β can interact *in vivo* we developed two antibodies, p5 and p6, which are specific for PIP4K β and one antibody, p19, which is specific for PIP4K α (supplementary data Figure 2). As the molecular weights of PIP4K α and PIP4K β are identical, HEK293 cell lines, in which PIP4K α or PIP4K β expression was stably suppressed by RNAi, were used for co-immunoprecipitation studies to further ensure specificity. The ShRNAi construct targeting PIP4K α decreased PIP4K α protein levels to 54%, while targeting PIP4K β decreased PIP4K β protein levels to 15% (Figure 3A). Pre-immune antibodies did not immunoprecipitate either PIP4K α or β . Both p5 and p6 immunoprecipitated endogenous PIP4K β and co-immunoprecipitated endogenous PIP4K α . The specificity was demonstrated by the lack of co-immunoprecipitation of PIP4K α when its expression was suppressed using RNAi (Figure 3B). Immunoprecipitation of endogenous PIP4K α also co-immunoprecipitated endogenous PIP4K β and the interaction was specific as RNAi mediated suppression of PIP4K α reduced the co-immunoprecipitation of PIP4K β (Figure 3C). The data show that PIP4K α and PIP4K β interact directly and that the interaction occurs *in vivo* between endogenous PIP4K α and PIP4K β .

PIP4K β has 2000 times less PIP4K activity compared to PIP4K α .

PIP4K α and β phosphorylate the same substrate to generate the same product and so it is questionable why they would interact with each other. The interaction may be fortuitous, as the two proteins are highly related or alternatively, the interaction may modulate each others activities and/or their localisation. In order to rationalise why PIP4K α and PIP4K β interact with each other, their enzymatic activity towards PtdIns5P was reassessed. GST-PIP4K α and β were expressed in bacteria, purified and quantitated by SDS-PAGE and coomassie blue staining (Figure 4A). Both enzymes phosphorylated PtdIns5P on the 4-position, to generate PtdIns(4,5)P₂, as demonstrated by dephosphorylation of the product with a specific recombinant PtdIns(4,5)P₂-5-phosphatase (data not shown). Therefore, both PIP4K α and β are *bona fide* PtdIns5P-4-kinases. However, PIP4K α phosphorylated PtdIns5P approximately 434 fold better than PIP4K β (Figure 4A). As the difference in activity between the PIP4K α and β could be a consequence of their expression in bacteria, Myc-tagged proteins, expressed in and purified from HEK293 cells, were tested for PIP4K activity. As observed with the bacterially expressed proteins, PIP4K α had approximately 207 times more activity than PIP4K β (Figure 4B). To determine more

accurately the difference in PIP4K activity between PIP4K α and β , we assessed the activity of bacterially expressed PIP4K α and β at different concentrations of PtdIns5P presented in phosphatidylserine liposomes (Figure 4C). The data show that while both enzymes had approximately the same K_m with respect to PtdIns5P, the V_{max} of PIP4K α was 2000 fold higher than PIP4K β (see table for quantitation). To assess whether PIP4K α and PIP4K β have the same activity *in vivo*, cellular PtdIns5P levels were measured after the co-expression of each PIP4K isoform with a PtdIns(4,5)P₂ 4-phosphatase (IpgD), which increases the level of PtdIns5P *in vivo* [29]. The co-expression of PIP4K α partially attenuated an increase in the level of cellular PtdIns5P induced by the expression of GFP-IpgD. In contrast, expression of an equivalent amount of PIP4K β did not attenuate the IpgD induced increase in PtdIns5P (Figure 4D). This was not a consequence of the nuclear localisation of PIP4K β as we show, here (Figure 6C) that overexpressed PIP4K β is localised in the cytoplasm, nucleus and at the plasma membrane in HeLa cells. These data show that *in vitro* and *in vivo*, PIP4K β is much less active compared to PIP4K α .

PIP4K α provides the majority of PIP4K activity in PIP4K β immunoprecipitations.

To determine if PIP4K α provides the majority of PIP4K activity in a heteromeric complex, PIP4K activity was assessed in complexes containing either wild type or kinase inactive EE-PIP4K α . Overexpression of EE-PIP4K α led to a dramatic increase in PIP4K activity in the EE-immunoprecipitate that, as expected, was not present when an inactive EE-PIP4K α kinase was expressed (Figure 5A). Co-overexpression of wild-type HA-PIP4K β did not lead to any changes in PIP4K activity associated with the EE-immunoprecipitation but as expected HA-PIP4K β was co-immunoprecipitated as assessed by western blotting (Figure 5A). Overexpression of HA-PIP4K β induced a very small increase in PIP4K activity associated with the HA-immunoprecipitate, however, when EE-PIP4K α was co-expressed with HA-PIP4K β there was a 20 fold increase in PIP4K activity associated with the HA-PIP4K β immunoprecipitate (Figure 5A). Co-overexpression of the kinase inactive PIP4K α actually decreased the amount of PIP4K activity associated with the HA-immunoprecipitate. The level of HA-PIP4K β in the various immunoprecipitates was similar (21163, 16891 and 15280 arbitrary imager units) suggesting that the changes in PIP4K activity were a consequence of co-immunoprecipitation of active EE-PIP4K α .

To determine to what extent of the PIP4K activity associated with PIP4K β is due to its interaction with PIP4K α , we stably suppressed the expression of PIP4K α and assessed the PIP4K activity in an immunoprecipitation of endogenous PIP4K β . RNAi-targeting of PIP4K decreased the level of PIP4K α protein by approximately 50% (Figure 3A) and PIP4K activity was also decreased by about 50-70% in isolated membrane, cytosol and nuclear fractions (Figure 5B). When endogenous PIP4K β was immunoprecipitated from the cytosol/membrane or nuclear fractions of cells suppressed for the expression of PIP4K α , the associated PIP4K activity decreased by about 50% (Figure 5C), although the amount of PIP4K β immunoprecipitated remained similar (6601, 9288, 7838 and 7227 (arbitrary image units) respectively in the four separate immunoprecipitations). We also carried out a similar analysis using cells transiently transfected with siRNA oligos targeting either PIP4K α or β . Suppression of PIP4K α decreased PIP4K α protein (6314 to 487 arbitrary imager

units) assessed by western blotting, while PIP4K activity in a PIP4K α immunoprecipitate decreased to 27% of the control (Figure 5D). Similarly, suppression of PIP4K α decreased the PIP4K activity in a PIP4K β immunoprecipitate to 34% of control. Suppression of PIP4K β as expected diminished the PIP4K protein levels (33205 in the control to 5554 (arbitrary imager units) in the siRNA PIP4K β) and the PIP4K activity in the PIP4K β immunoprecipitate to 28% of the control (Figure 5D). These data are consistent with the PIP4K α subunit providing the majority of PIP4K activity present in a PIP4K β immunoprecipitate.

To investigate if active PIP4K β is required in the complex, we measured the PIP4K activity of either active or inactive Myc-PIP4K β in complex with the endogenous PIP4K α . Suppression of the expression of PIP4K α reduced the PIP4K activity associated with the immunoprecipitate of Myc-PIP4K β by 75% showing that the majority of activity comes from the endogenous PIP4K α subunit (Figure 5E). Strikingly, the PIP4K activity associated with kinase inactive Myc-PIP4K β was also reduced to 24% compared to that associated with the active Myc-PIP4K β (Figure 5E). Immunoblotting showed that wild type and kinase inactive Myc-PIP4K β co-immunoprecipitated the same amount of endogenous PIP4K α (3363 in the wild type PIP4K β immunoprecipitate compared with 3574 (arbitrary units) in the kinase inactive PIP4K β immunoprecipitate). The specificity of the co-immunoprecipitation of endogenous PIP4K α was demonstrated by the decrease in the PIP4K α band in the knockdown (505 and 400 arbitrary units). Both the wild type and the kinase inactive PIP4k β were immunoprecipitated to similar extents (21878 and 24723 in the pRetro-super cells compared with 20903 and 23537 (arbitrary imager units) in the RNAiPIP4K α cells). These data suggest that PIP4K α can either activate PIP4K β in the complex or that the kinase activity of PIP4K β is required for optimal PIP4K α activity.

PIP4K β can modulate the localisation of PIP4K α .

To determine whether PIP4K β can modulate the total amount of nuclear PIP4K α , the level of endogenous PIP4K α was determined in cytosol and nuclear fractions from HEK293 cells stably suppressed for PIP4K β expression. The majority of PIP4K α was cytosolic with a small amount that was nuclear. Although RNAi suppressed the expression of PIP4K β to 15% of the control level, the total amount of PIP4K α present in the nucleus was not reduced significantly. This suggests that translocation of PIP4K α into the nucleus may not be solely dependent on its interaction with PIP4K β (Figure 6A). As the antibodies against PIP4K α cannot be used to immunolocalise the endogenous protein, Myc-PIP4K α was expressed alone or together with HA-PIP4K β and their subcellular distribution was determined using confocal microscopy. Overexpressed Myc-PIP4K α was found predominantly in the cytosol with some staining in the nucleus (Figure 6B). PIP4K β on the other hand was present both in the cytoplasm and in nuclear speckles (Figure 6C). When the two enzymes were co-expressed the localisation of PIP4K β remained unchanged but PIP4K α could be clearly observed in nuclear speckles that co-localised with PIP4K β (see merge in Figure 6D). These data indicate that while PIP4K β may not control the overall nuclear import of PIPK α , it may modulate the sub-nuclear localisation of PIP4K α .

Although it is difficult to determine the absolute amounts of PIP4K α and β using antibodies and western blotting, we investigated the amount of PIP4K α associated with PIP4K β in each subcellular fraction. Western blotting of PIP4K β immunoprecipitates from the membrane fraction, revealed that the molecular weights of PIP4K α and β were smaller when compared to their cytosolic counterparts (Figure 6E). These bands are the correct proteins as they are not present in the RNAi PIP4K β knockdown lines. The decrease in molecular weight may be a consequence of post-translational modification or alternatively, of proteolytic processing. Strikingly, the ratio of PIP4K α to PIP4K β in the PIP4K β immunoprecipitate from nuclear extracts (1.55) was 6 fold higher when compared to the immunoprecipitate from the cytosol (0.21) (Figure 6E). This suggests that there is a larger percentage of PIP4K α /PIP4K β complex in the nucleus compared to the cytosol. The result is not just a reflection of the total levels of PIP4K α in the different sub-cellular fractions as the majority (80%) of PIP4K α is cytosolic (Figure 6A). How the PIP4K α /PIP4K β complex becomes enriched in the nuclear fraction is not clear. The nucleus may preferentially sequester the PIP4K α /PIP4K β complex compared to the PIP4K β homodimers or the nuclear environment might favour exchange of subunits to generate the hetero-complex.

PIP4K α affects the ubiquitination of PIP4K β by the SPOP/CUL3 complex.

As PIP4K α can interact with PIP4K β , PIP4K α should play a role in functions that are primarily associated with PIP4K β . The nuclear SPOP/CUL3 ubiquitination complex (CUL3, SPOP and RBX1 (ring-box 1)) can interact with and ubiquitinate PIP4K β . Furthermore, the ubiquitination activity of the complex appears to be stimulated by an increase in the level of PtdIns5P [24]. How does overexpression of wild type PIP4K β , which should decrease the level of PtdIns5P, increase its own ubiquitination? We considered that overexpression of PIP4K β will increase the level of the PIP4K β homodimer, which will have much less PIP4K activity than the PIP4K α /PIP4K β complex, and therefore could potentially act as a dominant negative kinase when recruited to the SPOP/CUL3 complex. This could increase the local level of PtdIns5P and therefore increase SPOP/CUL3 mediated ubiquitination of PIP4K β . If so, then overexpression of PIP4K α should rebalance the ratio of the PIP4K α /PIP4K β complex and therefore reduce PIP4K β ubiquitination. Furthermore, overexpression of a kinase inactive PIP4K α would not be expected to suppress PIP4K β ubiquitination.

As shown previously [24], ubiquitination of Myc-PIP4K β was only observed when it was co-expressed with all three CUL complex proteins (Figure 7A, compare lane 1 with 2 and 7 with 8) and ubiquitination was enhanced when the kinase inactive PIP4K β was expressed (Figure 7A, compare lane 2 with 8). Strikingly, co-expression of the wild type PIP4K α enzyme suppressed ubiquitination of PIP4K β (Figure 7A, compare lane 2 with 4 and 8 with 10), however, co-expression of the kinase inactive PIP4K α (EE-PIP4K α KD) did not (compare lane 2 with 6 and 8 with 12). To test if nuclear PIP4K α is required to suppress PIP4K β ubiquitination, PIP4K α was targeted to the plasma membrane by a myristoylation sequence and PIP4K β ubiquitination was assessed. When co-expressed with PIP4K β , Myr-PIP4K α was not present in the nucleus, localised predominantly on the membrane and did not influence the nuclear localisation of PIP4K β (supplementary figure 3). Myr-PIP4K α is active and *in vivo* can attenuate IpgD induced PtdIns5P generation to the same extent as the wild type

PIP4K α (data not shown). Overexpression of the Myr-PIP4K α however, did not suppress the ubiquitination of the PIP4K β (Figure 7B), suggesting that the interaction with PIP4K β and targeting of PIP4K α to the nucleus is required to suppress SPOP/CUL3 mediated PIP4K β ubiquitination.

Discussion.

PIP4K β exists as a dimer through the interaction between two anti-parallel β -sheets at the N-terminus of the protein [28;30]. When expressed in cells, we also found that PIP4K β forms dimers as assessed by sucrose density centrifugation studies. Under these conditions, no monomers were detected. The dimer appeared to be extremely stable as extraction with RIPA buffer containing 0.2% SDS before cross-linking did not prevent dimer formation. The dimeric surface creates a large flat positively charged surface able to interact electrostatically with the membrane [30]. The interaction of PIP4K β with the membrane occurs in order to present the enzyme to its substrate PtdIns5P, which is then phosphorylated to generate PtdIns(4,5)P₂. However, our studies show that when compared to the PIP4K α isoform, the PIP4K β dimer is 2000 fold less active *in vitro*. This led us to question whether PIP4K β is less active *in vivo* than PIP4K α . Overexpression of IpgD, a bacterial PtdIns(4,5)P₂ 4-phosphatase, induced PtdIns5P generation which could be partially attenuated by the expression of PIP4K α , but not by an equivalent level of expression of PIP4K β , showing that PIP4K β activity is also much lower *in vivo* than PIP4K α . The inability of PIP4K β to decrease IpgD induced PtdIns5P levels is not due to its nuclear localisation as we show that PIP4K β is present in the cytosol, membrane and in the nucleus.

If the role of both PIP4K β and PIP4K α is to regulate the level of PtdIns5P, then the much reduced PIP4K activity of PIP4K β is surprising. However, the unexpected discovery that PIP4K β interacts with and targets PIP4K α provides an explanation as to how the low activity PIP4K β isoform can regulate PtdIns5P levels. They also suggest that the level of nuclear PtdIns5P may depend on nuclear PIP4K α . Whilst we show that PIP4K α and β can interact with each other, the nature of the interaction is not clear. It is possible that it exists as a heterodimer, or that a PIP4K α monomer may interact with the PIP4K β dimer in a trimeric complex. Indeed, PIP4K purified from erythrocytes showed a native molecular weight of 150000 suggesting the presence of a trimer [31]. Quantitative proteomic studies of PIP4K α and PIP4K β in chicken DT40 cells suggest that the complex of α and β is a dimer. Furthermore, the amino-acid sequence of the β -sheet that forms the dimer interface in PIP4K β is identical in PIP4K α and *in silico* modelling studies confirm the suggestion that PIP4K α and β could form hetero-dimers [32].

The much lower PIP4K activity of PIP4K β compared to PIP4K α begs the question as to whether there is a role for active PIP4K β in the complex. We show that the PIP4K activity is higher when active PIP4K β , compared to the inactive PIP4K β , is complexed with endogenous PIP4K α . These data suggest either that PIP4K α can dramatically stimulate the activity of PIP4K β or that PIP4K β kinase activity is required to activate PIP4K α in the complex. The conformation of the active

PIP4K β may also be different to the inactive kinase, which may be important for the regulation of the activity of the PIP4K α subunit. In the absence of specific inhibitors against each isoform and crystal structures of the various complexes it is difficult to conclude the exact role of the active PIP4K β subunit in the complex.

It is also pertinent to question whether the formation of the PIP4K α/β complex is regulated. If the affinities of interaction between PIP4K β homodimers and the hetero-complex are similarly equal then the formation of the hetero-complex may just be controlled by the ratio of the levels of expression of PIP4K α to PIP4K β . In this case changes in the ratio of expression of PIP4K α to PIP4K β will modulate the amount of PIP4K α/β complex, and therefore the amount of PIP4K activity, targeted by PIP4K β . Physiologically, the ratio of the levels of PIP4K α and PIP4K β differ dramatically between tissue types. Perhaps more importantly, the gene encoding PIP4K β is located in the ERBB2 amplicon that is often amplified in human breast tumours, which can lead to increased expression of PIP4K β [33]. The increased expression of PIP4K β could: Increase the concentration of the homodimer, which may have a specific function within the cell, independent of PIP4K α ; Increase targeting of PIP4K α to decrease PtdIns5P levels; Increase the homodimer concentration, which may act in a dominant negative manner and thereby lead to an increase in the level of PtdIns5P. Clearly, what the outcome is will very much depend on the absolute ratio of PIP4K α to PIP4K β . Being able to control the expression of PIP4K β together with measurement of the PtdIns5P level in both the cell and in the nucleus, should clarify some of these issues. Quantitative mass spectrometric evidence for random dimerisation of PIP4K α and β in chicken DT40 cells are presented in another study [32].

Alternatively or additionally, the hetero-complex formation may be regulated. PIP4K β is extensively post-translationally modified (our unpublished data) and these modifications may be able to regulate complex formation. We previously showed that p38 MAP kinase phosphorylates PIP4K β at serine 326 and induces a decrease in its PIP4K activity [15]. As PIP4K α accounts for the majority of PIP4K activity in a PIP4K β immunoprecipitate, we considered that the decrease in activity might be due to phosphorylation dependent inhibition of the interaction between PIP4K α and β . We assessed this and found that UV stimulation, okadaic acid, etoposide, doxorubicin or H₂O₂ treatment did not change the interaction between PIP4K α and PIP4K β (supplementary figure 4), although they did induce serine 326 phosphorylation of PIP4K β (data not shown). Phosphorylation of serine 326 on PIP4K β may lead to a conformational change that inhibits the activity of the associated PIP4K α .

Interestingly, in HEK293 cells and in MEL cells (data not shown) the PIP4K α/β complex appears to be more prevalent in nuclear extracts than in the cytosolic fraction. This does not reflect the total level of PIP4K α in the two fractions as the majority of PIP4K α is cytosolic. Notwithstanding that during the nuclear isolation procedure there may be a preferential loss of the PIP4K β dimer or of the PIP4K α enzyme, this may suggest that hetero-complex formation is regulated in the nucleus. We do not believe that there is significant redistribution of the enzymes on sub-cellular fractionation as immunofluorescence analysis of overexpressed PIP4K α or β show a similar subcellular distribution to subcellular fractionation. Why then is the

PIP4K α / β complex more prevalent in the nucleus? If the PIP4K α isoform can only enter the nucleus as a consequence of its interaction with PIP4K β , we would expect that the nuclear PIP4K α /PIP4K β ratio would reflect the cytosolic PIP4K α /PIP4K β ratio. The PIP4K α /PIP4K β complex, however, may be preferentially imported into the nucleus or preferentially sequestered in the nucleus compared to the PIP4K β homo-dimer. Alternatively, if PIP4K α can be imported into the nucleus independently of PIP4K β then the nuclear environment may favour rapid exchange of subunits to generate the PIP4K α /PIP4K β complex. This may be a direct consequence of sub-cellular specific post-translational modification of PIP4K β . PIP4K α may be imported into the nucleus independently of PIP4K β as, compared to control cells, we were unable to detect significant differences in the total amount of PIP4K α present in the nucleus after PIP4K β expression was suppressed by RNAi.

It is clear, however, that control of the nuclear localisation of both PIP4K β and α is complex. Previous studies on the overexpression of PIP4K β in HeLa cells [16] and on the localisation of flag-tagged endogenous PIP4K β in Chicken DT40 cells [34] suggested that the majority of PIP4K β was nuclear. However, in most cells that we have studied, overexpressed PIP4K β localises to the nucleus, the cytosol and the plasma membrane as observed by others [24] and in this study in HeLa cells. However, in MCF7 cells the majority of PIP4K β appears nuclear while in T47D, another breast cancer cell line, PIP4K β is mainly cytosolic (data not shown). The reason for these differences is not clear but may be a consequence of differential expression of import factors or post-translational modification of PIP4K β . In the case of PIP4K α in HT1080 cells, overexpressed PIP4K α is predominantly cytosolic. Co-overexpression of PIP4K α and PIP4K β leads to a clear increase in total PIP4K α targeted to the nucleus (supplementary Figure 5) suggesting that in some cells, overexpressed PIP4K β will target more PIP4K α into the nucleus. Our data also show that PIP4K β can target PIP4K α to nuclear speckles and that PIP4K β mediated targeting of PIP4K α is functional and can regulate the ubiquitination activity of the SPOP/CUL3 complex.

The ability of inactive mutant proteins to localise or modulate active counterparts has been reported in numerous signalling cascades and has been well characterised in the myotubularin family. Myotubularins are a family of lipid phosphatases some of which are inactive, such as MTMR13, due to a mutation in a critical active site cysteine residue [35]. MTMR13 interacts with and activates MTMR2 [36] and mutation in either MTMR2 or the inactive MTMR13 can induce Charcot Marie tooth syndrome 4B [37]. These data exemplify the importance of how the interaction between the active PIP4K α and less active PIP4K β may impinge on phosphoinositide regulation in physiological and pathological conditions.

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We would like to note that while this work was in progress we became aware that another group independently discovered the association between PIP4K α and PIP4K β [32]. We would like to acknowledge previous colleagues at the NKI and the Dutch cancer society for funding. IpgD was from B. Payrastre, CUL3, RBX and SPOP were from M. Van Lohuizen and pSuper and pRetroSuper plasmids from R. Agami. We thank all members of the inositide laboratory for helpful discussions during the preparation of this manuscript and to members of Leukaemia biology. We would also like to acknowledge the efforts of the Duncan Smith and the mass spectrometry unit and all core facilities at the PICR.

Figure legends

Figure 1

A. Nuclear extracts from HEK293 cells expressing HA-PIP4K β were separated by sucrose density centrifugation and each fraction was separated by SDS-PAGE and the gel was stained with silver. The western blot below shows the presence of the BRG1 complex (>500000 kDa) in fractions 12-15. 2 μ l of every fraction from the sucrose density centrifugation of cytosolic or nuclear fraction was spotted onto nitrocellulose and probed with the anti-HA antibody and relevant fractions containing HA immunoreactivity were analysed by western blotting. B. MEL cell nuclei were treated with increasing concentrations of the irreversible cross-linking agent DSS. Nuclear extracts were then immunoprecipitated with either a pre-immune serum or a PIP4K β specific serum (p5 or p6) and western blotted with an anti-PIP4K rat monoclonal antibody (Antibody 3). Increasing concentrations of DSS induced the formation of the cross linked dimer (arrow). C. Nuclei were treated as shown, extracted and immunoprecipitated with the indicated antibodies. The western blot was probed with a specific rat monoclonal antibody against PIP4K (Antibody 3). The data shown are representative of at least two different experiments.

Figure 2.

A. HA-PIP4K β was purified from HEK293 cells expressing HA-PIP4K β using HA-immunoaffinity chromatography. Eluted proteins were separated by SDS-PAGE and the coomassie stained gel was cut into 60 slices and each slice was analysed by mass spectrometry. The figure shows the peptides which uniquely defined the presence of PIP4K α (top) or PIP4K β (bottom), in fractions 33 and 34. B. The lysate from HEK293 cells expressing Myc-PIP4K β (lane 1) was used for affinity purification with either GST (lane 2), GST-PIP4K α (lane 3), GST-PIP4K β (lane 4) or GST-PIP4K γ (lane 5). Bound proteins were analysed by western blotting using an anti-Myc antibody. Lane 1 shows 10% of the lysate input used for each affinity purification. C. EE-PIP4K α and HA-PIP4K β were expressed in HEK293 cells, as indicated (below) and immunoprecipitated and western blotted with the indicated antibodies. The experiment is representative of three separate experiments. D. HA-PIP4K β purified from HEK293 cells was combined with purified bacterially expressed PIP4K α , after removal of the GST-tag by thrombin cleavage, incubated at room temperature for one hour and then immunoprecipitated using the anti-HA antibody. Immunoprecipitates were analysed by western blot and were probed first for PIP4K α (top panel) after which the blot was stripped and reprobed with an anti-HA antibody (lower panel). 10% of the total protein input was also western blotted. The arrow denotes PIP4K α co-immunoprecipitated in the HA-immunoprecipitate.

Figure 3.

A. Lysates, from HEK293 stably expressing the indicated pRetroSuper construct, were probed using the antibodies shown. RNAi to PIP4K α suppressed its expression to 54% of the control value while the RNAi to PIP4K β suppressed its expression to 15% of the control value. B. Lysates from HEK293 stably expressing the indicated RNAi construct were

immunoprecipitated with the antibodies shown and the western blot was probed with an anti-PIP4K α antibody. The blot was stripped and reprobed with an anti-PIP4K β antibody. C. Lysates from HEK293 stably expressing the indicated RNAi construct were immunoprecipitated as indicated and the western blot was probed for the presence of PIP4K β . The blot was stripped and reprobed for PIP4K α . The data are representative of three separate experiments.

Figure 4

- A. Purified GST-PIP4K α and β were quantitated by SDS-PAGE and coomassie blue staining (top panel). Matched quantities of protein were then assayed for PIP4K activity (autoradiograph middle panel and quantitation in the graph and box). B. Myc-PIP4K α and β were purified from HEK293 cells and quantitated by western blots probed with the anti-Myc antibody (top panel). Matched inputs were assayed for PIP4K activity (autoradiograph middle panel and quantitation in the graph and box). C PIP4K α (left) or PIP4K β (right) activity was assayed using liposomes containing PtdSer (50nmol) and increasing amounts of PtdIns5P. The data is represented graphically and the kinetic parameters (K_m and V_{max}) were determined and are shown in the table. Note that for the graph the phosphoimaging exposure time for the PIP4K α was 5 minutes while the exposure time for PIP4K β was 90 minutes. D. PtdIns5P was measured in HEK293 cells transfected as indicated. Expression of the various constructs are shown in the top panels while PtdIns5P levels are shown in the graph.

Figure 5

HEK293 cells were transfected with the constructs shown and the lysates were divided and immunoprecipitated using an antibody against the EE (left panel) or HA-tag (right panel). Immunoprecipitates were split and PIP4K activity was assessed (graph) and the rest was analysed by western blotting using the indicated antibodies (top blot). The blot was stripped and then reprobed with the indicated antibodies (bottom blot). It appears that PIP4K α KD interacts less well with PIP4K β , however this is a consequence of its lower expression (see left blot EE-IP WB PIP4K α). The ratio of PIP4K α /PIP4K α KD in the input is 3.95 and is similar in the PIP4K β immunoprecipitate (4.4). B. Membrane, cytosol and nuclear fractions were isolated from HEK293 cells expressing either pRetroSuper or RNAi PIP4K α and lysates from these fractions were immunoprecipitated with p19 (anti-PIP4K α) and PIP4K activity was measured. C. Cytosol/membrane and nuclear fractions from the above cell lines were immunoprecipitated with p6 (anti-PIP4K β). The immunoprecipitates were divided and assessed for PIP4K activity (graph), and analysed by western blotting with antibodies to endogenous PIP4K α (upper right panel). The blot was stripped and reprobed for PIP4K β (lower right panel). D. HEK293 cells were transfected with the indicated siRNA oligos and lysates were immunoprecipitated as indicated, split and assayed for PIP4K activity (numbers on the graph indicate % of control) and western blotted with the indicated antibodies. E. Wild type or kinase inactive (D278A) Myc-PIP4K β was expressed in control cells (pRetroSuper) or cells suppressed for the expression of PIP4K α (RNAi PIP4K α). Cell lysates were immunoprecipitated

with the anti-Myc antibody and assayed for PIP4K activity (graph) and western blotted for the co-IP of endogenous PIP4K α (Arrow upper panel). The blot was then stripped and reprobred for Myc-PIP4K β (lower panel). The data are representative of at least two separate experiments.

Figure 6.

- A. Lysates from cytosol and nuclear fractions, isolated from HEK293 expressing the indicated RNAi constructs, were immunoprecipitated with an antibody specific for PIP4K α (p19) and western blotted using the antibody against PIP4K α . B. HeLa cells were transfected with Myc-PIP4K α , fixed and stained using the anti-Myc antibody. C. Cells were transfected with HA-PIP4K β and stained using the anti-HA antibody. D. Cells were co-transfected with Myc-PIP4K α and HA-PIP4K β , fixed and stained with rabbit anti-Myc antibody (left micrograph) and mouse anti-HA (middle micrograph) and the merge is shown on the right. E. Lysates from HEK293 cells stably expressing the RNAi constructs indicated, were immunoprecipitated with p6 (anti-PIP4K β) and western blotted for PIP4K α (top panel). The blot was stripped and reprobred with p6 (anti-PIP4K β). The data show that the ratio of PIP4K α to PIP4K β in a PIP4K β immunoprecipitate is higher in the nuclear extracts (1.55) compared to the cytosol (0.21). The arrows denote the different molecular weights of PIP4K α in the membrane, cytosol and nuclear fraction. The data are representative of at least two separate experiments

Figure 7.

- A. HEK293 cells were transfected as indicated and His-tagged proteins were affinity purified using nickel sepharose and analysed by western blotting with an anti-Myc antibody to visualise Myc-PIP4K β and its ubiquitinated products. The arrow denotes where non-ubiquitinated Myc-PIP4K β would migrate. Total cell lysates were also separated by SDS-PAGE and probed as shown. B. His-tagged proteins were purified from lysates of cells expressing the indicated constructs and analysed by western blotting using a Myc-antibody. Total cell lysates were also western blotted with the indicated antibodies. Note all cells received the three CUL complex proteins (CUL, RBX and SPOP). The data are representative of three separate experiments.

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Figure 1

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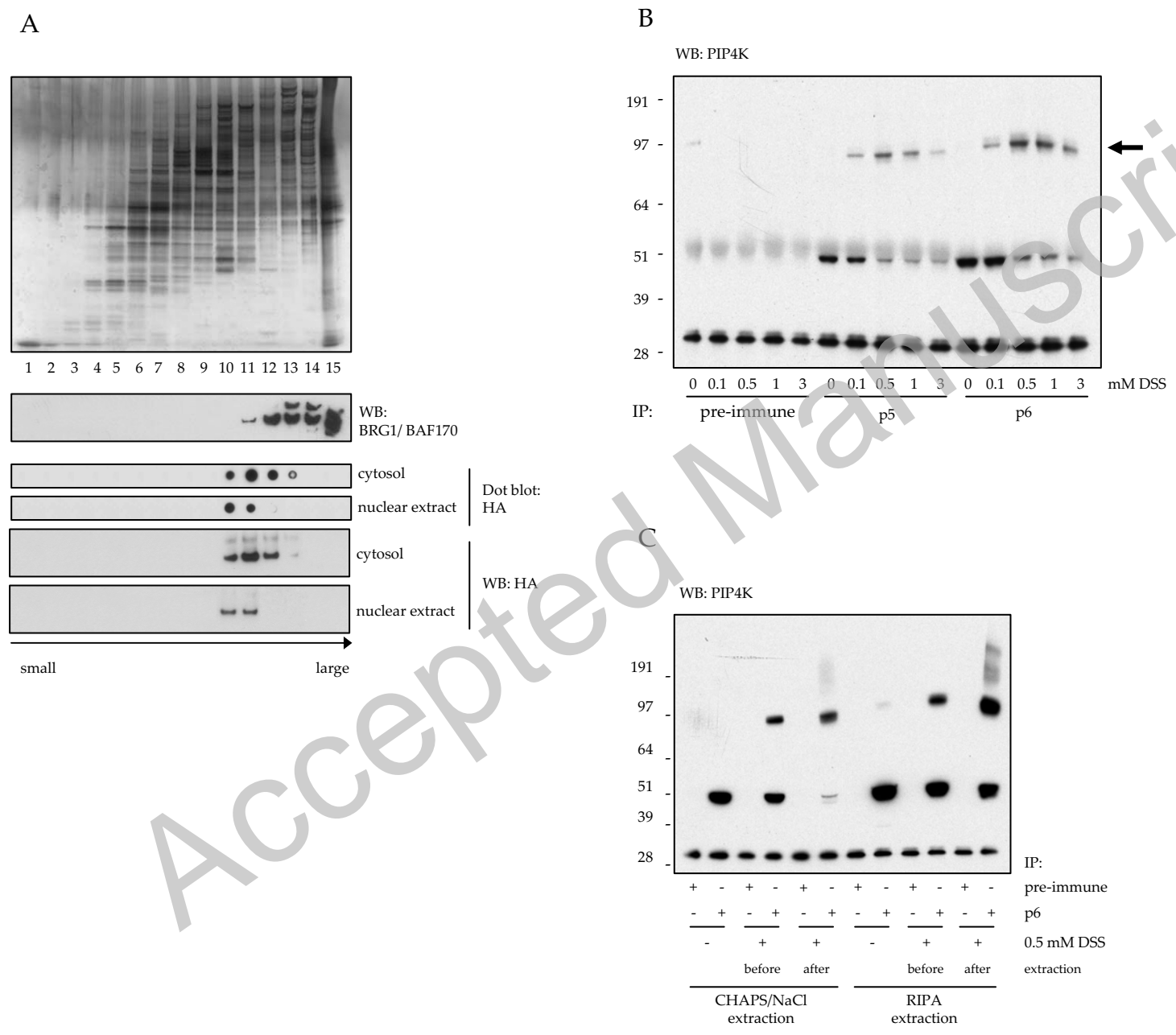
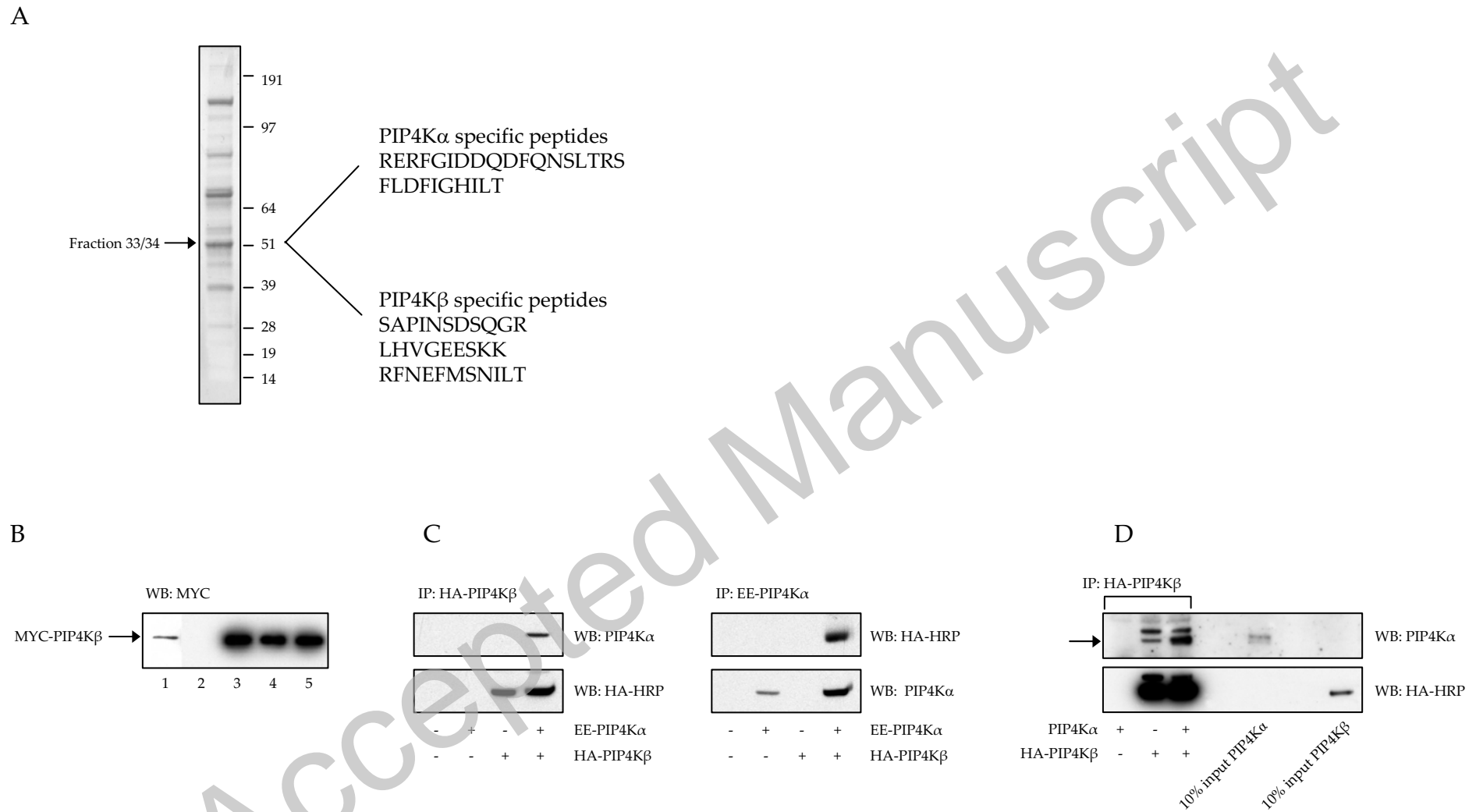


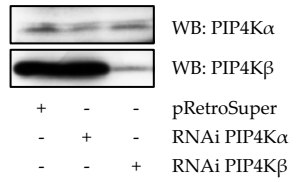
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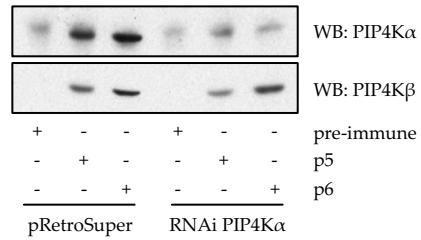
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Figure 3

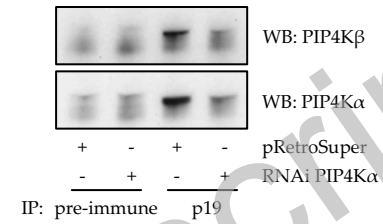
A



B



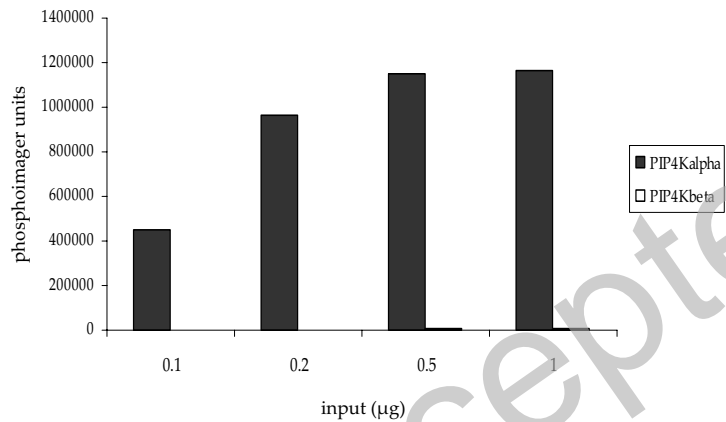
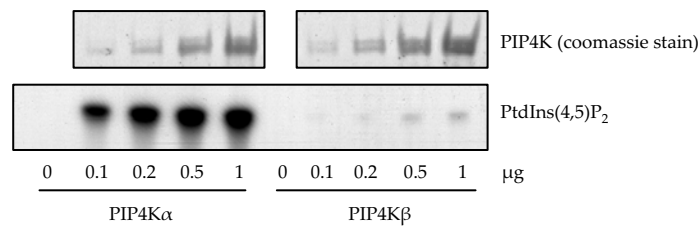
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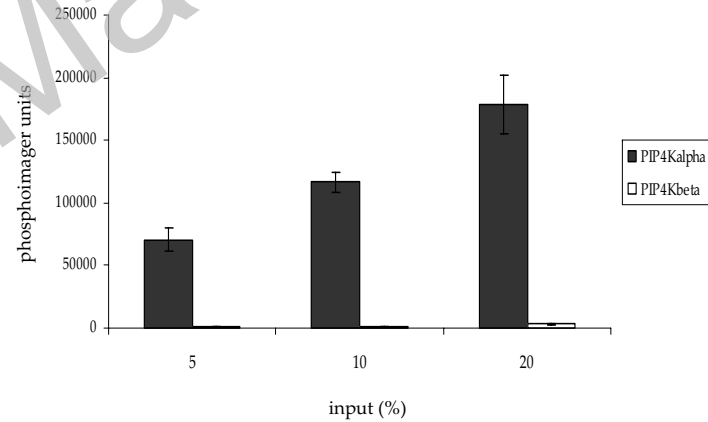
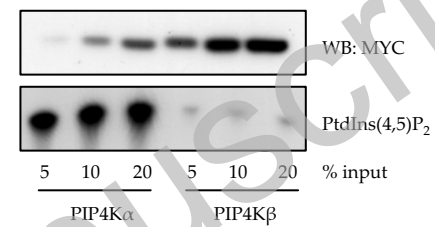
Figure 4

A



	Specific activity (counts/ μg protein)	fold
GST-PIP4Kα	4662.2 ± 228.9 *10 ³	434
GST-PIP4Kβ	10.8 ± 2.4 *10 ³	1

B

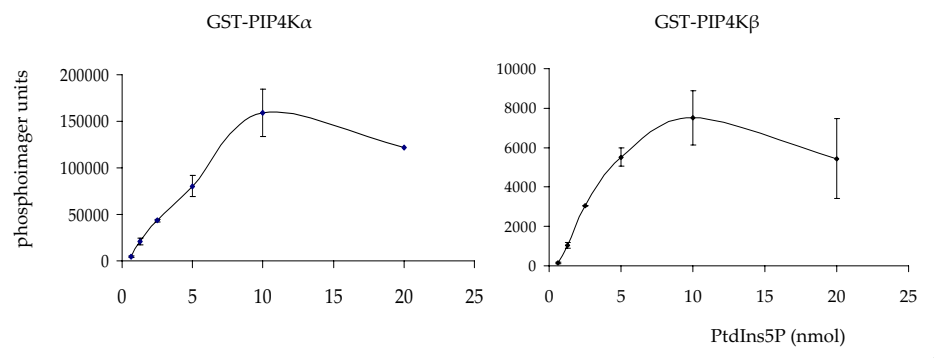


	Specific activity (counts/protein)	fold
MYC-PIP4Kα	1139.8 ± 277.8	207
MYC-PIP4Kβ	5.5 ± 3.2	1

Figure 4

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C



	Vmax (pmol/min/μg)	Km (μM)
GST-PIP4Kα	466.00 ± 30.56	50
GST-PIP4Kβ	0.202 ± 0.014	30

D

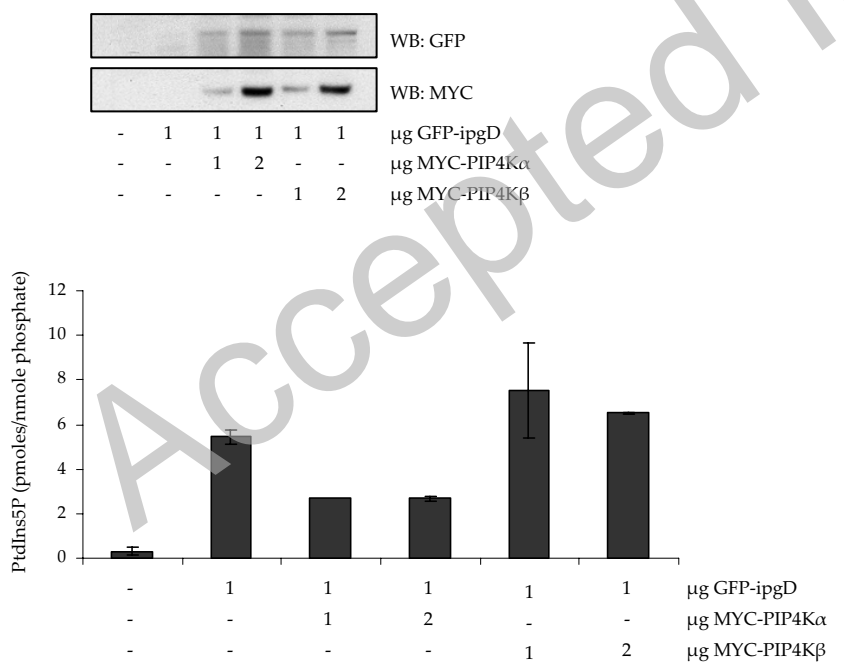
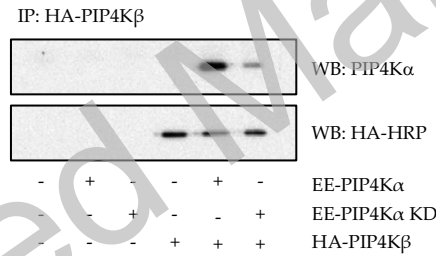
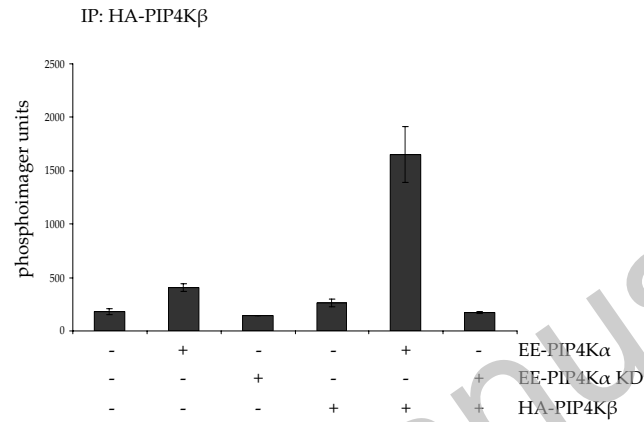
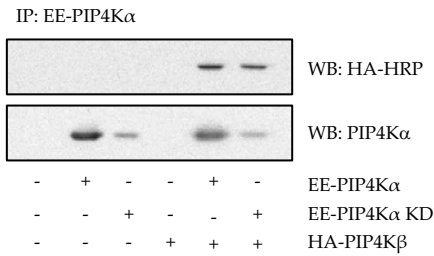
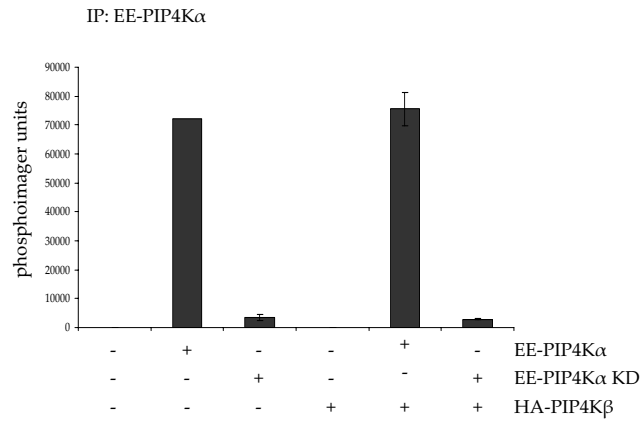
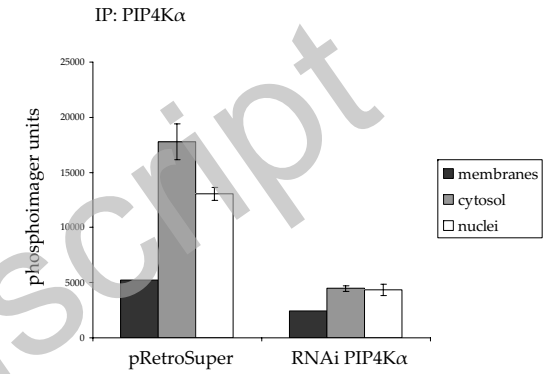


Figure 5

A



B



C

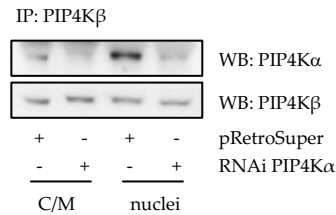
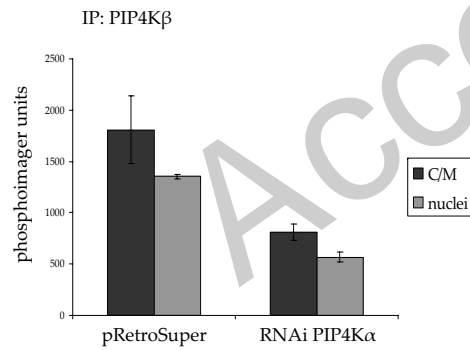
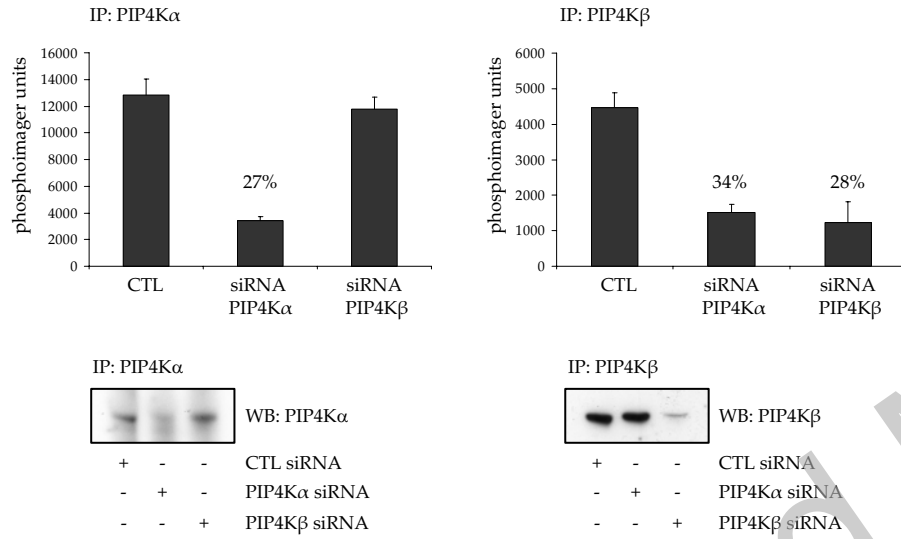
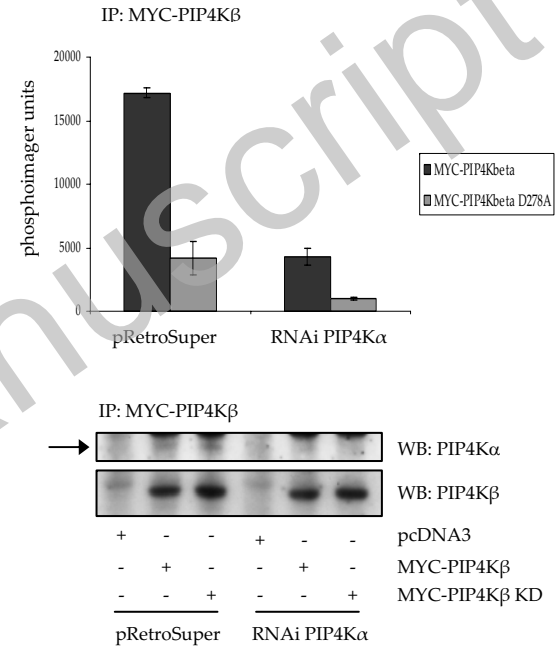


Figure 5

D

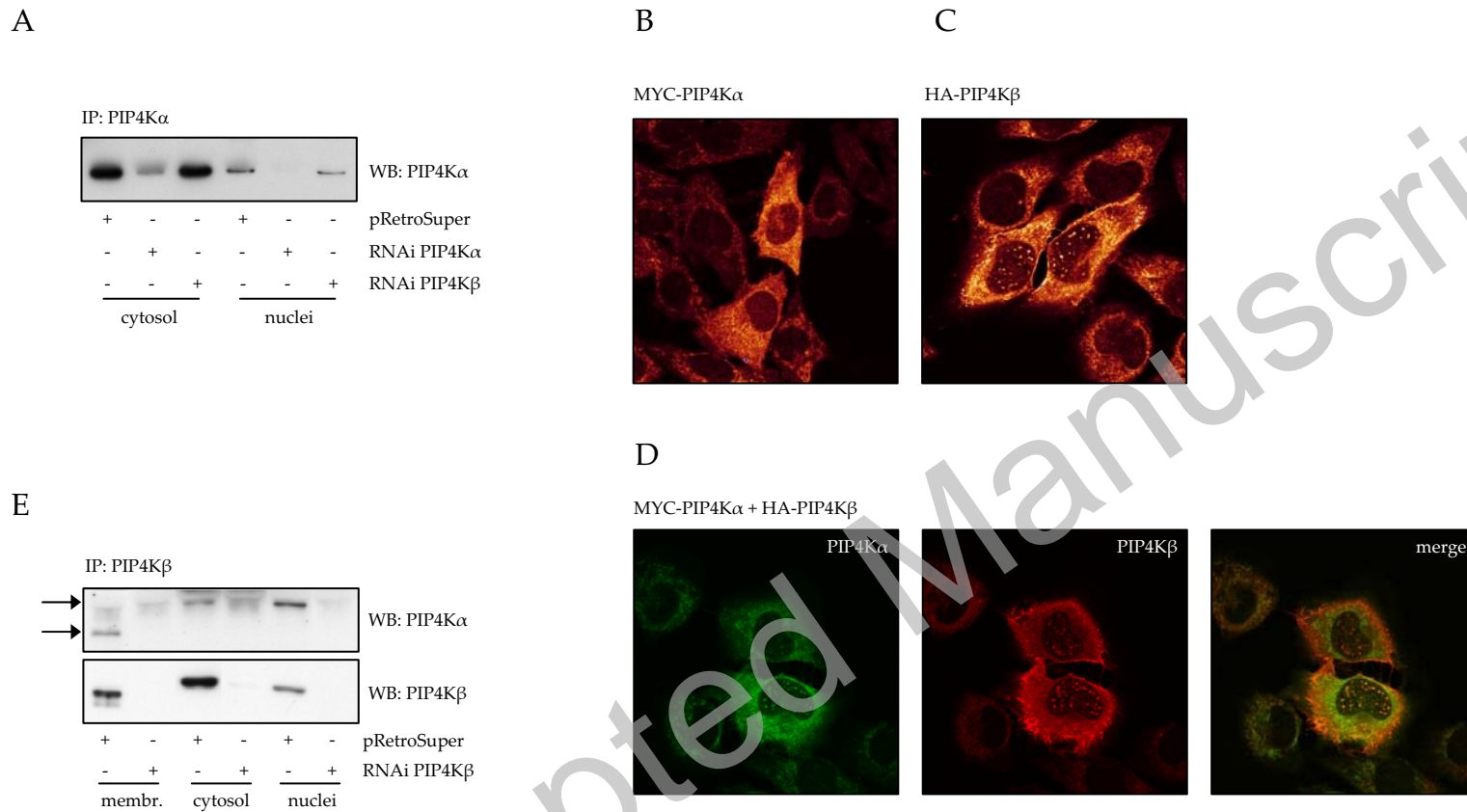


E



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Figure 6

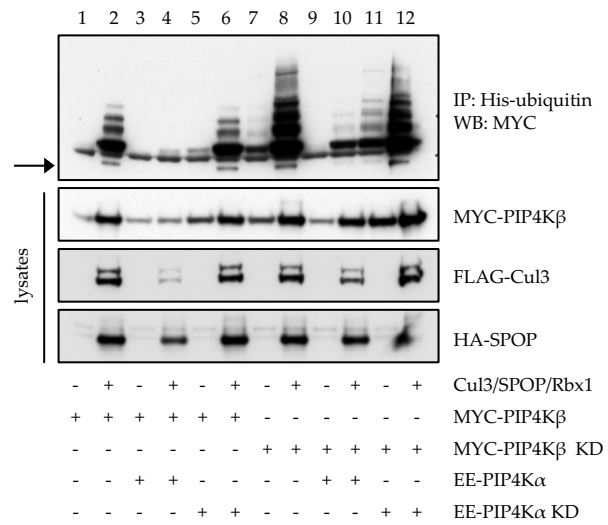


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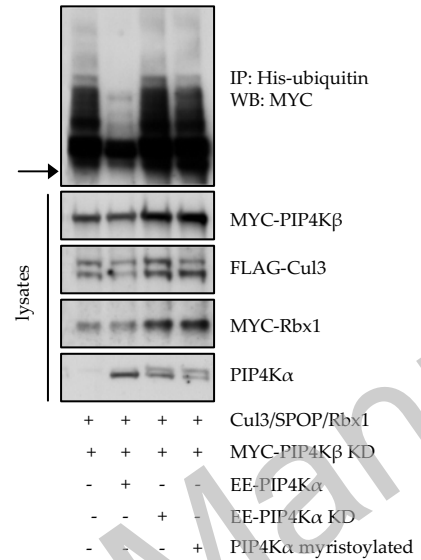
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Figure 7

A



B



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