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# Genomic tagging reveals a random association of endogenous PtdIns5P 4-kinases II $\alpha$ and II $\beta$ and a partial nuclear localisation of the II $\alpha$ isoform

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Phosphatidylinositol 5-phosphate 4-kinases (PtdIns5P 4-kinases) II $\alpha$  and II $\beta$  are respectively cytosolic or nuclear when transfected into cells, including DT40 cells (Richardson et al Cellular Signalling 19, 1309-1314; 2007). Here we have genomically tagged both PtdIns5P 4-kinase II isoforms in DT40 cells, and immunoprecipitation of either isoform from tagged cells followed by mass spectrometry showed that they are associated directly with each other, most likely by heterodimerisation. We quantified the cellular levels of the PtdIns5P 4-kinase II mRNAs by real-time quantitative PCR, and the absolute amount of each isoform in immunoprecipitates by mass spectrometry using selective reaction monitoring with  $^{14}\text{N}^{13}\text{C}$ -labelled internal standard peptides. The data suggest that the dimerisation is complete and random, governed solely by the relative concentrations of the two isoforms. While PtdIns5P 4-kinase II $\beta$  is > 95% nuclear as expected, the distribution of PtdIns5P 4-kinase II $\alpha$  is 60% cytoplasmic (all bound to membranes) and 40% nuclear. *In vitro*, PtdIns5P 4-kinase II $\alpha$  was 2000 times more active as a PtdIns5P 4-kinase than the II $\beta$  isoform. Overall the data suggest a function of PtdIns5P 4-kinase II $\beta$  may be to target the more active II $\alpha$  isoform into the nucleus.

## **Introduction**

Amongst the many known intracellular roles of inositol lipids, the best understood are those in the cytoplasm. However, there are also a number of less well studied functions for these lipids within the nucleus (for reviews see [1-4]). There is clear evidence in the nucleus for the minor route of PtdIns(4,5) $P_2$  synthesis [5], the 4-phosphorylation of PtdIns5P by Type II PtdIns5P 4-kinases (PtdIns5P 4-kinases II). The most likely major route of PtdIns5P synthesis in animals is by 4-dephosphorylation of PtdIns(4,5) $P_2$  [6, 7], with the re-phosphorylation of PtdIns5P by PtdIns5P 4-kinases II serving to remove it. Thus the PtdIns(4,5) $P_2$  4-phosphatases and the PtdIns5P 4-kinases II can form a reversible pathway regulating the levels of PtdIns5P (see Lecompte et al [8] for evolutionary arguments for this pathway). PtdIns5P is present in the nucleus [9] and evidence has been presented that it regulates the activity of the transcription factor ING-2 [10]. Nuclear levels of PtdIns5P increase during the cell cycle [9] or when cells are stressed [11], and it is generally accepted that a key regulator of PtdIns5P levels in the nucleus is PtdIns5P 4-kinase II $\beta$  whose activity in stressed cells is decreased as a result of it being phosphorylated, and thus inhibited, by p38 MAP kinase [11]. This, together with an increase in nuclear PtdIns(4,5) $P_2$  4-phosphatase [7], causes the increase in nuclear PtdIns5P. Nuclear PtdIns5P 4-kinase II $\beta$  has also been reported to associate with and regulate the activity of Cul3 SPOP ubiquitin ligase [12].

The nuclear localisation of PtdIns5P 4-kinase II $\beta$  is mediated by a novel nuclear localisation sequence [13] consisting of an acidic  $\alpha$ -helix 17 amino acids long, numbered  $\alpha$ -helix 7 in the PtdIns5P 4-kinase II $\beta$  structure described by Rao et al [14]. We have shown that any disruption of this  $\alpha$ -helix in PtdIns5P 4-kinase II $\beta$  leads to a cytosolic localisation [13, 15]. On the other hand, PtdIns5P 4-kinase II $\alpha$ , which is cytosolic when transfected, lacks this  $\alpha$ -helix 7 altogether, and merely introducing that helix into PtdIns5P 4-kinase II $\alpha$  is sufficient to target the enzyme to the nucleus [13]. However, there is evidence, though not yet definitive, that some endogenous PtdIns5P 4-kinase II $\alpha$  may be nuclear [13, 16, 17].

Recently we established that endogenous PtdIns5P 4-kinase II $\beta$  is indeed mostly nuclear by genomic tagging of the enzyme in DT40 cells [18]. We also showed that when PtdIns5P 4-kinase II $\alpha$  is acutely transfected into DT40 cells it is, as in other cells, cytosolic [18]. Here we have used the specificity of genomic tagging together with quantitative mass spectrometry with internal peptide standards to show that endogenous PtdIns5P 4-kinases II $\alpha$  and II $\beta$  associate, probably by random heterodimerisation, *in vivo*, and that approximately 40% of the endogenous II $\alpha$  isoform is nuclear.

## **Materials and methods**

The culturing of DT40 cells, extraction of genomic DNA, Western blotting, protein purification and immunoprecipitation of tagged proteins were all performed as in Richardson *et al.* [18].

### Tagging PtdIns5P 4-kinase II $\alpha$

This was accomplished using the same strategy as for the tagging of PtdIns5P 4-kinase II $\beta$ , described in refs [18] and [19]. Primers were used for the PCR amplification of genomic DNA upstream and downstream of the stop codon of the *PIP5K2A* (5' construct; (forward) GCATGGTACCTCAGAGTCATGTTAGCTGTG, (reverse) GCATTCTAGACGTCAAGATGTTGGCAATAAAG, 3' construct; (forward) CATGGATCCTCCCCTCATGTACACCGGACAG, (reverse) GCATGCGGCCGCGTCTGTCAGTGCTACAGAAGTG). The subsequent insertion of the puromycin selection cassette, plasmid linearisation, transfection and selection of DT40 lines which had incorporated the insert, and PCR confirmation of the correct incorporation, were all as described in Richardson *et al.* [18].

### Fractionation of DT40 cells

DT40 cells were swollen and disrupted by syringing exactly as in ref [18], but the subsequent fractionation was modified as follows. Samples were layered onto 3 ml of 1.8 M sucrose solution and spun at 116,000 g for 1 h on a SW55Ti rotor using a Beckman Optima L-100 XP Ultracentrifuge (Supplementary Fig S3). After spinning, two separate volumes were taken from above the buoyant membranes (Fraction C; top 0.4 ml, and Fraction P; remaining volume). The cytoplasmic membranes (Fraction M) were added to 0.5 ml of Lysis Buffer P1 (0.1 % (v/v) Triton X-100 and 0.5 % (v/v) protease inhibitor cocktail in PBS buffer). The nuclei were collected, and their purity determined by brightfield microscopic inspection after staining in trypan blue solution or by fluorescent microscopic inspection using a wheat germ agglutinin (alexa 555) plasma membrane stain in combination with DAPI nuclear staining. The nuclei were lysed in 0.8 ml of Lysis Buffer (50 mM sodium phosphate buffer pH 7.4 with 300 mM NaCl, 0.1 % (v/v) Triton X-100 and 0.5 % (v/v) protease inhibitor cocktail) on ice for 20 min, and the supernatant (Fraction N) was collected after centrifugation. Identical proportions of each fraction were taken for SDS-PAGE and immunoblotting with anti-histone or anti- $\alpha$ -actin antibodies, and the remainder was used for affinity purification of the FLAG-(His6)<sub>2</sub>-tagged proteins using TALON beads, followed by anti-FLAG western blotting [18].

### Pull-down using anti-FLAG antibody

Anti-FLAG M2 monoclonal antibody (Stratagene) was coupled to protein G Sepharose beads using dimethyl pimelimidate dihydrochloride, and these were incubated for 2h at 4°C with 400  $\mu$ l of DT40 cell lysate [18]. Beads were pelleted by centrifugation and washed twice in PBS buffer, and bound protein eluted with 3x FLAG peptide buffer (100  $\mu$ g/ml 3x FLAG peptide (Sigma) and 0.5 % (v/v) protease inhibitor cocktail in TBS).

### Lipid kinase assays

PtdIns5P 4-kinase assays were carried out as described previously [20] with slight adaptations. Substrate (300 pmoles PtdIns5P) lipid was dried under vacuum, and micelles made by sonication in kinase buffer (50mM Tris pH7.4, 10mM MgCl<sub>2</sub>, 80mM KCl, 2mM EGTA). Recombinant human PtdIns5P 4-kinases II, cloned and purified as described in

ref [21], were added to the reaction mixture with 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP for 90 min at 30°C. Lipids were extracted and separated by silica-gel thin layer chromatography [20] and detected by autoradiography. Radioactivity was quantified by scintillation counting of labelled PtdIns(4,5) $P_2$ .

### Quantitative PCR

Total mRNA was extracted from approximately  $3 \times 10^7$  DT40 cells from three different cultures of each clone tested (SV Total RNA Isolation System, Promega) and cDNA libraries were constructed from each preparation by RT-PCR (Sprint RT Complete kit; Clontech). Primers for singleplex quantitative PCR were designed using Primer3 [22]. Due to high sequence similarity between *PIP5K2A* and *PIP5K2B*, primer pairs were designed spanning exon 10 and the 3'-untranslated region (*PIP5K2A* forward: 5'-TCAAAGCGCTTCTTGGACTT-3' and reverse: 5'-CTACCCTCGTGGTCACTGCT-3', *PIP5K2B* forward: 5'-GCAGTACTCCAAACGCTTCA-3' and reverse: 5'-GGCAAGTAGCCCTCTTCTC-3'). Primer pairs specific to the tagged genes were generated from exon 10 and the inserted tag sequence (*PIP5K2A-tag* forward: 5'-CGCAGAAATTTCAACCGTTA-5', *PIP5K2B-tag* forward: 5'-AGAGATCTCCACCGTGAACC-3', common reverse: 5'-CTTGTCATCGTCGTCCTTGT-3'). A primer set was also designed to the chicken  $\beta$ -actin housekeeping gene, spanning exons 1 and 2 (forward: 5'-TGGCAATGAGAGGTTTCAGGT-3' and reverse: 5'-CGGATATCCACATCACACTTCA-3'). All primers were obtained from Sigma-Aldrich and produced single PCR products in conventional PCR reactions. PCR amplification was performed over 40 cycles (95°C for 15 sec, 60°C for 30 sec) in a 96-well plate using SYBR Green I master mix (Applied Biosystems, Warrington, UK) and threshold cycle ( $C_T$ ) values were collected using a CFX96 real-time PCR detection system (Bio-Rad). Dissociation curves for each primer set indicated a single product, and no-template controls were negative after 40 cycles. Control reactions using RNA from each extraction as template were negative, showing that the preparations were free from genomic DNA contamination. Primer and template concentrations were optimised to give  $C_T$  values of 20-27. Validation experiments showed equivalent relative amplification efficiencies between products from *PIP5K2* genes and the chicken  $\beta$ -actin reference gene (Supplementary Fig S2). Normalised expression ratios were calculated by the  $2^{-\Delta\Delta C_T}$  method [23].

### Mass Spectrometry

Absolute protein quantification of PtdIns5P 4-kinase II isoforms was achieved by isotope dilution and selective reaction monitoring (SRM). Briefly, proteotypic peptides unique to each isoform were selected as internal standards giving consideration for mass spectra, size and residue composition (see below and Table 1). Internal standards were synthesised (Anaspec) incorporating a single  $^{13}\text{C}_6\ ^{15}\text{N}_4$  Arginine at the C-terminus and each independently quantified by amino acid analysis [24].

For quantifying total protein in unfractionated cells, 7 mgs of lysate digest spiked with 30 pmoles of internal standards was separated by strong cation exchange (0-150 mM  $\text{KCl}_2$ ,



90 minutes, NEST column 200 mm, 2.1 mm internal diameter). Subsequent fractions were each dried, resuspended in 0.1% formic acid and analysed by UPLC-SRM. To determine the ratio of PtdIns5P 4-kinase II $\alpha$  and II $\beta$  isoforms in pull-downs, protein precipitates were digested by incubation overnight with 40 ngs of trypsin, spiked with 100 or 200 fmoles of internal standards, and analysed by nano-UPLC-SRM as appropriate.

Nano-flow (300 nl/min) UPLC was used for separation of pulldown digests and normal-flow (400  $\mu$ l/min) UPLC used for the second dimension of separation for lysate digests. In both cases peptides were eluted from preformed 1.7 mm diameter BEH packed columns (Waters; nanoUPLC [150 mm, 75 mm i.d.] and UPLC [100 mm, 2.1 mm i.d.]) using gradients of linearly increasing acetonitrile concentration from 0 to 45% over 40 minutes (nano-flow) or 10 minutes (normal-flow).

SRM was conducted using a Quattro Premier XE (Waters). For each peptide monitored by SRM, up to four transitions were configured to monitor the four most intense fragment ions (Supplementary Table S1). Transition dwell times were 5 ms for 2D-UPLC-SRM and 50 ms for nanoUPLC-SRM. Signals derived from internal standards and analytes of interest were mean smoothed (window of 2 with 1 iteration) and integrated using QuantLynx (Waters). Ratios of standards and analyte peak integrals were calculated and analyte concentration determined.

## **Results and Discussion**

### **Immunoprecipitation of genomically tagged PtdIns5P 4-kinase II $\beta$**

We planned to use the DT40 cell line JPR3, in which PtdIns5P 4-kinase II $\beta$  is genomically tagged with a FLAG-(His6)<sub>2</sub> tag [18], to identify protein partners that associate with this enzyme. As a proof of principle that we could specifically pull down and detect endogenous PtdIns5P 4-kinase II $\beta$  by mass spectrometry, we conducted a series of pull-downs from 3 litres of cells using anti-FLAG antibody beads, followed by three washes of the beads and elution with 3x-FLAG peptide. We performed this on triplicate samples from wild type (WT) and JPR3 cells in parallel, and mass spectrometric analysis revealed that PtdIns5P 4-kinase II $\beta$  is the major protein pulled down, and that it was detectable only in pull-downs from JPR3 and not from WT cells (data not shown). With one exception (below), the next 20 most abundant proteins identified (mostly contaminants) were present in much lower amounts and were common to WT and JPR3 pull-downs, establishing that this stringent protocol is highly specific for the tagged PtdIns5P 4-kinase II $\beta$ .

However, unexpectedly we also detected PtdIns5P 4-kinase II $\alpha$ , which was identified as the other major protein present, and was again found only in pull-downs from JPR3 cells and not WT cells; the quantitative data below (Table 2) confirm that this co-precipitation of PtdIns5P 4-kinase II $\alpha$  with the II $\beta$  is a highly reproducible. Given that only the II $\beta$  is FLAG-tagged in JPR3 cells, these data unambiguously establish that the two PtdIns5P 4-

kinase II isoforms associate with each other, and the absence of any detectable third protein suggests that their association is direct. As both PtdIns5P 4-kinase II $\beta$  [14] and II $\alpha$  [25] are homodimers in solution and the proposed dimerisation interface is conserved between the two isoforms [14], it is likely that this association reflects a heterodimerisation (see Supplemental Fig S1), but as yet we have no data directly addressing the structure of the interaction.

#### Cellular ratio of PtdIns5P 4-kinase IIs

There are many examples of different isoforms of the same enzymic activity heterodimerising to regulate localisation and function, such as the myotubularins [26]. But these phenomena are usually based on associations of transfected proteins and the actual extent of association of endogenous species, and how this may be regulated, have not been quantified. Our genomic tagging approach gave us a unique opportunity to address these issues, and prompts three questions: (1) Is this PtdIns5P 4-kinase II association random and complete, that is, dictated only by the relative mass levels of the two isoforms? (2) Given that PtdIns5P 4-kinase II $\beta$  is mostly nuclear [18], are there therefore significant levels of endogenous PtdIns5P 4-kinase II $\alpha$  in the nucleus? (3) What is the physiological relevance of these phenomena?

To answer the first question, we needed to know both the mass ratio of the enzymes in the JPR3 DT40 line, and their relative levels in the pull-downs from JPR3 cells. Both PtdIns5P 4-kinase II isoforms are expressed at very low levels in DT40s, and were at the limit of accurate quantification by mass spectrometry, with PtdIns5P 4-kinase II $\alpha$  being the predominant isoform (see below). Additionally we quantified the mRNA levels by QT-PCR of WT and JPR3 DT40 cells, and the data in Figs 1 and S2 show that the ratio of PtdIns5P 4-kinase II $\beta$  to II $\alpha$  mRNA is about 0.23, and is similar in WT and JPR3 cells. Moreover, the data also suggest that the tagged and untagged PtdIns5P 4-kinase II $\beta$  alleles are expressed in approximately equal amounts. If these proportions are reflected in protein, and an association between the isoforms were random, then we can define the cellular PtdIns5P 4-kinase II $\beta$ :II $\alpha$  ratio as  $x$ , and the proportion of PtdIns5P 4-kinase II $\beta$  that is tagged as  $t$ , so the ratio of PtdIns5P 4-kinase II $\beta$  to II $\alpha$  in a pull-down from PtdIns5P 4-kinase II $\beta$ -tagged cells will be  $(2-t)x-1$  (see Supplementary calculations). Thus if we take  $x$  as 0.23 and  $t$  as 0.5, random association would give a predicted PtdIns5P 4-kinase II $\beta$ :II $\alpha$  ratio in the pull-downs from JPR3 cells of 1.36:1.

#### Quantification of PtdIns5P 4-kinase IIs by quadrupole mass spectrometry

Mass spectrometry was applied to quantify PtdIns5P 4-kinase II isoforms at the protein level using an absolute quantification (AQUA) [27] isotope dilution strategy. Given the high sequence identity, suitable proteotypic candidate peptides were limited. Unique candidates were ranked according to their perceived fitness for quantification by considering the quality of empirical data and residue composition. The two best proteotypic peptides for each isoform were selected and AQUA peptides for use as internal standards synthesised (Table 1).



Initially the cellular PtdIns5P 4-kinases II $\alpha$  and II $\beta$  in whole cell lysates were analysed; 7 mgs of lysate digest from  $2 \times 10^{10}$  cells, spiked with 30 pmoles of AQUA peptides, were separated by two dimensional liquid chromatography and peptides were quantified by selective reaction monitoring using a triple quadrupole mass spectrometer. Preliminary experiments indicated which first dimension fractions the AQUA (and analyte) peptides would elute in. Two transitions were monitored for each peptide across the selected first dimension fractions by UPLC-SRM and quantifiable signals with acceptable signal to noise response were integrated (Fig 2). Signals were within the linear response range of the instrument (data not shown) enabling direct calculation of the analyte from the known amount of internal standards. Both diagnostic peptides for PtdIns5P 4-kinase II $\alpha$  were detected and quantified the protein at 1.5 pmoles/mg total protein, but no quantifiable signals were detected for PtdIns5P 4-kinase II $\beta$ .

We then turned to analysis of the anti-FLAG immunoprecipitations from JPR3 cells, and good quantification of both isoforms was achieved. Pre-elution wash steps did not affect the PtdIns5P 4-kinase II $\beta$ :II $\alpha$  ratio observed but provided a better signal to noise response allowing more reliable quantification, and the data from four independent experiments using two different sets of internal AQUA peptides (Table 2) yielded a mean PtdIns5P 4-kinase II $\beta$ :II $\alpha$  ratio in the immunoprecipitates of  $1.39 \pm 0.10$  (S.E.M). This is close enough to the ratio of 1.36 predicted above to suggest strongly that the association between PtdIns5P 4-kinases II $\alpha$  and II $\beta$  is random and complete.

#### Tagging PtdIns5P 4-kinase II $\alpha$

To gain independent data of this association, and to answer directly the second question posed above (the likelihood of some endogenous PtdIns5P 4-kinase II $\alpha$  being nuclear), we knocked the same FLAG-(His6)<sub>2</sub> tag into the 3' end of one allele of the *PIP5K2A* gene by a strategy identical to that which we used previously for the *PIP5K2B* gene [18] – note that the gene nomenclature for PtdIns5P 4-kinases II is *PIP5K2*. This proved more difficult to do than for the *PIP5K2B* gene, and once we had achieved it, expression of tagged protein in several DT40 lines, including the one we studied most (MW2), declined during the first 8 passages (assessed by Western blotting), stabilising at a level per cell about 5 times lower than the tagged PtdIns5P 4-kinase II $\beta$  expression in JPR3 cells (not shown). Moreover, PCR analysis of the MW2 cells revealed unequal transcription of the tagged and untagged alleles (Fig 1), and an increase in overall PtdIns5P 4-kinase II $\alpha$  transcription, presumably to compensate for the low expression of tagged protein. Clearly there are problems with tagging this isoform that do not apply for PtdIns5P 4-kinase II $\beta$  in JPR3 cells, for which expression of tagged protein was always stable, and where no distortion of transcription is evident compared with WT cells (Fig 1). This distortion of both transcription and translation is probably due to a gene-specific effect of the tag, particularly of the puromycin selection cassette in the 3' UTR. Overall these factors limit the quantification that can be achieved, but the MW2 line nevertheless yielded quantitative data to complement data from JPR3 cells.

In two independent immunoprecipitation experiments where quantification was clear the PtdIns5P 4-kinase II $\alpha$ :II $\beta$  ratios were 11:1 and 8.8:1. As discussed above, our analysis by

Western blotting suggested that the level of total tagged PtdIns5P 4-kinase II $\alpha$  protein in MW2 cells was about 0.2 that of II $\beta$  in JPR3 cells. So if we assume that the untagged PtdIns5P 4-kinase II $\alpha$  allele was translated into protein with the same efficiency in MW2 cells as in WT and JPR3 cells, then from Fig 1 we can to a first degree of approximation estimate that  $x = 0.2$  and  $t = 0.05$  in MW2 cells. These parameters lead to a predicted PtdIns5P 4-kinase II $\alpha$ :II $\beta$  ratio in pull-downs from MW2 cells of 10.3:1, which is consistent with the mean of 9.9:1 from the above experiments.

### Nuclear PtdIns5P 4-kinase II $\alpha$

Immunocytochemistry in these tagged DT40 cell lines is not possible with such low expression levels [18], and so we previously used cell fractionation to show that most of the genomically tagged PtdIns5P 4-kinase II $\beta$  is nuclear [18]. Here we quantified the cytoplasm-to-nuclear ratio of the PtdIns5P 4-kinases II by another fractionation protocol (Fig S3 and Methods) that is more quantitative than that used previously [18] in that we quantified all fractions to give a complete balance sheet of the distribution of the enzymes and markers. Fig 3A gives representative images of nuclei obtained from this protocol (see Methods section). The data (Fig 3B) confirm our earlier observation [18] that >95% of PtdIns5P 4-kinase II $\beta$  is nuclear. Note that the fractionation protocol has caused significant leakage of histones (as exemplified by H1) from the nuclei (Fig 3 B-D), indicating some damage to the nuclear membrane. The retention of most of the PtdIns5P 4-kinase II $\beta$  in the nucleus (Fig 3B) in contrast to this histone leakage suggests that the enzyme is not freely diffusible within the nucleus but is interacting with nuclear structures.

Examination of the nuclear fraction with DAPI staining revealed <1% nuclei with any detectable cytoplasmic debris attached (Fig 3A). We also probed for remaining plasma membrane using WGA, and again found <1% of nuclei had any detectable signal (Fig 3A). Additionally we quantified cytoplasmic contamination of the nuclei by blotting for actin, and found it to be low (Fig 3D) – indeed, the levels of actin (10% of total) that are detected may be mostly accounted for by *bona fide* intranuclear actin [28] and/or F-actin closely associated with the nucleus [29]. This protocol is therefore apparently effective enough to remove >90% of the cytoplasm from the nuclei, yet fractionation of MW2 cells showed that 40% of the tagged PtdIns5P 4-kinase II $\alpha$  is nuclear, and 60% cytoplasmic (Fig 3C,D). This suggests that in contrast to transfected PtdIns5P 4-kinase II $\alpha$  [13,18], the endogenous enzyme is significantly nuclear. It also is important to emphasise another difference – we found that endogenous cytoplasmic PtdIns5P 4-kinase II $\alpha$  is in the membranous fraction, not in the cytosol (Fig 3C,D). We are not sure to which membrane PtdIns5P 4-kinase II $\alpha$  is attached, but from our current knowledge we suggest that it is most likely to be the plasma membrane, to which it could be targeted by its interaction with Type I PtdIns4P 5-kinases [30].

### Lipid kinase activity of PtdIns5P 4-kinase II $\alpha$ and II $\beta$

The simplest interpretation of our data overall is that the PtdIns5P 4-kinases II $\alpha$  and II $\beta$  associate randomly, and one consequence may be that PtdIns5P 4-kinase II $\beta$  therefore

targets the II $\alpha$  to the nucleus. The third question posed above then remains: What is the physiological significance of these events? One possibility is that the two isoforms could be differentially regulated within the nucleus – for example, we have shown that PtdIns5P 4-kinase II $\alpha$  is phosphorylated by casein kinase II on a residue that is unique to that isoform [31, 32]. However, another (not mutually exclusive) possibility is that the two isoforms have very different enzymic activities. This idea is made more likely by our previous demonstration of an association between PtdIns5P 4-kinases II $\alpha$  and II $\gamma$  – the latter had negligible activity when we assayed it in comparison with the former, and II $\gamma$  may target II $\alpha$  to the vesicular compartment in which it (II $\gamma$ ) is localised [21, 33]. To address the issue directly we assayed the PtdIns5P 4-kinase activity of recombinant human PtdIns5P 4-kinases II $\alpha$  and II $\beta$  in parallel (see Supplementary Fig S4 and Supplementary Table S2). The specific activity in  $\mu\text{moles/min/mg}$  protein of PtdIns5P 4-kinase II $\alpha$  is  $3.25 \times 10^{-2}$  (S.E.M.  $\pm 1.9 \times 10^{-4}$ ,  $n = 4$ ), and that of II $\beta$  is  $1.57 \times 10^{-5}$  (S.E.M.  $\pm 7.9 \times 10^{-7}$ ,  $n = 4$ ). Thus, strikingly, PtdIns5P 4-kinase II $\alpha$  is 2000 times more active than the II $\beta$  isoform, which means that in a PtdIns5P 4-kinase II $\alpha$ /II $\beta$  heterodimer the activity of the PtdIns5P 4-kinase II $\alpha$  is going to be by far the most significant.

### Conclusion

We should note that our suggestion that PtdIns5P 4-kinase II $\beta$  may target II $\alpha$  to the nucleus could throw a new light on experiments where PtdIns5P 4-kinase II $\beta$  has been increased by transfection [34] or decreased in a knockout mouse [35], both of which cause alterations in (cytoplasmic) insulin sensitivity, an effect primarily evident in muscle and liver. One consequence of manipulating PtdIns5P 4-kinase II $\beta$  might be to alter significantly the amount of the II $\alpha$  isoform present in the cytoplasm, and this would be especially evident in muscle and liver – of the tissues we analysed these have the highest PtdIns5P 4-kinase II $\beta$ /II $\alpha$  ratios [21].

More importantly, the apparently complete and random association (probably heterodimerisation) of endogenous proteins that we have revealed and then quantitatively examined here, plus the much greater enzymic activity of PtdIns5P 4-kinase II $\alpha$  versus II $\beta$ , make it reasonable to pose the question: is a (or even the) major function of PtdIns5P 4-kinase II $\beta$  simply to act as a II $\alpha$  nuclear-targeting protein? This may not be the whole story – for example the requirement for PtdIns5P 4-kinase II $\beta$  to be catalytically active to fulfil at least some of its nuclear functions is suggested by a striking dominant-negative effect of a kinase-dead PtdIns5P 4-kinase II $\beta$  construct on Cul3 SPOP ubiquitin ligase [12]. As previously discussed, there are precedents for inactive (or nearly so) isoforms of proteins targeting active isoforms to cellular locations, an example taken from phosphoinositide metabolism being the myotubularins [26]. Much of the evidence for this idea is based on association of transfected proteins, and the degree, regulation and extent of association and targeting *in vivo* is not always clear. Here we have combined the powers of DT40 genomic tagging with mass spectrometry to place this type of functional relationship, at least for PtdIns5P 4-kinases II $\alpha$  and II $\beta$ , on a new level of quantitative clarity.

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## References

- 1 Irvine, R. F. (2003) Nuclear lipid signalling. *Nat. Rev. Mol. Cell Biol.* **4**, 349-360.
- 2 Cocco, L., Faenza, I., Fiume, R., Maria Billi, A., Gilmour, R. S. and Manzoli, F. A. (2006) Phosphoinositide-specific phospholipase C (PI-PLC) beta1 and nuclear lipid-dependent signaling. *Biochim. Biophys. Acta* **1761**, 5095-5021
- 3 York, J. D. (2006) Regulation of nuclear processes by inositol polyphosphates. *Biochim. Biophys. Acta* **1761**, 552-559
- 4 Bunce, M. W., Bergendahl, K. and Anderson, R. A. (2006) Nuclear PI(4,5)P<sub>2</sub>: a new place for an old signal. *Biochim. Biophys. Acta* **1761**, 560-569
- 5 Rameh, L. E., Tolias, K. F., Duckworth, B. C. and Cantley, L. C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. *Nature* **390**, 192-196
- 6 Ungewickell, A., Hugge, C., Kisseleva, M., Chang, S. C., Zou, J., Feng, Y., Galyov, E. E., Wilson, M. and Majerus, P. W. (2005) The identification and characterization of two phosphatidylinositol-4,5-bisphosphate 4-phosphatases. *Proc. Natl. Acad. Sci. U.S.A* **102**, 18854-18859
- 7 Zou, J., Marjanovic, J., Kisseleva, M. V., Wilson, M. and Majerus, P. W. (2007) Type I phosphatidylinositol-4,5-bisphosphate 4-phosphatase regulates stress-induced apoptosis. *Proc. Natl. Acad. Sci. USA* **104**, 16834-16839
- 8 Lecompte, O., Poch, O. and Laporte, J. (2008) PtdIns5P regulation through evolution: roles in membrane trafficking? *Trends Biochem. Sci.* **33**, 453-460
- 9 Clarke, J. H., Letcher, A. J., S., D. C., Halstead, J. R., Irvine, R. F. and Divecha, N. (2001) Inositol lipids are regulated during cell cycle progression in the nuclei of murine erythroleukaemia cells. *Biochem. J.* **357**, 905-910.
- 10 Gozani, O., Karuman, P., Jones, D. R., Ivanov, D., Cha, J., Lugovskoy, A. A., Baird, C. L., Zhu, H., Field, S. J., Lessnick, S. L., Villasenor, J., Mehrotra, B., Chen, J., Rao, V. R., Brugge, J. S., Ferguson, C. G., Payastre, B., Myszka, D. G., Cantley, L. C., Wagner, G., Divecha, N., Prestwich, G. D. and Yuan, J. (2003) The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. *Cell* **114**, 99-111.
- 11 Jones, D. R., Bultsma, Y., Keune, W. J., Halstead, J. R., Elouarrat, D., Mohammed, S., Heck, A. J., D'Santos, C. S. and Divecha, N. (2006) Nuclear PtdIns5P as a Transducer of Stress Signaling: An In Vivo Role for PIP4Kbeta. *Mol. Cell* **23**, 685-695
- 12 Bunce, M. W., Boronenkov, I. V. and Anderson, R. A. (2008) Coordinated Activation of the Nuclear Ubiquitin Ligase Cul3-SPOP by the Generation of Phosphatidylinositol 5-Phosphate. *J. Biol. Chem.* **283**, 8678-8686



- 13 Ciruela, A., Hinchliffe, K. A., Divecha, N. and Irvine, R. F. (2000) Nuclear targeting of the  $\beta$  isoform of Type II phosphatidylinositol phosphate kinase (phosphatidylinositol 5-phosphate 4-kinase) by its  $\alpha$ -helix 7. *Biochem. J.* **364**, 587-591
- 14 Rao, V. D., Misra, S., Boronenkov, I. V., Anderson, R. A. and Hurley, J. H. (1998) Structure of type IIbeta phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. *Cell* **94**, 829-839
- 15 Clarke, J. H., Richardson, J. P., Hinchliffe, K. A. and Irvine, R. F. (2007) Type II PIP kinases: location, regulation and function. *Biochem.Soc. Symp.* **74**, 149-159
- 16 Divecha, N., Rhee, S. G., Letcher, A. J. and Irvine, R. F. (1993) Phosphoinositide signalling enzymes in rat liver nuclei: phosphoinositidase C isoform beta 1 is specifically, but not predominantly, located in the nucleus. *Biochem J* **289**, 617-620
- 17 Boronenkov, I. V., Loijens, J. C., Umeda, M. and Anderson, R. A. (1998) Phosphoinositide signaling pathways in nuclei are associated with nuclear speckles containing pre-mRNA processing factors. *Mol. Biol. Cell* **9**, 3547-3560
- 18 Richardson, J. P., Wang, M., Clarke, J. H., Patel, K. J. and Irvine, R. F. (2007) Genomic tagging of endogenous type IIbeta phosphatidylinositol 5-phosphate 4-kinase in DT40 cells reveals a nuclear localisation. *Cell Signal.* **19**, 1309-1314
- 19 Mosedale, G., Niedzwiedz, W., Alpi, A., Perrina, F., Pereira-Leal, J. B., Johnson, M., Langevin, F., Pace, P. and Patel, K. J. (2005) The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. *Nat. Struct. Mol. Biol.* **12**, 763-771
- 20 Hinchliffe, K. A., Irvine, R. F. and Divecha, N. (1998) Regulation of PtdIns4P 5-kinase C by thrombin-stimulated changes in its phosphorylation state in human platelets. *Biochem J* **329**, 115-119
- 21 Clarke, J. H., Emson, P. C. and Irvine, R. F. (2008) Localization of phosphatidylinositol phosphate kinase IIgamma in kidney to a membrane trafficking compartment within specialized cells of the nephron. *Am. J. Physiol. Renal Physiol.* **295**, F1422-1430
- 22 Rozen, S. and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132**, 365-386
- 23 Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408
- 24 Spackman, D. H., Stein, W. H. and Moore, S. (1958) Automatic recording apparatus for use in the chromatography of amino acids. *Analyt. Chem.* **30**, 1190-1206
- 25 Hinchliffe, K., Ciruela, A., Morris, J. A., Divecha, N. and Irvine, R. F. (1999) The Type II PIPkins (PtdIns5P 4-kinases): enzymes in search of a function? *Biochem. Soc. Trans* **27**, 657-661
- 26 Lorenzo, O., Urbe, S. and Clague, M. J. (2006) Systematic analysis of myotubularins: heteromeric interactions, subcellular localisation and endosome related functions. *J. Cell Sci.* **119**, 2953-2959
- 27 Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W. and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6940-6945
- 28 Rando, O. J., Zhao, K. and Crabtree, G. R. (2000) Searching for a function for nuclear actin. *Trends Cell. Biol.* **10**, 92-97

- 29 Khatau, S. B., Hale, C. M., Stewart-Hutchinson, P. J., Patel, M. S., Stewart, C. L., Searson, P. C., Hodzic, D. and Wirtz, D. (2009) A perinuclear actin cap regulates nuclear shape. *Proc. Natl. Acad. Sci. U S A* **106**, 19017-19022
- 30 Hinchliffe, K. A., Giudici, M. L., Letcher, A. J. and Irvine, R. F. (2002) Type II $\alpha$  phosphatidylinositol phosphate kinase associates with the plasma membrane via interaction with type I isoforms. *Biochem. J.* **363**, 563-570.
- 31 Hinchliffe, K. A., Ciruela, A., Letcher, A. J., Divecha, N. and Irvine, R. F. (1999) Regulation of type II $\alpha$  phosphatidylinositol phosphate kinase localisation by the protein kinase CK2. *Curr. Biol.* **9**, 983-986
- 32 Hinchliffe, K. A. and Irvine, R. F. (2003) CK2 phosphorylation of Type II $\alpha$  PIPkin. *Curr. Biol.* **13**, 178
- 33 Clarke, J. H., Wang, M. and Irvine, R. F. (2010) Phosphatidylinositol 5-phosphate 4-kinases: Localization, regulation and function of Type II phosphatidylinositol 5-phosphate 4-kinases. *Adv. Enz. Reg.* **50**, 12-18
- 34 Carricaburu, V., Lamia, K. A., Lo, E., Favereaux, L., Payrastra, B., Cantley, L. C. and Rameh, L. E. (2003) The phosphatidylinositol (PI)-5-phosphate 4-kinase type II enzyme controls insulin signaling by regulating PI-3,4,5-trisphosphate degradation. *Proc. Natl. Acad. Sci. U S A* **100**, 9867-9872
- 35 Lamia, K. A., Peroni, O. D., Kim, Y. B., Rameh, L. E., Kahn, B. B. and Cantley, L. C. (2004) Increased insulin sensitivity and reduced adiposity in phosphatidylinositol 5-phosphate 4-kinase beta $^{-/-}$  mice. *Mol. Cell. Biol.* **24**, 5080-5087
- 36 Bultsma, Y., Keune, J. K. and N., D. (2010) PIP4K $\beta$  interacts with and modulates nuclear localisation of the high activity PtdIns5P-4-kinase isoform, PIP4K $\alpha$ . (Submitted)



## Tables

Table 1 Proteotypic peptides selected for mass spectrometry internal standards

<b>Protein</b>	<b>AQUA Peptide</b>	
PtdIns5P 4-kinase II $\alpha$	SAPLANDSQAR*	[1128.6/1138.6*] (565/570*)
PtdIns5P 4-kinase II $\alpha$	FGIDDQDFQNSLTR*	[1654.8/1664.8*] (828/833*)
PtdIns5P 4-kinase II $\beta$	SAPVNSDSQGR*	[1116.5/1126.5*] (559/564*)
PtdIns5P 4-kinase II $\beta$	FGIDDQDYQNSVTR*	[1656.7/1666.7*] (829/834*)

Two peptides were selected that were unique to each PtdIns5P 4-kinase II isoform. \* denotes stable isotope charge; [] denotes the molecular weight (Da) of unlabelled and labelled peptides respectively; and () denotes the mass to charge ratio of the doubly charged precursor ions (Th).

Table 2 PtdIns5P 4-kinase II isoform ratios determined using two separate sets of internal peptides

	<u>Ratio II<math>\beta</math>:II<math>\alpha</math></u>	
	559/565	829/828
JPR3 Exp 1	1.39	0.92
JPR3 Exp 2	ND	1.41
JPR3 Exp 3	1.37	ND
JPR3 Exp 4	1.75	1.47

Ratios of PtdIns5P 4-kinase II $\beta$ :II $\alpha$  in anti-FLAG pull-downs from JPR3 cells measured by the relevant peptide sets (559/565 and 829/828) and quantified by internal labelled standards (see Table 1).

## Figure legends

### Figure 1 Differential expression of PtdIns5P 4-kinase II isoforms from endogenously tagged alleles

Expression from tagged and untagged *PIP5K2A* and *PIP5K2B* alleles determined by QT-PCR using the comparative threshold cycle ( $C_T$ ) method. Normalised expression is presented as relative fold increase above untagged *PIP5K2B* in WT cells ( $n = 9$ ). PCR was performed on cDNA made from RNA extracts of WT, JPR3 and MW2 cells as described in the Methods section.

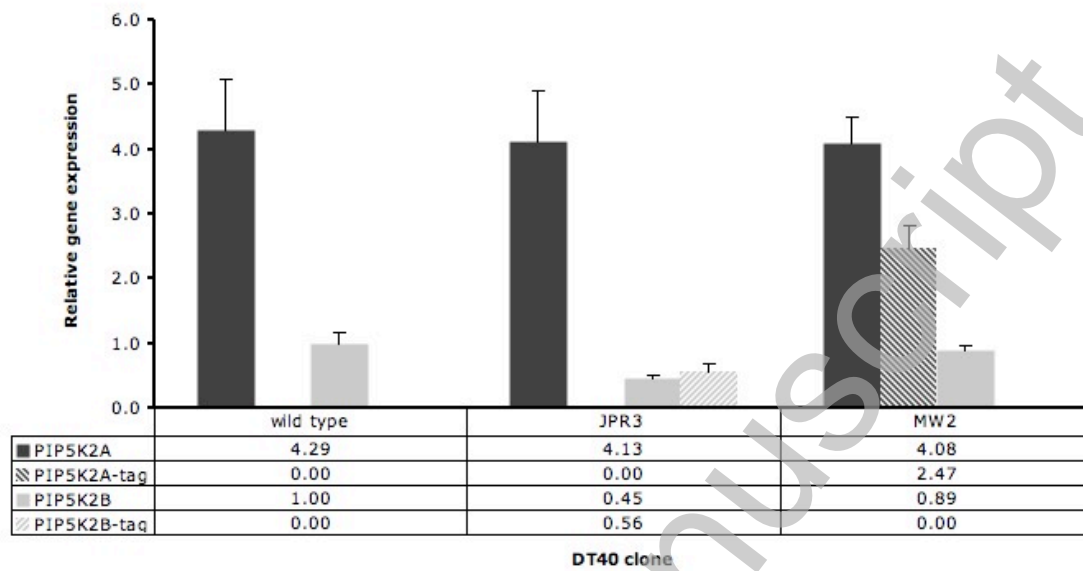
### Figure 2 Detection of AQUA and analyte peptides by mass spectrometry

Integrated signals derived from two selected transitions (see Supplementary Table 1) for internal AQUA standard peptide (A: 570.38 > 770.87, top panel; 570.38 > 699.89, lower panel) and corresponding analyte peptide (B: 565.38 > 760.87, top panel; 565.38 > 689.99, lower panel) for quantification of PtdIns5P 4-kinase II $\alpha$  in a whole cell lysate by selective reaction monitoring.

### Figure 3 Fractionation and Western blotting of DT40 cell lysates

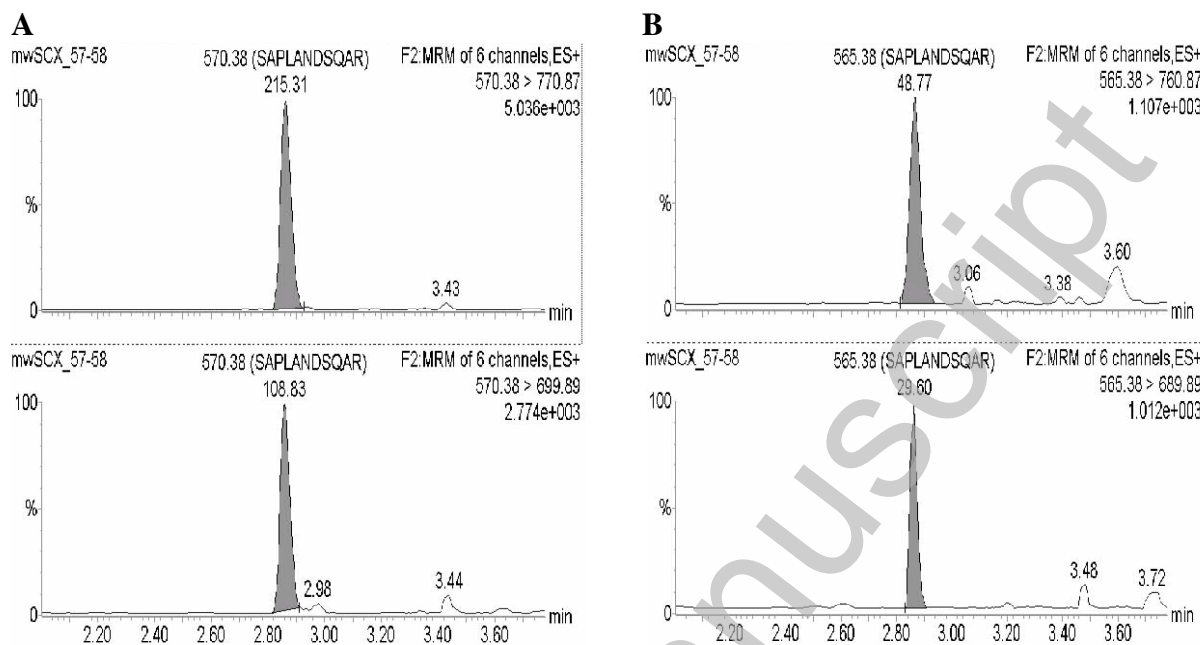
(A) Purity of isolated nuclei were assessed by adhering whole cells or nuclei (prepared as in the Methods section) to coverslips using cell-tak and decorating with a combination of wheat germ agglutinin plasma membrane stain (Alexa 555-conjugated) and DAPI nuclear stain. Scale bar = 5  $\mu$ m. (B) Representative Western blot for fractionation of JPR3 or (C) MW2 cell lysates. The same proportion of each cell lysate fraction (see Supplementary Fig S3) was either Western blotted for actin or Histone H1, or used for immunoprecipitation and detection of FLAG-tagged protein as described in the Materials and Methods section. (D) Quantification of PtdIns5P 4-kinase II $\alpha$ , actin and Histone H1 in blots of MW2 cell lysate fractionations (cytosolic fractions C and P were combined) with mean and S.E.M. ( $n = 4$  for PtdIns5P 4-kinase II $\alpha$ ,  $n = 3$  for actin and Histone H1). C+P; cytosol, M; cytoplasmic membranes, S; sucrose cushion, N; extracted nuclei.

Figure 1



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**Figure 2**

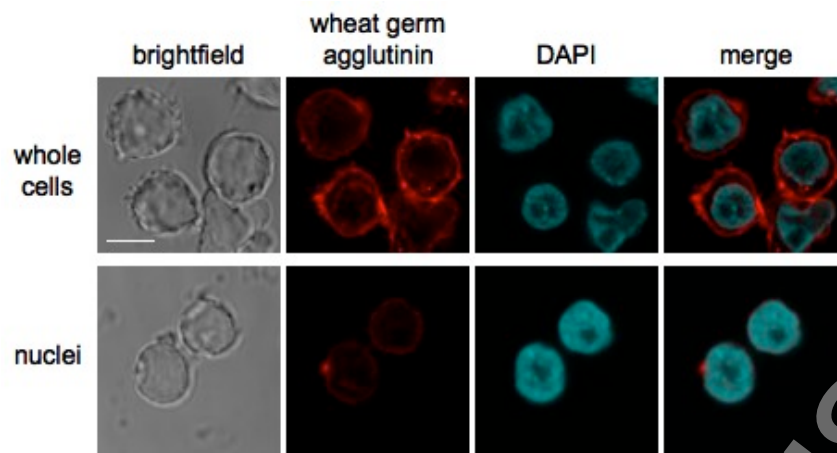


THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20100340

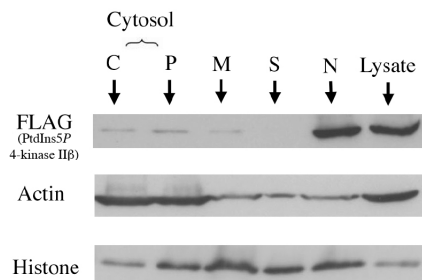
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Figure 3

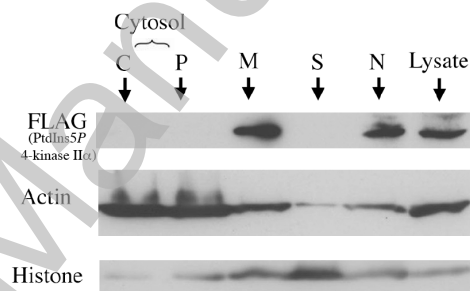
A



B



C



D

