

In B cells, phosphatidylinositol 5-phosphate 4-kinase- α synthesizes PI(4,5)P₂ to impact mTORC2 and Akt signaling

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Phosphatidylinositol 5-phosphate 4-kinases (PI5P4Ks) are enigmatic lipid kinases with physiological functions that are incompletely understood, not the least because genetic deletion and cell transfection have led to contradictory data. Here, we used the genetic tractability of DT40 cells to create cell lines in which endogenous PI5P4K α was removed, either stably by genetic deletion or transiently (within 1 h) by tagging the endogenous protein genomically with the auxin degron. In both cases, removal impacted Akt phosphorylation, and by leaving one PI5P4K α allele present but mutating it to be kinase-dead or have PI4P 5-kinase activity, we show that all of the effects on Akt phosphorylation were dependent on the ability of PI5P4K α to synthesize phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P₂] rather than to remove PI5P. Although stable removal of PI5P4K α resulted in a pronounced decrease in Akt phosphorylation at Thr308 and Ser473, in part because of reduced plasma membrane PIP₃, its acute removal led to an increase in Akt phosphorylation only at Ser473. This process invokes activation primarily of mammalian target of rapamycin complex 2 (mTORC2), which was confirmed by increased phosphorylation of other mTORC2 substrates. These findings establish PI5P4K α as a kinase that synthesizes a physiologically relevant pool of PI(4,5)P₂ and as a regulator of mTORC2, and show a phenomenon similar to the “butterfly effect” described for phosphatidylinositol 3-kinase α [Hart JR, et al. (2015) *Proc Natl Acad Sci USA* 112(4):1131–1136], whereby through apparently the same underlying mechanism, the removal of a protein’s activity from a cell can have widely divergent effects depending on the time course of that removal.

phosphatidylinositol 5-phosphate 4-kinase | phosphatidylinositol 5-phosphate | phosphatidylinositol (4,5)-bisphosphate | Akt | mTOR

The phosphatidylinositol 5-phosphate 4-kinases (PI5P4Ks) are an enigmatic family of three (PI5P4K α , β , and γ) with cellular functions that remain poorly understood (1–3). In general, their principal activity is believed to be to remove and thus, regulate the levels of their substrate phosphatidylinositol 5-phosphate (PI5P). Phenotypes from knockout mice have highlighted that PI5P4Ks have important roles to play in physiology and pathology, including links between PI5P4Ks and the Akt signaling pathway, and other studies have pointed to roles in the generation of cancer (3). A number of key questions, however, remain unanswered. Knocking proteins out or down (by RNAi) is a long-term strategy that may lead to indirect changes, such as, for example, those highlighted in the recent study by Hart et al. (4) concerning the indirect long-term consequences of a point mutation in phosphatidylinositol 3-kinase- α (PI3K α). Another issue still unresolved for PI5P4Ks is whether removal of PI5P is their only function or whether their ability to synthesize phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P₂] may also be important (5, 6).

We have recently used the high homologous recombination frequency of DT40 cells to tag endogenous PI5P4Ks and thus, bypass the interpretational problems associated with cell transfection, and

this has led to the demonstration of heterodimerization of α and β and a nuclear localization of endogenous PI5P4K β (7–9). DT40s, being of avian origin, do not have a PI5P4K γ gene, which facilitates the study of the functions and interrelationship of the α - and β -isoforms (8). Here, we have more fully exploited the genetic power of DT40s to gain unprecedented insight into the physiological functions of PI5P4K α .

Results

Long-Term Loss of PI5P4K α by Gene Disruption Results in Compromised Akt Phosphorylation. Karyotype analysis of the DT40 cells with which we were working revealed trisomy on 2 (*SI Appendix, Fig. S1*), the chromosome on which *PIP4K2A* resides. For three main reasons, we chose to delete a 2.8-kb segment of the gene encompassing exons 8 and 9. First, with this short deletion, we hoped to avoid off-target effects, like those we saw in *PIP4K2B*^{−/−} cells (*SI Appendix, SI Results and Discussion* and *Figs. S2 and S3*); second, these exons encode large portions of the PI5P and ATP binding sites of the kinase, and therefore, any N-terminal fragment is unlikely to possess any function relying on ATP or PI5P binding.

Significance

Investigating enzyme function by genetic knockout is often complicated by indirect and compensatory changes, whereas supraphysiological levels of protein can compromise overexpression. These pitfalls have made it difficult to understand the functions of the enigmatic phosphatidylinositol 5-phosphate 4-kinases (PI5P4Ks); we are not even sure what lipid phosphorylation they catalyze in vivo. Here, we have used the unique genetic power of DT40 cells to genomically delete PI5P4K α or remove the endogenous protein acutely (within 60 min). We used similar approaches to manipulate the endogenous catalytic activity of the enzyme. From this approach, we have gained unique and unexpected insights into the physiological role of PI5P4K α and the ways in which it interacts with the Akt signaling pathway.

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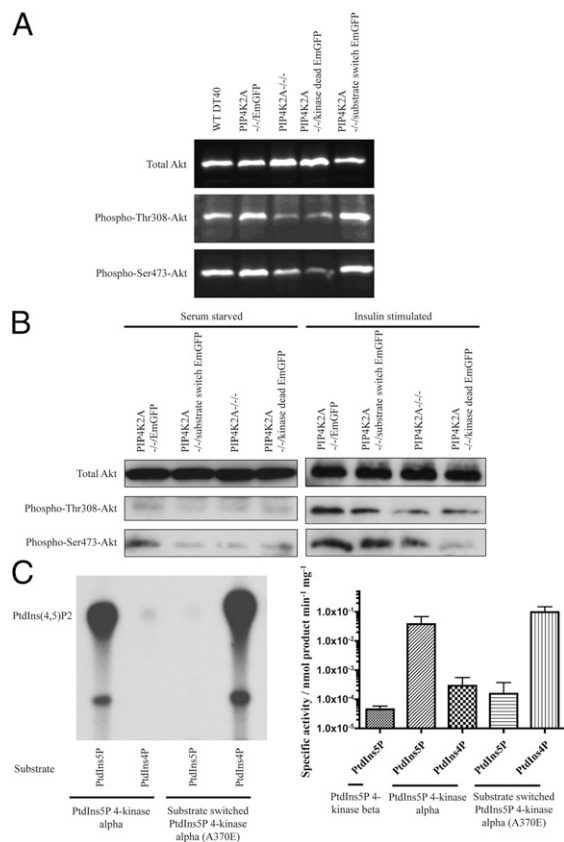


Fig. 1. The effect of endogenous *PIP4K2A* mutations on Akt phosphorylation at Thr308 and Ser473. (A) Cells were synchronized at the same density in exponential growth before analysis. Complete *PIP4K2A* deletion results in reduced phospho-Akt at both Thr308 and Ser473. Deletion of two of three *PIP4K2A* alleles, while leaving the third unaffected aside from tagging with EmGFP, has no effect on Akt phosphorylation. This process acts as a control for the fourth and fifth lanes, where the undeleted allele is either rendered kinase-dead or mutated to encode a PI4P5K (*SI Appendix, SI Materials and Methods*) along with EmGFP tagging in both cases. A kinase-dead third allele does not support normal Akt phosphorylation in the way that a kinase-live one does, and it makes no difference to Akt phosphorylation whether the PI(4,5)₂ here is generated from PI5P or PI4P. Representative blot from three biological replicates. Note that these blots were captured electronically by a program that generates a negative image. To avoid any unnecessary image manipulation, the original negative image is presented. (B) The cell lines shown in A were serum starved and insulin stimulated (*SI Appendix, SI Materials and Methods*). The pattern of deficient Akt phosphorylation was found to be the same as when cells were in exponential growth. (C) Substrate specificity of wild-type PI5P4K α and the A370E (substrate switch) mutant. Recombinant enzymes were assayed as described in *SI Appendix, SI Materials and Methods* using either PI5P or PI4P substrates. Note the logarithmic y axis. Wild-type PI5P4K α has two orders of magnitude less activity toward PI4P than toward PI5P, whereas the A370E mutant prefers PI4P by almost three orders of magnitude. The activity of chicken PI5P4K β is included for comparison. The activity of chicken PI5P4K α toward PI5P is about three orders of magnitude greater than that of chicken PI5P4K β , very similar to the human enzymes (3). Quantification is of four technical replicates.

Third, in cells with two of three alleles deleted, we could mutate the catalytic activity of the third. PIP4K2A^{-/-} cells have a similar growth rate to controls (*SI Appendix, Fig. S1*) and unchanged PI5P4K β message (*SI Appendix, Fig. S4*). Analysis of the phenotype of PIP4K2A^{-/-} cells revealed a significant decrease in Akt phosphorylation at both Thr308 and Ser473 (human numbering) both in synchronized exponential growth and on insulin stimulation (Fig. 1 *A* and *B*). Given this consistency between assays, we performed additional experiments using the former protocol only. As an independent test of the phenotype of PIP4K2A^{-/-} cells,

we knocked down PI5P4K α by stable shRNA and found the same effect (*SI Appendix, Fig. S5*).

To see the Akt phenotype, all three alleles of *PIP4K2A* had to be deleted. If we left one allele intact, the Akt phosphorylation levels were indistinguishable from those in wild-type cells. This process gave us the chance to explore the mechanism behind this phenotype. The remaining wild-type allele was mutated to encode a kinase-dead enzyme (D272K) (10). The allele was simultaneously tagged genomically at the C terminus with EmGFP, and as a control, we knocked in an EmGFP tag alone to the third allele. The data show that, whereas a remaining kinase-live third allele can support normal Akt phosphorylation, one that is kinase-dead cannot (Fig. 1). Note that these experiments are very tightly controlled, because *PIP4K2A*^{-/-}/kinase-dead EmGFP cells and their controls (*PIP4K2A*^{-/-}/EmGFP) differ by only one amino acid.

To explore whether the function of PI5P4K α here is to remove PI5P, generate PI(4,5)P₂, or both, we used the finding in the work by Kunz et al. (11) that a single mutation (A381E) in the activation loop of human PI5P4K β changes its specificity from a PI5P4K

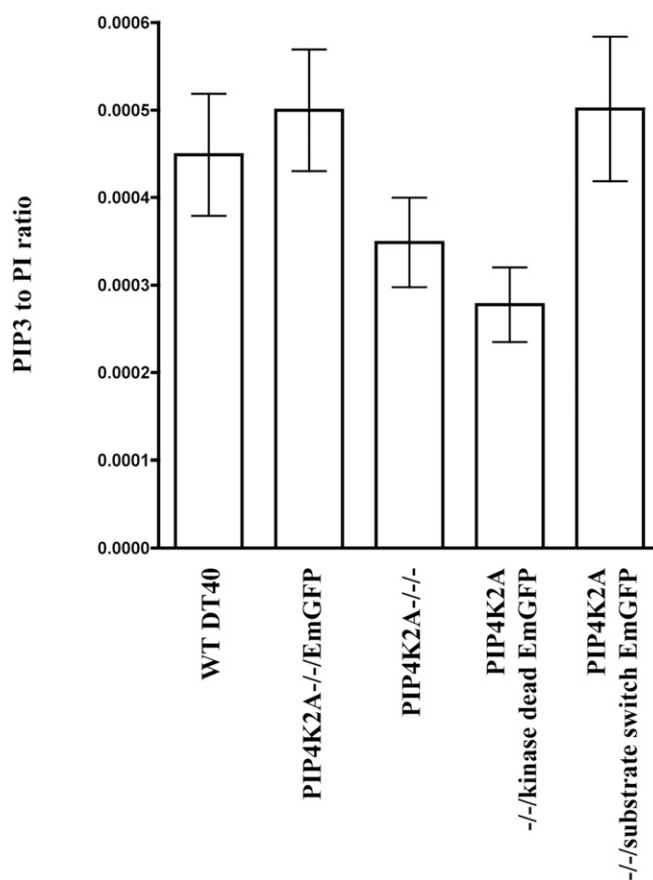


Fig. 2. The effect of endogenous *PIP4K2A* mutations on PIP_3 mass levels. Cells were synchronized at the same density in exponential growth before analysis by MS. PIP_3 levels are expressed relative to phosphatidylinositol (PI) to correct for cell number. PIP_3 and PI internal standards are present in the assay. Data were combined for 38:4, 38:3, and 36:2 PIP_3 species. The chart displays means and 95% confidence intervals. Bonferroni's multiple comparison tests are as follows: WT DT40 vs. *PIP4K2A*^{-/-}; $P = 0.065$; *PIP4K2A*^{-/-}EmGFP vs. *PIP4K2A*^{-/-}kinase dead EmGFP; $P < 0.001$; and *PIP4K2A*^{-/-}EmGFP vs. *PIP4K2A*^{-/-}substrate switch EmGFP; $P = 0.97$. Pooled data are from three replicates. The difference in PIP_3 levels between WT and complete *PIP4K2A* knockout cells does not reach statistical significance, despite the central estimate of PIP_3 levels in the latter cell lines being 25% lower than that in the former. This result may be because of an inadequate number of replicates, and therefore, we also pooled data for the cell lines exhibiting normal Akt phosphorylation and compared these with pooled data for the cell lines exhibiting abnormal Akt phosphorylation (Sf Appendix, Fig. S6).

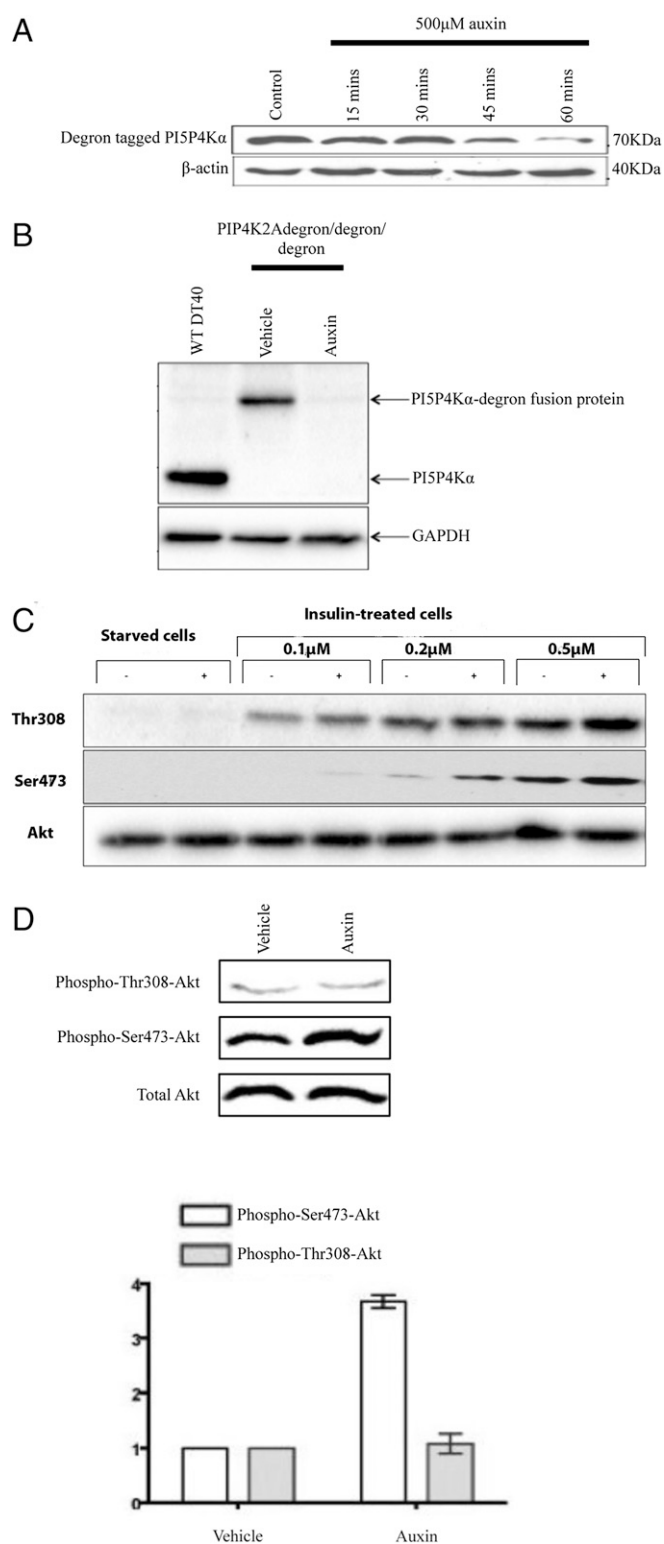


Fig. 3. Acute removal of PI5P4K α with the auxin degon system. (A) Cells (PIP4K2A^{degron/degron/degron}) were treated with auxin as indicated, and any remaining PI5P4K α degon fusion protein was immunoprecipitated against its FLAG tag and blotted against its poly-His tag. (B) Whole-cell lysates blotted with an anti-PI5P4K α antibody. (C) PIP4K2A^{degron/degron/degron} cells were serum starved with or without 500 μ M auxin for 60 min to remove PI5P4K. Insulin was added at the concentrations shown; after 10 min, the cells were lysed, and lysates were blotted sequentially (stripping in-between) for Akt phosphorylation at Thr308 and Ser473. (D) PIP4K2A^{degron/degron/degron} cells were synchronized in exponential growth and treated with either 500 μ M

to a phosphatidylinositol 4-phosphate 5-kinase (PI4P5K). If PI5P removal is the main function of PI5P4K α in this context, such a mutation introduced into the nondeleted PIP4K2A allele should see the Akt phenotype emerge; however, if PI(4,5)P₂ synthesis is its function, because there is plenty of phosphatidylinositol 4-phosphate (PI4P) present in most cellular membranes (1), the mutant should support normal Akt phosphorylation. We tested directly using recombinant enzymes that the substrate specificity switch of human PI5P4K β (11) is valid in the chicken PI5P4K α . The data (Fig. 1C) show that a marked (>98%) substrate specificity switch occurs (note the logarithmic y axis). Fig. 1 shows that, if the third PIP4K2A allele in PIP4K2A^{-/-wt} cells is mutated (A370E) to encode a PI4P5K, normal Akt phosphorylation is maintained. Our interpretation of these experiments is that PI5P4K α synthesizes a pool(s) of PI(4,5)P₂ required for sustained phosphorylation of Akt.

By generating PIP4K2B null cells, we excluded a similar role for PI5P4K β in the regulation of Akt phosphorylation. However, complete deletion of PIP4K2B did result in indirect down-regulation of PI5P4K α (SI Appendix, Fig. S2). The consequent Akt phenotype was rescued only by overexpression of PI5P4K α (not of PI5P4K β) and then, only if the PI5P4K α was kinase-live, irrespective of whether it was mutated to a PI4P5K (SI Appendix, SI Results and Discussion and Figs. S2 and S3).

PI5P4K α Is Necessary for Maintenance of Normal Plasma Membrane PIP₃ Levels in DT40 Cells. Given that PI5P4K α is a lipid kinase, the simplest explanation of decreased Akt phosphorylation is that phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) synthesis may be compromised (see the introductory paragraphs). We, therefore, quantified PIP₃ levels by MS (12) in the cell lines shown in Fig. 1. A decrease in the mass of PIP₃ was evident that followed the pattern of loss of Akt phosphorylation (Fig. 2 and SI Appendix, Fig. S6), suggesting that at least part of this Akt phenotype is because of decreased PIP₃ synthesis. We confirmed these findings by overexpression of the fluorescent 3-phosphoinositide reporter EmGFP-PH-Akt (13), which was recruited less to the plasma membrane in cells with deficient Akt phosphorylation (SI Appendix, Fig. S3C).

In light of these findings, we wondered if the PI5P4K α -generated PI(4,5)P₂ was acting as a substrate for class 1 PI3Ks to synthesize PIP₃. Quantification of total PIP₂ levels [which will be mostly PI(4,5)P₂] in PIP4K2A^{-/-} cells showed no change from the WT (SI Appendix, Fig. S6B), although this is not surprising given that the PI(4,5)P₂ required for PIP₃ synthesis is a very small fraction of total cellular PI(4,5)P₂. However, in cells where all of the endogenous PI5P4K α can be removed within 1 h by the addition of auxin (below), no change in PIP₃ levels on acute removal of PI5P4K α was detected, which would not be expected if PI5P4K α -generated PI(4,5)P₂ acted as an immediate precursor for PIP₃. The simplest interpretation of these data suggests that the PI(4,5)P₂ pool generated by PI5P4K α regulates PIP₃ synthesis indirectly over a time course longer than a few hours rather than by acting as its lipid precursor, but the latter possibility cannot be ruled out with our data.

Auxin-Inducible Degradation System for PI5P4K α Reveals a Contrasting Phenotype of Akt Phosphorylation. As just discussed, the simplest interpretation of the above data is that a pool of PI(4,5)P₂ synthesized by PI5P4K α is required for full PIP₃ synthesis, but it is difficult to take this further in exploring what exactly is the primary defect using a knockout strategy. However, the power of DT40 genetics also gave us a unique opportunity to try a completely different approach but now looking at the effect of acute removal of the PI5P4K α protein. To do this, we used the auxin degon system, which has been used in a number of cells by transfection of degon-tagged protein, including DT40s (14). Here, we tagged the

auxin for 60 min to remove PI5P4K α or vehicle. The effect on Akt phosphorylation at Thr308 and Ser473 sites was examined. Quantitation of four such blots by densitometry is shown in the graph. Bars show means and SEs.

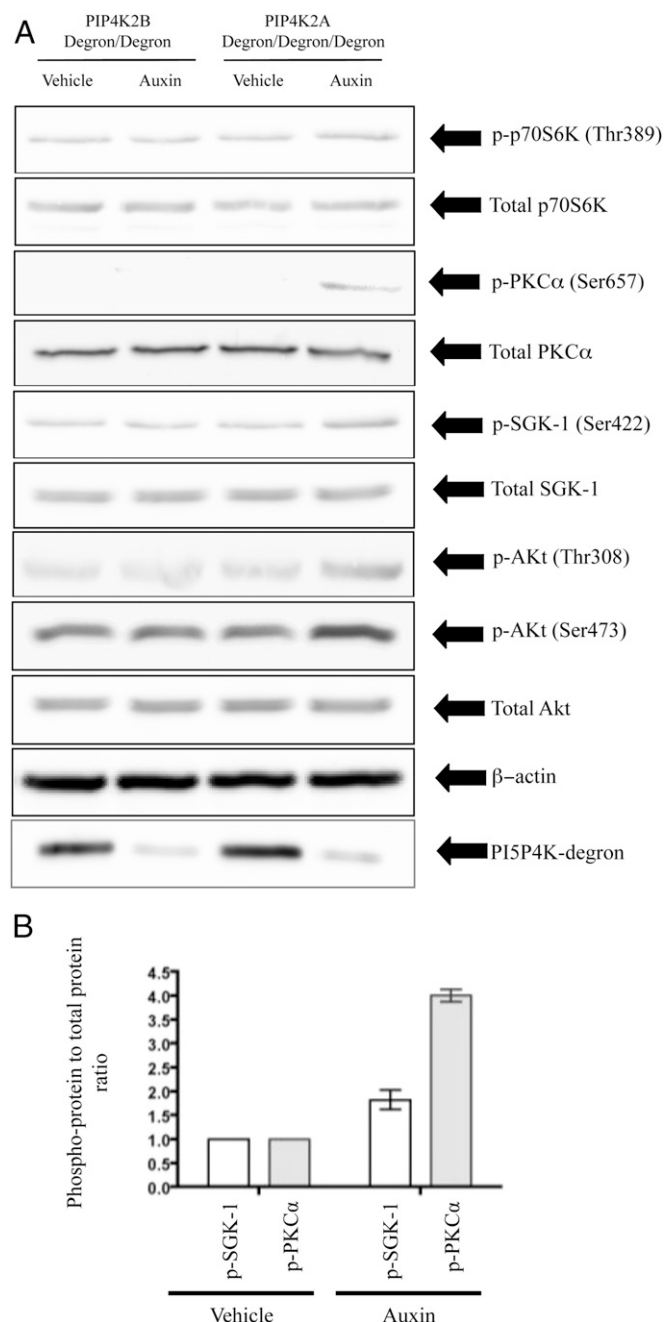


Fig. 4. The effect of acute removal of PI5P4K α or PI5P4K β on mTORC1 and mTORC2 phosphorylation targets. (A) Cells were synchronized at the same density in exponential growth before being treated for 60 min with 500 μ M auxin and harvested for analysis. All blots use total cell-free extract, except those against the PI5P4K degron fusion protein, which are antipoly-His blots of anti-FLAG immunoprecipitates as in Fig. 3A. (B) Quantification of the phosphorylation status of the mTORC2 substrates SGK-1 and PKC α in response to acute PI5P4K α removal by densitometry measurements from three such blots as in A. Results normalized to the phosphorylation status of these substrates in the presence of PI5P4K α .

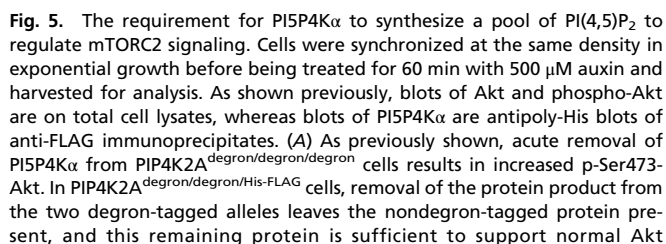
PIP4K2A alleles directly with the auxin degron tag in a DT40 cell line that we had already stably transfected with the TIR1 protein (14) that is necessary for the auxin degron system to work. We also degron-tagged both alleles of PI5P4K β to make another cell line (PIP4K2B^{degron/degron}), and this not only served as a control for any nonspecific effects of the manipulations but also, highlighted the specific role here of PI5P4K α .

For PI5P4K α , initially, we generated a cell line where all three alleles of the gene were degron-tagged (PIP4K2A^{degron/degron/degron}). By trying various strategies, we found that placing the degron tag at the C terminus of the protein followed by an (His)₆-FLAG tag (as in ref. 8) worked best in that C-terminal tagging is easier than N-terminal tagging in this protocol, the tagged proteins could easily be quantified by immunoprecipitation and blotting, and the degron tag still worked effectively to target the protein for degradation. In both PIP4K2A^{degron/degron/degron} (Fig. 3) and PIP4K2B^{degron/degron} (SI Appendix, Fig. S7) cells, addition of auxin led to the complete or near-complete removal of the tagged protein within 60 min, a process that is reversible (SI Appendix, Fig. S7). Blotting whole-cell lysates with an anti-PI5P4K α antibody shows that no wild-type PI5P4K α remains in PIP4K2A^{degron/degron/degron} cells (Fig. 3). The presence of the degron tag may reduce expression of PI5P4K α slightly (Fig. 3), but we could detect no phenotypic difference in the cells.

Because we did not see the same changes in PIP₃ mass levels on acute PI5P4K α removal compared with PIP4K2A deletion (see above), we felt it important to reexplore the Akt signaling phenotype in these new cells. As with the knockout cell lines, we examined Akt phosphorylation under conditions of both insulin stimulation (Fig. 3C) and exponential growth in full medium (Figs. 3D and 4). After 1 h of serum starvation, cells not concurrently exposed to auxin exhibited insulin-induced Akt phosphorylation at both Thr308 and Ser473 as expected (15) in a dose-dependent fashion (Fig. 3C). In cells depleted of PI5P4K α , the insulin-induced phosphorylation of Thr308 was unchanged or slightly increased, whereas the phosphorylation of Ser473 was significantly enhanced (Fig. 3C). We saw the same effect of increased Ser473 phosphorylation when cells were in exponential growth in full medium (Figs. 3D and 4A). This finding was confirmed to be a PI5P4K α -specific effect, because acute removal of PI5P4K β from PIP4K2B^{degron/degron} cells had no effect on Akt phosphorylation (SI Appendix, Fig. S8). We wondered whether the enhancement of Akt phosphorylation at Ser473 was stimulus-specific, and therefore, we treated our serum-starved PIP4K2A^{degron/degron/degron} B cells with the B-cell receptor agonist M4. Although as expected (16), M4 treatment resulted in increased phospho-Akt, this effect was not enhanced by removal of PI5P4K α (SI Appendix, Fig. S9). There is, therefore, stimulus selectivity in the regulation of Akt Ser473 phosphorylation by PI5P4K α .

PI5P4K α Is an Acute Negative Regulator of Mammalian Target of Rapamycin Complex 2. The above data suggest that acute loss of PI5P4K α results in enhanced Ser473 phosphorylation of Akt, with little or no effect on Thr308. Because the kinase primarily responsible for Ser473 phosphorylation is mammalian target of rapamycin complex 2 (mTORC2) (15), it seems most likely that PI5P4K α is acting as a negative regulator of mTORC2 either directly or indirectly. To investigate this further, we examined the phosphorylation states of the mTORC2 targets SGK-1 and PKC α (15) and found phosphorylation of both to be increased on acute removal of PI5P4K α but not of PI5P4K β (Fig. 4). In contrast and consistent with the lack of effect on Thr308 Akt phosphorylation (above), the phosphorylation state of the mTORC1 target p70S6K (15) is not affected by acute removal of PI5P4K α (Fig. 4).

An alternative explanation for these findings is that PI5P4K α removal inhibits the phosphatases responsible for dephosphorylating the three mTORC2 substrates. To address this idea we first confirmed that inhibition of TORC2 with the drug torin is successful in preventing phosphorylation of Akt at Ser473 in DT40 cells (SI Appendix, Fig. S11A). We then attempted to assay the kinetics of dephosphorylation of Ser473-Akt and Ser422-SGK1 in PIP4K2A^{degron/degron/degron} cells with or without auxin-induced removal of PI5P4K α (SI Appendix, Fig. S11B and C). Regardless of whether PI5P4K α is depleted, complete dephosphorylation of the substrates examined occurs by the first time point at which we can reliably perform an assay (5 min). This finding rules out a fully quantitative experiment, but nevertheless, these findings coupled with the fact that different phosphatases are apparently responsible



phosphorylation. (B) When PIP4K2A^{degron/degron/kinase-dead} cells are treated with auxin so as to leave the cells with only kinase-dead P15P4K α , an increase in p-Ser473-Akt results. P15P4K α , therefore, has to be kinase-*in* to promote normal Akt phosphorylation. (C) By a similar strategy, treating PIP4K2A^{degron/degron/substrate switch} cells with auxin so that the cells are left with P15P4K α that has been mutated into a P14P5K has no effect on Akt phosphorylation. Therefore, synthesis of PI(4,5)P₂ rather than P15P removal is the most likely function of P15P4K α here.

both the stable knockout and acute depletion of PI5P4K α , we established clear phenotypes to provide “readouts” of enzyme function, and in both situations, it was clear that the role of PI5P4K α was to synthesize PI(4,5)P₂ rather than to deplete PI5P. Of course, in other settings, PI5P depletion by PI5P4K α may well be of physiological importance. Furthermore, the PI5P4K α -generated PI(4,5)P₂ has a regulatory influence over mTORC2 and PI3K-Akt signaling.

It is striking, although perhaps not entirely unexpected, that very different phenotypes are identified on chronic and acute depletion of PI5P4K α . Given that, in simple terms, increased Akt phosphorylation is seen with acute PI5P4K α removal and reduced Akt phosphorylation is seen chronically, it is tempting to speculate that the long-term phenotype is an adaptive consequence of the initial perturbation. As discussed in the Introduction, a profound change in cell physiology stemming from a single-point mutation in type I PI3K α has been shown by Hart et al. (4), which they called the “butterfly effect,” and a similar set of events may have taken place here.

In other studies, the impact of the PI5P4Ks on Akt signaling is not consistent between different organisms and different tissues using different techniques, and our demonstration of temporal variability may help to reconcile some of these data. For example, stable knockout of *Drosophila dPIP4K* (orthologous to the high-activity *PIP4K2A* in higher species) results in a dramatic attenuation of Akt phosphorylation (20), whereas acute depletion of *PIP4K2A* in human leukocytes by RNAi results in increased Akt phosphorylation (19). No Akt phenotype was found in *PIP4K2A* knockout mice (21), but as far as we are aware, cells of the hematopoietic lineage were not studied there; another study from the same group elegantly showed the tissue variability of gene knockouts, with *PIP4K2B* knockout mice having enhanced insulin-induced Akt phosphorylation in skeletal muscle and liver but not in white fat (22). Certainly, it is not unreasonable to suggest a particular role of PI5P4K α in blood cells given that these are the only cells reported so far to have an excess of this enzyme over the other isoforms (23). Our data from exploring a simple, single-cell type system highlight how difficult it can be to interpret primary protein function from knockout phenotypes. More importantly, we suggest that the effects that we see in the degra-tagged cells, a strategy that gives the cells no chance to adapt around changes caused by the removal of protein, may be revealing a primary and

previously unsuspected cellular function of PI5P4K α related to mTORC2 regulation.

At this stage, it is unclear what mechanisms are leading to the different phenotypes, and clarification will take extensive additional work. This need is particularly so for the knockout phenotype because of its indirect nature. For the acute phenotype, it is interesting to note that the cellular location of endogenous PI5P4K α in DT40s is largely cytoplasmic (*SI Appendix, Fig. S10*), with all of it being attached to membranes (8), raising the possibility that it is localized to the endoplasmic reticulum, the same location in which mTORC2 has been reported to function (15). PI(4,5)P₂ is at very low levels in intracellular membranes (1), and therefore, any synthesized here by PI5P4K α could have a disproportionate impact. Indeed, synthesizing a localized pool of PI(4,5)P₂ by a different metabolic pathway from most of the cell's (PI4P5K-generated) PI(4,5)P₂ is an attractive concept for controlling localized functions of this highly multifunctional (1) lipid. Note that, if this is so, simplistically, the PI(4,5)P₂ pool made by PI5P4K α that we have invoked here must have a negative effect on mTORC2 activity. [Superficially, this might serve as the precursor for the PIP₃ pool suggested recently by Liu et al. in mTORC2 regulation (24), although because this appears to be stimulatory to mTORC2, the relationship of the observations by Liu et al. with our observations is unclear.] There are two proteins reported as inhibiting mTORC2, DEPTOR (25) and XPLN (26), the latter being mTORC2-specific and thus, potentially, an endoplasmic reticulum protein, and both of these proteins have the theoretical potential to bind PI(4,5)P₂ through their PDZ or PH domains, respectively; these considerations point to one way in which the negative influence of PI5P4K α on mTORC2 activity might be mediated.

Materials and Methods

All methodology pertinent to this study, including cell line generation, signaling assays, and fluorescent and MS-based lipid assays, can be found in *SI Appendix, SI Materials and Methods*.

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1. Balla T (2013) Phosphoinositides: Tiny lipids with giant impact on cell regulation. *Physiol Rev* 93(3):1019–1137.
2. Viaud J, Boal F, Tron  re H, Gaits-Iacovoni F, Payrastra B (2014) Phosphatidylinositol 5-phosphate: A nuclear stress lipid and a tuner of membranes and cytoskeleton dynamics. *BioEssays* 36(3):260–272.
3. Bulley SJ, Clarke JH, Droubi A, Giudici ML, Irvine RF (2015) Exploring phosphatidylinositol 5-phosphate 4-kinase function. *Adv Biol Regul* 57:193–202.
4. Hart JR, et al. (2015) The butterfly effect in cancer: A single base mutation can remodel the cell. *Proc Natl Acad Sci USA* 112(4):1131–1136.
5. Rozenvayn N, Flaumenhaft R (2001) Phosphatidylinositol 4,5-bisphosphate mediates Ca²⁺-induced platelet alpha-granule secretion: Evidence for type II phosphatidylinositol 5-phosphate 4-kinase function. *J Biol Chem* 276(25):22410–22419.
6. Rozenvayn N, Flaumenhaft R (2003) Protein kinase C mediates translocation of type II phosphatidylinositol 5-phosphate 4-kinase required for platelet alpha-granule secretion. *J Biol Chem* 278(10):8126–8134.
7. Richardson JP, Wang M, Clarke JH, Patel KJ, Irvine RF (2007) Genomic tagging of endogenous type IIbeta phosphatidylinositol 5-phosphate 4-kinase in DT40 cells reveals a nuclear localization. *Cell Signal* 19(6):1309–1314.
8. Wang M, et al. (2010) Genomic tagging reveals a random association of endogenous PtdIns5P 4-kinases IIalpha and IIbeta and a partial nuclear localization of the IIalpha isoform. *Biochem J* 430(2):215–221.
9. Bultma Y, Keune WJ, Divecha N (2010) PIP4Kbeta interacts with and modulates nuclear localization of the high-activity PtdIns5P 4-kinase isoform PIP4Kalpha. *Biochem J* 430(2):223–235.
10. Hinchliffe KA, Giudici ML, Letcher AJ, Irvine RF (2002) Type IIalpha phosphatidylinositol phosphate kinase associates with the plasma membrane via interaction with type I isoforms. *Biochem J* 363(Pt 3):563–570.
11. Kunz J, Fuelling A, Kolbe L, Anderson RA (2002) Stereo-specific substrate recognition by phosphatidylinositol phosphate kinases is swapped by changing a single amino acid residue. *J Biol Chem* 277(7):5611–5619.
12. Clark J, et al. (2011) Quantification of PtdInsP3 molecular species in cells and tissues by mass spectrometry. *Nat Methods* 8(3):267–272.
13. Kwon Y, Hofmann T, Montell C (2007) Integration of phosphoinositide- and calmodulin-mediated regulation of TRPC6. *Mol Cell* 25(4):491–503.
14. Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, Kanemaki M (2009) An auxin-based degra-tag system for the rapid depletion of proteins in nonplant cells. *Nat Methods* 6(12):917–922.
15. Gaubitz C, Prouteau M, Kusmider B, Loewer R (2016) TORC2 structure and function. *Trends Biochem Sci* 41(6):532–545.
16. Gold MR, et al. (1999) The B cell antigen receptor activates the Akt (protein kinase B)/glycogen synthase kinase-3 signaling pathway via phosphatidylinositol 3-kinase. *J Immunol* 163(4):1894–1905.
17. Gao T, Furnari F, Newton AC (2005) PHLPP: A phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell* 18(1):13–24.
18. Chao CC, Ma YL, Lee EHY (2007) Protein kinase CK2 impairs spatial memory formation through differential cross talk with PI-3 kinase signaling: Activation of Akt and inactivation of SGK1. *J Neurosci* 27(23):6243–6248.
19. Jude JG, et al. (2015) A targeted knockdown screen of genes coding for phosphoinositide modulators identifies PIP4K2A as required for acute myeloid leukemia cell proliferation and survival. *Oncogene* 34(10):1253–1262.
20. Gupta A, et al. (2013) Phosphatidylinositol 5-phosphate 4-kinase (PIP4K) regulates TOR signaling and cell growth during *Drosophila* development. *Proc Natl Acad Sci USA* 110(15):5963–5968.
21. Emerling BM, et al. (2013) Depletion of a putatively druggable class of phosphatidylinositol kinases inhibits growth of p53-null tumors. *Cell* 155(4):844–857.
22. Lamia KA, et al. (2004) Increased insulin sensitivity and reduced adiposity in phosphatidylinositol 5-phosphate 4-kinase beta-/- mice. *Mol Cell Biol* 24(11):5080–5087.
23. Clarke JH, Emson PC, Irvine RF (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(5):F1422–F1430.
24. Liu P, et al. (2015) PtdIns(3,4,5)P3-dependent activation of the mTORC2 kinase complex. *Cancer Discov* 5(11):1194–1209.
25. Peterson TR, et al. (2009) DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* 137(5):873–886.
26. Khanna N, Fang Y, Yoon MS, Chen J (2013) XPLN is an endogenous inhibitor of mTORC2. *Proc Natl Acad Sci USA* 110(40):15979–15984.