Major classification

Biological sciences.

Minor classification

Cell biology

Title

In B cells Phosphatidylinositol 5-phosphate 4-kinase α synthesizes $PI(4,5)P_2$ to impact on mTORC2 and Akt signalling.

Short title

PI5P4Kα generates a PIP_2 pool that regulates Akt.

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Keywords
Phosphatidylinositol 5-phosphate 4-kinase
Phosphatidylinositol 5-phosphate
Phosphatidylinositol (4,5) bisphosphate
Akt
Protein kinase B
mTOR

Abstract
Phosphatidylinositol 5-phosphate 4-kinases (PI5P4Ks) are enigmatic lipid kinases whose physiological functions are incompletely understood, not 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 PI5P4Kα was removed either stably by genetic deletion, or transiently (within one hour) by tagging the endogenous protein genomically with the auxin degron. In both cases removal impacted upon Akt phosphorylation, and by leaving one PI5P4Kα allele present but mutating it to be kinase-dead or to have PI4P 5-kinase activity we show that all the effects on Akt phosphorylation were dependent upon the ability of PI5P4Kα to synthesise $PI(4,5)P_2$ rather than to remove PI5P. Whilst stable removal of PI5P4Kα resulted in a pronounced decrease in Akt phosphorylation at Thr308 and Ser473, due in part to reduced plasma membrane PIP_3, its acute removal led to an increase in Akt phosphorylation only at Ser473. This invokes activation primarily of mTORC2, which was confirmed by increased phosphorylation of other mTORC2 substrates. These findings establish PI5P4Kα as a kinase that synthesizes a physiologically relevant pool of $PI(4,5)P_2$ and as a novel regulator of mTORC2. They also show a phenomenon similar to the 'butterfly effect' described for PI 3-kinase Iα (Hart JR et al PNAS 112 1131-1136; 2015) whereby via apparently the same underlying mechanism

the removal of a protein's activity from a cell can have widely divergent effects, depending upon the time course of that removal.

Significance Statement

Investigating enzyme function by genetic knock out is often complicated by indirect and compensatory changes, whilst supra-physiological levels of protein can compromise overexpression. These pitfalls have made it difficult to understand the functions of the enigmatic PI5P4Ks; we are even not sure what lipid phosphorylation they catalyse *in vivo*. Here we have employed the unique genetic power of DT40 cells to genomically delete PI5P4Kα or to remove the endogenous protein acutely (within 60 minutes). We employed similar approaches to manipulate the endogenous catalytic activity of the enzyme. From this novel approach we have gained unique and unexpected insights into the physiological role of PI5P4Kα and the ways in which it interacts with the Akt signaling pathway.

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Introduction

The phosphatidylinositol 5-phosphate 4-kinases (PI5P4Ks) are an enigmatic family of three (PI5P4Ks α, β and γ), whose cellular functions 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 knock-out 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) are long term strategies that may lead to indirect changes such as, for example, those highlighted in the recent study of Hart et al (4) concerning the indirect long term consequences of a point mutation in PI3Kα. 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 ($PI(4,5)P_2$) 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 PI5P4Kβ (7,8.9). DT40s, being of

avian origin, do not have a PI5P4Kγ gene, which facilitates the study of the functions and inter-relationship 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 PI5P4Kα.

Results

Long term loss of PI5P4Kα by gene disruption results in compromised Akt phosphorylation.

Karyotype analysis of the DT40 cells with which we were working revealed trisomy 2 (figure S1), the chromosome on which *PIP4K2A* resides. For three main reasons we chose to delete a 2.8kb segment of the gene encompassing exons 8 and 9: Firstly, with this short deletion we hoped to avoid off-target effects like those we saw in PIP4K2B$^{-/-}$ cells (see supporting text and figures S2 and S3); secondly, these exons encode large portions of the PI5P- and ATP-binding sites of the kinase so any N-terminal fragment is unlikely to possess any function relying on ATP or PI5P binding; and thirdly, in cells with two of three alleles deleted we could mutate the catalytic activity of the third (below). PIP4K2A$^{-/-/-}$ cells have a similar growth rate to controls (figure S1) and unchanged PI5P4Kβ message (figure S4). Analysis of the phenotype of PIP4K2A$^{-/-/-}$ cells revealed a significant decrease in Akt phosphorylation at both Thr308 and Ser473 (human numbering) in both synchronized exponential growth and upon insulin stimulation (figure 1A,B). Given this consistency between assays we performed further experiments using the former protocol only. As an independent test of the phenotype of PIP4K2A$^{-/-/-}$ cells we knocked down PI5P4Kα by stable shRNA and found the same effect (figure S5).

In order 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 gave us the chance to explore the mechanism behind this phenotype. Firstly, 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 whilst a remaining kinase-live third allele can support normal Akt phosphorylation, one that is kinase dead cannot (figure 1). Note that these

experiments are very tightly controlled as PIP4K2A$^{-/-/\text{kinase dead EmGFP}}$ cells and their controls (PIP4K2A$^{-/-/\text{EmGFP}}$) differ by only one amino acid.

To explore whether the function of PI5P4Kα here is to remove PI5P, generate PI(4,5)P_2, or both, we used Kunz et al's (11) finding that a single mutation (A381E) in the activation loop of human PI5P4Kβ changes its specificity from a PI5P 4-kinase to a PI4P 5-kinase. If PI5P removal is the main function of PI5P4Kα in the present context, such a mutation introduced into the non-deleted *PIP4K2A* allele should see the Akt phenotype emerge, but if PI(4,5)P_2 synthesis is its function then, because there is plenty of PI4P present in most cellular membranes (1), the mutant should support normal Akt phosphorylation. First we tested directly using recombinant enzymes that the substrate specificity switch of human PI5P4Kβ (11) is valid in the chicken PI5P4Kα. The data (figure 1C) show that a marked (> 98%) substrate specificity switch occurs (note the logarithmic Y axis). Figure 1 shows that if the third *PIP4K2A* allele in PIP4K2A$^{-/-/wt}$ cells is mutated (A370E) to encode a PI4P 5-kinase normal Akt phosphorylation is maintained. Our interpretation of these experiments is that PI5P4Kα synthesizes a pool(s) of PI(4,5)P_2 required for sustained phosphorylation of Akt.

By generating *PIP4K2B* null cells we excluded a similar role for PI5P4Kβ in the regulation of Akt phosphorylation. Howver, complete deletion of *PIP4K2B* did result in indirect downregulation of PI5P4Kα (Fig S2). The consequent Akt phenotype was recued only by overexpression of PI5P4Kα, not of PI5P4Kβ, and then only if the PI5P4Kα was kinase live, irrespective of whether or not it was mutated to a PI4P 5-kinase (see supplementary results and discussion and figures S2 and S3).

PI5P4Kα is necessary for maintenance of normal plasma membrane PIP_3 levels in DT40 cells

Given that PI5P4Kα is a lipid kinase, the simplest explanation of decreased Akt phosphorylation is that PIP_3 synthesis may be compromised (see Introduction). We therefore quantified PIP_3 levels by mass spectrometry (12) in the cell lines shown in figure 1. A decrease in the mass of PIP_3 was evident that followed the pattern of loss of Akt phosphorylation (figures 2 and S6), suggesting that at least part of this Akt

phenotype is due to decreased PIP_3 synthesis. We confirmed these findings by overexpression of the fluorescent 3-phosphoinositde reporter EmGFP-PH-Akt (13) which was recruited less to the plasma mebrane in cells with deficient Akt phosphorylation (figure S3C).

In light of these findings we wondered if the PI5P4Kα-generated PI(4,5)P2 was acting as a substrate for class 1 PI3-kinases to synthesise PIP_3. Quantification of total PIP_2 levels (which will be mostly $PI(4,5)P_2$) in PIP4K2A$^{-/-/-}$ cells showed no change from wild-type (figure S6B), although this is not surprising given that the $PI(4,5)P_2$ required for PIP_3 synthesis is a very small fraction of total cellular $PI(4,5)P_2$. However, in cells where all the endogenous PI5P4Kα can be removed within an hour by the addition of auxin (below) no change in PIP_3 levels upon acute removal of PI5P4Kα was detected, which would not be expected if PI5P4Kα-generated $PI(4,5)P_2$ acted as an immediate precursor for PIP_3. The simplest interpretion of these data suggests that the $PI(4,5)P_2$ pool generated by PI5P4Kα regulates PIP_3 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 current data.

An auxin-inducible degradation system for PI5P4Kα reveals a contrasting phenotype of Akt phosphorylation

As just discussed the simplest interpretation of the above data is that a pool of $PI(4,5)P_2$ synthesized by PI5P4Kα is required for full PIP_3 synthesis, but it is difficult to take this further in exploring what exactly is the primary defect using a knock-out 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 PI5P4Kα protein. To do this we employed the auxin degron system, which has been used in a number of cells by transfection of degron-tagged protein, including DT40s (14). Here we tagged the *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 PI5P4Kβ to make another cell line (PIP4K2B$^{degron/degron}$), and this not only served as a control for any non-specific effects of the manipulations but also highlighted the specific role here of PI5P4Kα.

For PI5P4Kα, 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 a $(His)_6$-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$^{degron/degron/degron}$ (figure 3) and PIP4K2B$^{degron/degron}$ (figure S7) cells addition of auxin led to the complete or near complete removal of the tagged protein within 60 minutes, a process that is reversible (figure S7). Blotting whole cell lysates with an anti-PI5P4Kα antibody shows that no wild type PI5P4Kα remains in PIP4K2A$^{degron/degron/degron}$ cells (figure 3). The presence of the degron tag may reduce expression of PI5P4Kα slightly (figure 3) but we could detect no phenotypic difference in the cells.

As we did not see the same changes in PIP_3 mass levels upon acute PI5P4Kα removal compared to *PIP4K2A* deletion (see above) we felt it important to re-explore the Akt signaling phenotype in these new cells. As with the knockout cell lines we examined Akt phosphorylation under conditions of both insulin stimulation (figure 3C) and exponential growth in full medium (figures 3D and 4). Following an hour 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 (figure 3C). In cells depleted of PI5P4Kα the insulin-induced phosphorylation of Thr308 was unchanged or slightly increased, while the phosphorylation of Ser473 was significantly enhanced (figure 3C). We saw the same effect of increased Ser473 phosphorylation when cells were in exponential growth in full medium (figures 3D and 4A). This was confirmed to be a PI5P4Kα-specific effect as acute removal of PI5P4Kβ from PIP4K2B$^{degron/degron}$ cells had no effect on Akt phosphorylation (figure 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. Whilst, as expected (16) M4 treatment resulted in increased phospho-Akt, this effect was not enhanced by removal of PI5P4Kα (figure S9). There is therefore stimulus-selectivity in the regulation of Akt

Ser473 phosphorylation by PI5P4Kα.

PI5P4Kα is an acute negative regulator of mTORC2

The above data suggest that acute loss of PI5P4Kα results in enhanced Ser473 phosphorylation of Akt, with little or no effect on Thr308. As the kinase primarily responsible for Ser473 phosphorylation is mTORC2 (15) it seems most likely that PI5P4Kα 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 PKCα (15), and found phosphorylation of both to be increased upon acute removal of PI5P4Kα but not of PI5P4Kβ (figure 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 PI5P4Kα (figure 4).

An alternative explanation for these findings is that PI5P4Kα removal inhibits the phosphatase(s) responsible for dephosphorylating the three mTORC2 substrates. In order to address this we first confirmed that inhibition of TORC2 with the drug torin is successful in preventing phosphorylation of Akt at Ser473 in DT40 cells (figure 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 PI5P4Kα (figures S11B and S11C). Whether or not PI5P4Kα is depleted, complete dephosphorylation of the substrates examined occurs by the first time point at which we can reliably perform an assay (5 minutes). This rules out a fully quantitative experiment, but nevertheless these findings, coupled with the fact that different phosphatases are apparently responsible for removing the Ser473 phosphate of Akt and the Ser422 phosphate of SGK1 (17, 18), support PI5P4Kα being a negative regulator of mTORC2 as more likely than a positive regulator of two different phosphatases.

Acute regulation of Akt by PI5P4Kα depends upon PI(4,5)P_2 generation

The unexpected finding that the Akt signaling phenotype of PI5P4Kα-deficient cells is completely different depending upon the time course of PI5P4Kα removal begs the question whether the mechanism by which PI5P4Kα acts is the same in both cases;

that is: is kinase activity required in both cases, and if so is PI5P removal or $PI(4,5)P_2$ generation the primary effect? In order to address this question we employed a similar strategy to that described above for the stable knockout lines. Starting with $PIP4K2A^{degron/degron/wt}$ cells we either mutated the third allele to be kinase dead or to have PI4P 5-kinase activity whilst simultaneously tagging this allele at the C-terminus with a $(His)_6$-FLAG tag in both cases to generate $PIP4K2A^{degron/degron/kinase\ dead}$ and $PIP4K2A^{degron/degron/substrate\ switch}$ cells respectively. As a control we generated a cell line where we simply knocked a C-terminal $(His)_6$-FLAG tag into the third allele ($PIP4K2A^{degron/degron/His\text{-}FLAG}$ cells). This strategy allows degron-tagged but otherwise wild type PI5P4Kα to be acutely removed from the cell whilst leaving behind either kinase live, kinase dead, or substrate switched PI5P4Kα. Note that despite the likely homodimerization (8, 9) of PI5P4Kα monomers the non-degron tagged protein is not trafficked for degradation with its degron-tagged counterpart (figure 5), presumably because the kinetics of dimer dissociation are fast enough to prevent this.

It should further be noted at this point that we were unable to achieve kinase dead and substrate switch mutations by the same strategies as previously: the single triplet changes required were repeatedly edited out, presumably by homologous recombination between alleles. We reasoned that more substantial coding changes might not be edited out, and found that if the kinase dead mutation was made by introducing a K366-368Q mutation into the activation loop, the equivalent of the K377-379Q substitution that Kunz et al (11) showed reduces the kinase activity of human PI5P4Kβ to less than 2% of wild type, the mutation was retained. The substrate switch was generated by the A370E (11) mutation as above, but in order to avoid repair by homologous recombination we had to introduce synonymous mutations into two neighboring triplets coding for K368 and A369.

Addition of auxin to the $PIP4K2A^{degron/degron/kinase\ dead}$ cells recapitulated the increase in Ser473 phosphorylation of Akt that was seen in $PIP4K2A^{degron/degron/degron}$ cells (figure 5), implying that the kinase activity of PI5P4Kα is required for it to fulfill this aspect of its function. Therefore the question again is whether PI5P removal or $PI(4,5)P_2$ generation is relevant; note that Jude et al (19) have reported that RNAi knockdown of PI5P4Kα leads to an increase in PI5P levels and mTOR activity. Adding auxin to

PIP4K2A$^{degron/degron/substrate\ switch}$ cells and therefore leaving the cell with PI4P 5-kinase activity did not result in an increase in p-Ser473-Akt (figure 5) suggesting that generation of a PI(4,5)P_2 pool by PI5P4Kα is crucial in this enzyme's role as a negative regulator of mTORC2.

Discussion

A crucial result of our experiments is to establish for the first time that a functional pool (or pools) of PI(4,5)P_2 is synthesized via a novel route; phosphorylation of PI5P rather than PI4P. For both the stable knockout and acute depletion of PI5P4Kα we established clear phenotypes to provide 'read-outs' of enzyme function and in both situations it was clear that the role of PI5P4Kα was to synthesize PI(4,5)P_2 rather than to deplete PI5P. Of course in other settings PI5P depletion by PI5P4Kα may well be of physiological importance. Furthermore, the PI5P4Kα-generated PI(4,5)P_2 has a regulatory influence over mTORC2 and PI3K-Akt signalling.

It is striking, although perhaps not entirely unexpected, that very different phenotypes are identified upon chronic and acute depletion of PI5P4Kα. Given that in simple terms increased Akt phosphorylation is seen with acute PI5P4Kα 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 pertubation. As discussed in the Introduction, a profound change in cell physiology stemming from a single point mutation in Type I PI3Kα has been shown by Hart et al, which they called the 'butterfly effect' (4), and 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 this 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), whilst acute depletion of *PIP4K2A* in human leukocytes by RNA interference 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 haematopoietic lineage were not studied there; another study from the same group elegantly

demonstrated 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 PI5P4Kα 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 knock-out phenotypes. More importantly, we suggest that the effects we see in the degron-tagged cells, a strategy which gives the cell no chance to adapt around changes caused by the removal of protein, may be revealing for the first time a primary and previously unsuspected cellular function of PI5P4Kα related to mTORC2 regulation.

At this stage it is unclear what mechanisms are leading to the different phenotypes, and clarification will take extensive further work. This is particularly so for the knock-out phenotype because of its indirect nature. For the acute phenotype it is interesting to note that the cellular location of endogenous PI5P4Kα in DT40s is largely cytoplasmic (figure S10), with all of it being attached to membranes (8), raising the possibility that it is localised to the endoplasmic reticulum, the same location in which mTORC2 has been reported to function (15). $PI(4,5)P_2$ is at very low levels in intracellular membranes (1), so any synthesized here by PI5P4Kα could have a disproportionate impact. Indeed, synthesizing a localized pool of $PI(4,5)P_2$ by a different metabolic pathway from most of the cell's (PI4P5K-generated) $PI(4,5)P_2$ is an attractive concept for controlling localised functions of this highly multi-functional (1) lipid. Note that if this is so, simplistically the $PI(4,5)P_2$ pool made by PI5P4Kα that we have invoked here must have a negative effect on mTORC2 activity. (Superficially this might serve as the precursor for the PIP_3 pool suggested recently by Liu et al in mTORC2 regulation (24), though as this appears to be stimulatory to mTORC2 the relationship of Liu et al's observations with ours 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 these proteins have the theoretical potential to bind $PI(4,5)P_2$ via their PDZ or PH domains respectively; these considerations point to one way in which the negative infuence of PI5P4Kα on mTORC2 activity might be mediated.

Materials and methods

All methodology pertinent to this study including cell line generation, signaling assays, and fluorescent and mass spectrometry based lipid assays can be found in supplementary materials and methods.

Acknowledgements

S.J.B. was supported by an A.J. Clark Studentship from the British Pharmacological Society, A.D. by Sidney Sussex College, the Cambridge Overseas Trust and the Säid Foundation, and J.H.C by the MRC (Grant RG64071). We thank Ashok Venkitaraman, Gerard Evan and Tony Jackson for generous gifts of reagents.

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Figure Legends

Figure 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 prior to analysis. Complete *PIP4K2A* deletion results in reduced phospho-Akt at both Thr308 and Ser473. Deletion of two of the three *PIP4K2A* alleles whilst leaving the third unaffected aside from tagging with EmGFP has no effect on Akt phosphorylation. This acts as a control for the fourth and fifth lanes where the undeleted allele is either rendered kinase dead or mutated to encode a PI4P 5-kinase (see materials and methods) along with EmGFP tagging in both cases. A kinase dead third allele does not support normal Akt phosphorylation in the way a kinase live one does, and it makes no difference to Akt phosphorylation whether the $PI(4,5)P_2$ 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. In order to avoid any unnecessary image manipulation the original negative image is presented.

B

The cell lines shown in figure 1A were serum starved and insulin stimulated (see 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 PI5P4Kα and the A370E (substrate switched) mutant. Recombinant enzymes were assayed as described in the materials and methods section using either PI5P or PI4P substrates. Note the logarithmic y-axis. Wild type PI5P4Kα has two orders of magnitude less activity towards PI4P than towards PI5P whilst the A370E mutant prefers PI4P by almost three orders of magnitude. The activity of chicken PI5P4Kβ is included for comparison. The activity of chicken PI5P4Kα towards PI5P is about three orders of magnitude greater than that

of chicken PI5P4Kβ, very similar to the human enzymes (3). Quantification is of four technical replicates.

Figure 2

The effect of endogenous *PIP4K2A* mutations on PIP_3 mass levels. Cells were synchronized at the same density in exponential growth prior to analysis by mass spectrometry. 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 combined for 38:4, 38:3 and 36:2 PIP_3 species. Chart displays means and 95% confidence intervals.

Bonferroni's multiple comparison test as follows:

WT DT40 versus $PIP4K2A^{-/-/-}$	P=0.065
$PIP4K2A^{-/-/EmGFP}$ versus $PIP4K2A^{-/-/kinase\ dead\ EmGFP}$	P<0.001
$PIP4K2A^{-/-/EmGFP}$ versus $PIP4K2A^{-/-/substrate\ switched\ EmGFP}$	P=0.97

Pooled data from 3 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 in the former. This may be due to an inadequate number of replicates, so 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 (figure S6).

Figure 3

Acute removal of PI5P4Kα with the auxin degron system.

A

Cells ($PIP4K2A^{degron/degron/degron}$) were treated with auxin as indicated and any remaining PI5P4Kα-degron fusion protein was immunoprecipitated against its FLAG tag and blotted against its poly-His tag.

B

Whole cell lysates blotted with an anti-PI5P4Kα antibody.

C

PIP4K2A$^{degron/degron/degron}$ cells were serum starved with or without 500 micromolar auxin for 60 minutes to remove PI5P4K. Insulin was added at the concentrations shown and after 10 mins the cells were lysed and lysates blotted sequentially (stripping in between) for Akt phosphorylation at Thr-308 and Ser-473.

D

PIP4K2A$^{degron/degron/degron}$ cells were synchronised in exponential growth and treated either with 500 micromolar auxin for 60 minutes to remove PI5P4Kα or with vehicle. The effect on Akt phosphorylation at Thr-308 and Ser-473 sites was examined. Quantitation of four such blots by densitometry is shown in the graph. Bars show mean and standard error.

Figure 4

The effect of acute removal of PI5P4Kα or PI5P4Kβ on mTORC1 and mTORC2 phosphorylation targets.

A

Cells were synchronized at the same density in exponential growth before being treated for 60 minutes with 500 micromolar auxin and harvested for analysis. All blots use total cell free extract except those against the PI5P4K-degron fusion protein which are anti-poly-His blots of anti-FLAG immunoprecipitates as in figure 3A.

B

Quantification of the phosphorylation status of the mTORC2 substrates SGK-1 and PKCα in response to acute PI5P4Kα removal by densitometry measurements from three such blots as in figure 4A. Results normalized to the phosphorylation status of these substrates in the presence of PI5P4Kα.

Figure 5

The requirement for PI5P4Kα to synthesize a pool of $PI(4,5)P_2$ in order to regulate mTORC2 signalling.
Cells were synchronized at the same density in exponential growth before being treated for 60 minutes with 500 micromolar auxin and harvested for analysis.
As previously blots of Akt and phospho-Akt are on total cell lysates whilst blots of PI5P4Kα are anti-poly-His blots of anti-FLAG immunoprecipitates.

A

As previously, acute removal of PI5P4Kα from $PIP4K2A^{degron/degron/degron}$ cells results in increased p-Ser473-Akt. In $PIP4K2A^{degron/degron/His\text{-}FLAG}$ cells removal of the protein product from the two degron tagged alleles leaves the non degron-tagged protein present and this remaining protein is sufficient to support normal Akt phosphorylation.

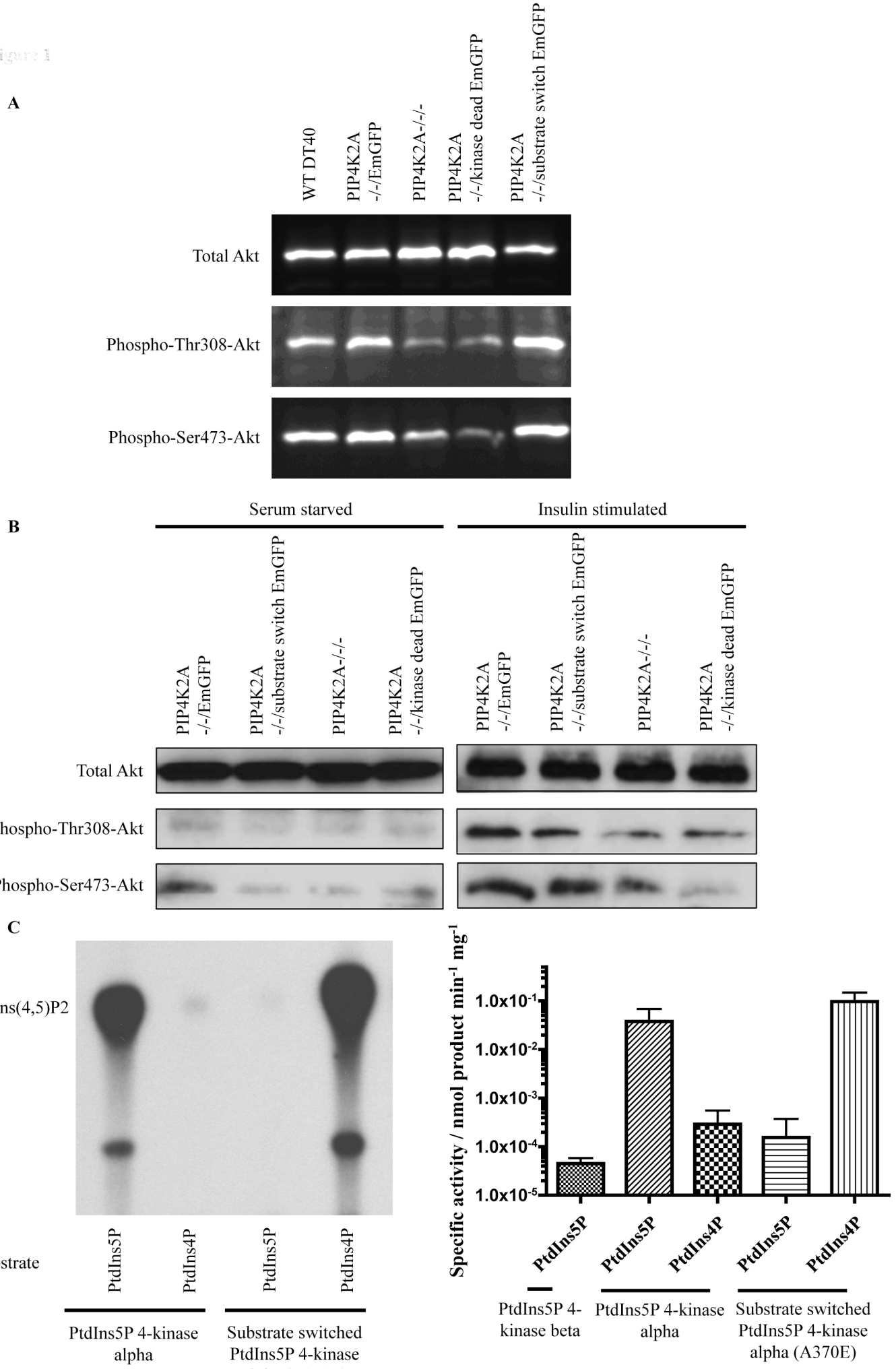
B

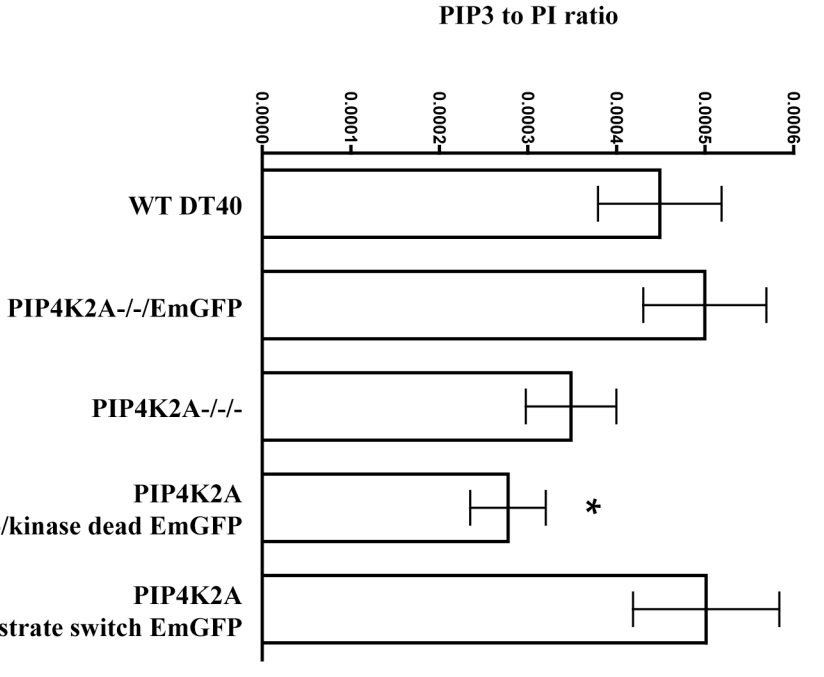
When $PIP4K2A^{degron/degron/kinase\ dead}$ cells are treated with auxin so as to leave the cells with only kinase dead PI5P4Kα an increase in p-Ser473-Akt results. PI5P4Kα therefore has to be kinase live in order to promote normal Akt phosphorylation.

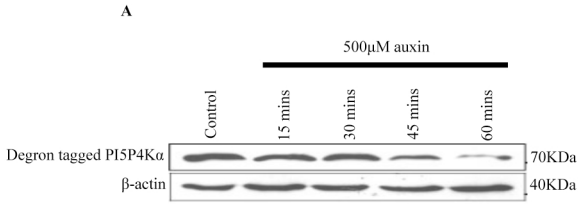
C

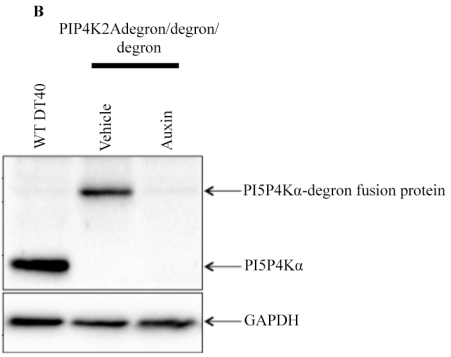
By a similar strategy, treating $PIP4K2A^{degron/degron/substrate\ switch}$ cells with auxin so the cells are left with PI5P4Kα that has been mutated into a PI4P 5-kinase has no effect on Akt phosphorylation. Therefore synthesis of $PI(4,5)P_2$ rather than PI5P removal is the most likely function of PI5P4Kα here.

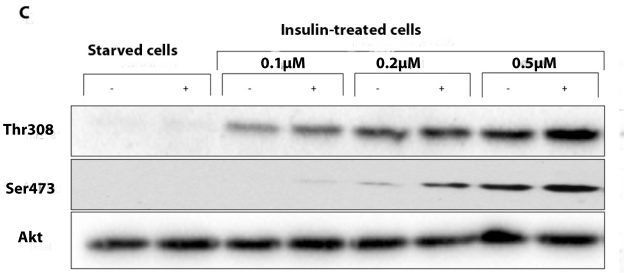
Figure 1

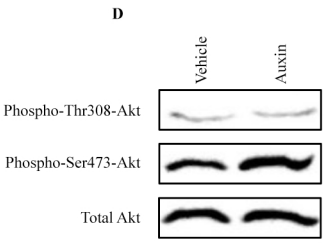

A
WT DT40
PIP4K2A -/-/EmGFP
PIP4K2A-/-/-
PIP4K2A -/-/kinase dead EmGFP
PIP4K2A -/-/substrate switch EmGFP
Total Akt
Phospho-Thr308-Akt
Phospho-Ser473-Akt
B
Serum starved
Insulin stimulated
PIP4K2A -/-/EmGFP
PIP4K2A -/-/substrate switch EmGFP
PIP4K2A-/-/-
PIP4K2A -/-/kinase dead EmGFP
Total Akt
Phospho-Thr308-Akt
Phospho-Ser473-Akt
C
PtdIns(4,5)P2
Substrate
PtdIns5P
PtdIns4P
PtdIns5P
PtdIns4P
PtdIns5P 4-kinase alpha
Substrate switched PtdIns5P 4-kinase alpha (A370E)
Specific activity / nmol product min-1 mg-1
1.0x10-1
1.0x10-2
1.0x10-3
1.0x10-4
1.0x10-5
PtdIns5P
PtdIns5P
PtdIns4P
PtdIns5P
PtdIns4P
PtdIns5P 4-kinase beta
PtdIns5P 4-kinase alpha
Substrate switched PtdIns5P 4-kinase alpha (A370E)

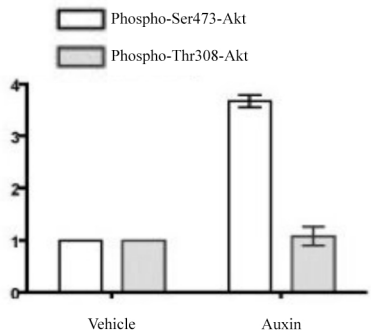

PIP3 to PI ratio
0.0000
0.0001
0.0002
0.0003
0.0004
0.0005
0.0006
WT DT40
PIP4K2A-/-/EmGFP
PIP4K2A-/-/-
PIP4K2A
-/-/kinase dead EmGFP
PIP4K2A
-/-/substrate switch EmGFP
*


A
500μM auxin
Control
15 mins
30 mins
45 mins
60 mins
Degron tagged PI5P4Kα
70KDa
β-actin
40KDa

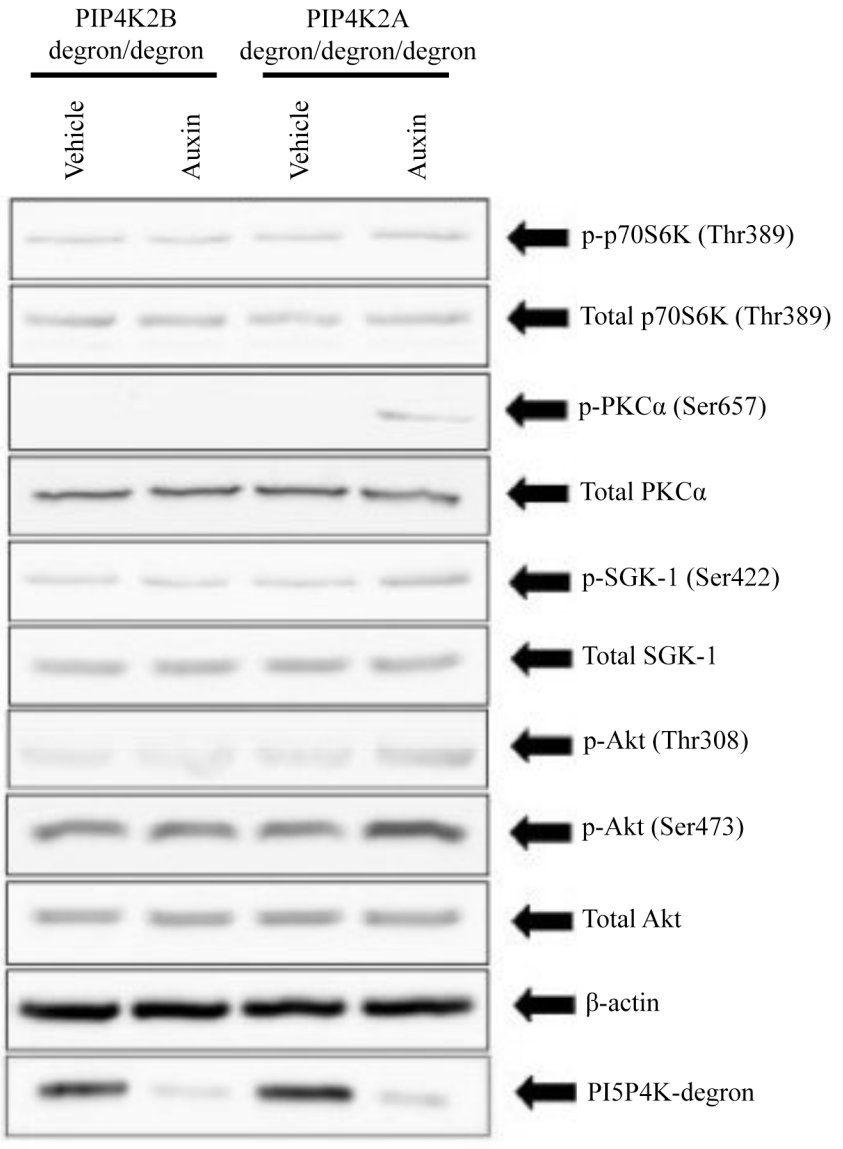

B
PIP4K2Adegron/degron/degron
WT DT40
Vehicle
Auxin
PI5P4Kα-degron fusion protein
PI5P4Kα
GAPDH


C
Insulin-treated cells
Starved cells
0.1μM
0.2μM
0.5μM
Thr308
Ser473
Akt


D
Vehicle
Auxin
Phospho-Thr308-Akt
Phospho-Ser473-Akt
Total Akt


Phospho-Ser473-Akt
Phospho-Thr308-Akt
4
3
2
1
0
Vehicle
Auxin

A


PIP4K2B degron/degron
PIP4K2A degron/degron/degron
Vehicle
Auxin
Vehicle
Auxin
p-p70S6K (Thr389)
Total p70S6K (Thr389)
p-PKCα (Ser657)
Total PKCα
p-SGK-1 (Ser422)
Total SGK-1
p-Akt (Thr308)
p-Akt (Ser473)
Total Akt
β-actin
PI5P4K-degron

B

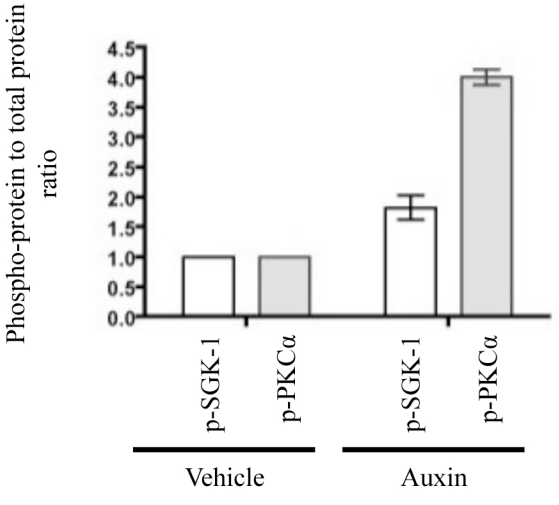
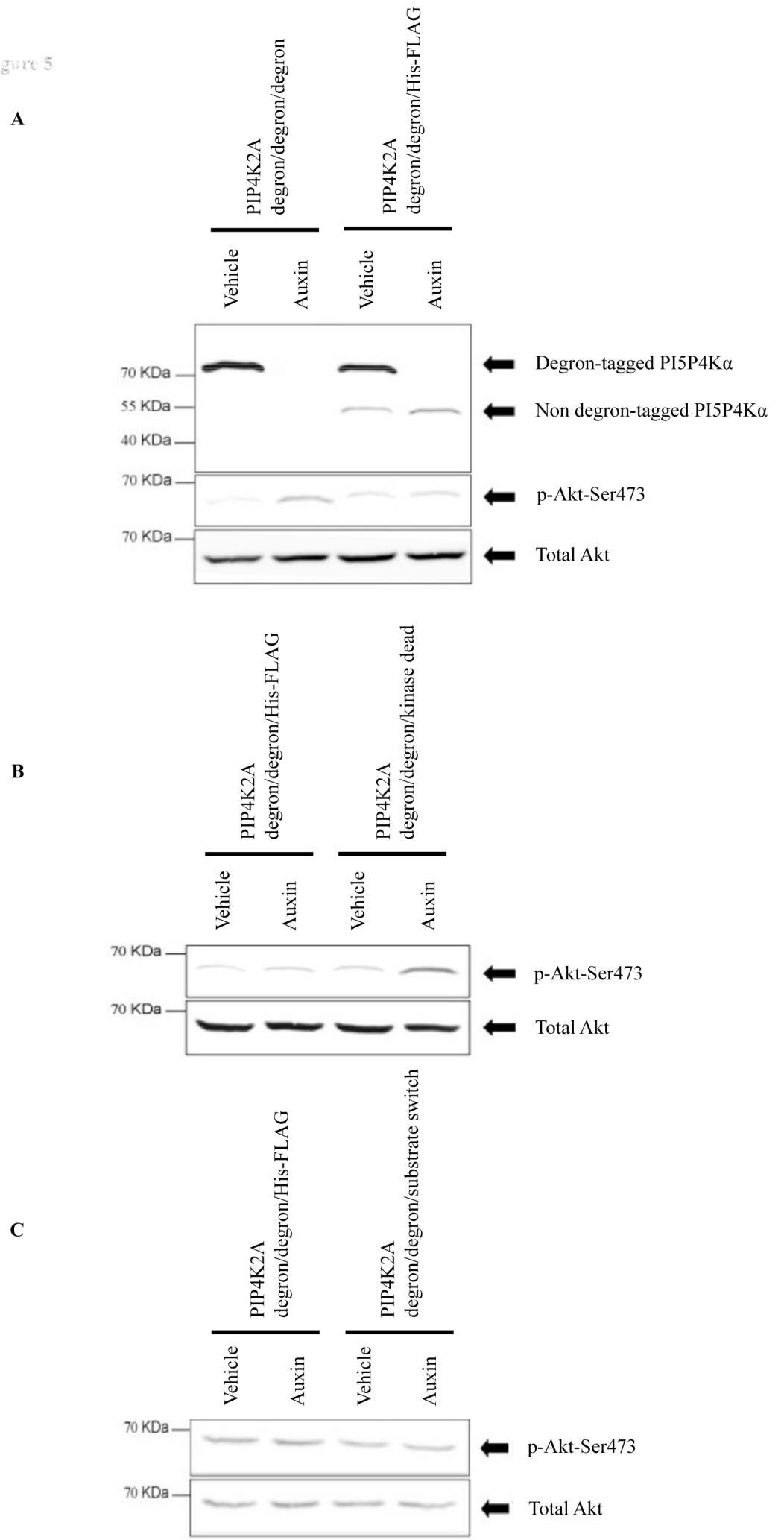

Phospho-protein to total protein ratio
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p-SGK-1
p-PKCα
p-SGK-1
p-PKCα
Vehicle
Auxin

Figure 5


A
PIP4K2A degron/degron/degron
PIP4K2A degron/degron/His-FLAG
Vehicle
Auxin
Vehicle
Auxin
70 KDa
55 KDa
40 KDa
70 KDa
70 KDa
Degron-tagged PI5P4Kα
Non degron-tagged PI5P4Kα
p-Akt-Ser473
Total Akt
B
PIP4K2A degron/degron/His-FLAG
PIP4K2A degron/degron/kinase dead
Vehicle
Auxin
Vehicle
Auxin
70 KDa
70 KDa
p-Akt-Ser473
Total Akt
C
PIP4K2A degron/degron/His-FLAG
PIP4K2A degron/degron/substrate switch
Vehicle
Auxin
Vehicle
Auxin
70 KDa
70 KDa
p-Akt-Ser473
Total Akt

Supplementary Information

Supplementary results and discussion

DT40 cells exhibit trisomy 2

Karyotype analysis of wild type DT40 cells by examination of DAPI stained metaphase spreads revealed trisomy for chromosome 2, the chromosome on which the *PIP4K2A* gene is located (figure S1). This is consistent with a previous karyotype analysis of DT40 cells (27) and means that three alleles have to be considered when designing experiments to endogenously manipulate the PI5P4Kα gene (see main manuscript).

Initial observations pointing to a link between PI5P4Kα and Akt phosphorylation

In addition to investigating the functions of PI5P4Kα we are also interested in elucidating the physiological roles of its isoenzyme PI5P4Kβ (encoded by the gene *PIP4K2B*). In light of this we generated PI5P4Kβ null cells (PIP4K2B$^{-/-}$) by stable genomic deletion from the DT40 line, and surprisingly it was from these cells that we gained our first insight into the role of PI5P4Kα in the regulation of Akt. We noted that these cells had reduced Akt phosphorylation at both Thr308 and Ser473 (figure S3), but this was in the setting of off-target downregulation of PI5P4Kα (figure S2) and therefore it was unclear which isoform was responsible for the Akt phenotype. Note that we interpret this off-target downregulation of PI5P4Kα as an epigenetic consequence of loss of the *PIP4K2B* locus for two reasons: Firstly, long-range PCR and sequencing showed that the *PIP4K2A* loci were still intact in the PIP4K2B$^{-/-}$ cells; secondly, in these cells we had deleted the entire *PIP4K2B* locus including some of the 5' untranslated region which is often responsible for epigenetic regulation (see supplementary material and methods); and thirdly, when we generated a *PIP4K2B* knockout in a less extreme way by introducing a stop codon into exon 1 and deleting exon 2 we saw no downregulation of PI5P4Kα. Whatever the details of this epigenetic mechanism these cells (PIP4K2B$^{-/-}$) afforded us an initial opportunity to investigate the isoform responsible for the downregulation of Akt phosphorylation before proceeding to the formal *PIP4K2A* knockout we describe in the main body of the manuscript.

We found that the deficit in Akt phosphorylation in PIP4K2B$^{-/-}$ cells was not rescued by stable overexpression of PI5P4Kβ, but was rescued by PI5P4Kα (figure S3). Importantly, overexpression of PI5P4Kβ in PIP4K2B$^{-/-}$ cells did not rescue the expression of PI5P4Kα from its suppressed levels (figure S2). Note also that our *PIP4K2B* null cells that did not show PI5P4Kα downregulation (see above) did not have an Akt phenotype. Consistent with our findings when endogenous PI5P4Kα was manipulated (see main results section), overexpressed PI5P4Kα could not rescue Akt phosphorylation in the present context when kinase dead, but remained able to do so when mutated to a PI4P 5-kinase (figure S3) suggesting that PI(4,5)P_2 generation is the important function of PI5P4Kα here. As discussed in the main text we also used these cells for independent confirmation of our finding of reduced PIP_3 levels in PIP4K2A$^{-/-/-}$ cells. We overexpressed the fluorescent 3-phosphoinositide reporter EmGFP-PH-Akt (see main manuscript) in PIP4K2B$^{-/-}$ cells and associated overexpression rescue lines and found a reduced plasma membrane to cytoplasm ratio of the PH domain of Akt following the pattern of loss of Akt phosphorylation (figure S3).

Before proceeding to endogenous manipulations of *PIP4K2A* (see main manuscript) we also, at this point, sought evidence for the link between PI5P4Kα and Akt phosphorylation by creating cells in which PI5P4Kα was knocked down by stable shRNA (PIP4K2A^{shRNA}). Figure S5 shows that these cells have 5.1% (95%CI 4.7% to 5.5%) of wild type *PIP4K2A* message levels, but also an off target effect on *PIP4K2B* message which is at 39.6% (95%CI 37.7% to 41.4%) of wild type levels. These cells do have compromised Akt phosphorylation at both Thr308 and Ser473 (figure S5). We decided that it was not worth refining the knockdown experiment to limit off target effects on PI5P4Kβ as at this stage we had enough data to convince us to proceed directly to the definitive experiments of manipulating endogenous *PIP4K2A* (see main manuscript).

How much PI5P4Kα is required to support normal Akt phosphorylation in stably manipulated cell lines?

The fact that Akt phosphorylation is reduced secondary to stable PI5P4Kα loss in a number of cell lines that must contain varying amounts of PI5P4Kα raises the

interesting question of whether we can say anything about the amount of PI5P4Kα required to maintain normal Akt phosphorylation. The short answer is that we cannot be precise because the signal on western blot from any antibody (whether to endogenous tags or native PI5P4Kα) on a whole cell lysate is too weak to be reliably quantifiable due to the low abundance of PI5P4Kα. Indeed we have to immunoprecipitate PI5P4Kα even to detect it by mass spectrometry (8), and reliable quantification to the precision required here following immunoprecipitation is difficult. There are some general conclusions we can draw, however, from the fluorescence signal given by endogenous PI5P4Kα EmGFP tags in our various cell lines.

The situation in PIP4K2A$^{-/-/-}$ cells is straightforward; there is no functional PI5P4Kα protein and reduced Akt phosphorylation (figure 1). In PIP4K2A^{shRNA} cells, which also have reduced Akt phosphorylation (figure S5), we have no way of assaying the PI5P4Kα that "leaks" through the knockdown but we can say that the encoding mRNA is at 5% of wild type levels (figure S5) and therefore the cells are likely to be severely crippled in terms of PI5P4Kα protein. Next we come to PIP4K2B$^{-/-}$ cells, which have compromised Akt phosphorylation secondary to off target down-regulation of PI5P4Kα (figures S2 and S3). These cells have 50% of the wild type level of PI5P4Kα message, but in these cells we also have a way of reading out PI5P4Kα protein by endogenously tagging one PI5P4Kα allele with EmGFP to create PIP4K2A$^{EmGFP/wt/wt}$PIP4K2B$^{-/-}$ cells. Figure S2 shows the confocal signal from these cells in comparison with PIP4K2A$^{EmGFP/wt/wt}$ cells where one allele of *PIP4K2A* is EmGFP tagged in a *PIP4K2B* wild type background. The confocal settings are the same in both sets of images in figure S2, and quantification of the background subtracted signal reveals that the mean fluorescence intensity in PIP4K2A$^{EmGFP/wt/wt}$PIP4K2B$^{-/-}$ cells is 16% (95% CI 10% to 22%) of that in PIP4K2A$^{EmGFP/wt/wt}$ cells. Therefore our best estimate is that in PIP4K2B$^{-/-}$ cells PI5P4Kα protein is reduced by 84% despite PI5P4Kα message only being reduced by 50%. This 84% reduction in protein is sufficient to result in compromised Akt phosphorylation. The final cell line that provides useful information here is PIP4K2A$^{-/-/EmGFP}$ in which two alleles of *PIP4K2A* are deleted with the third being unaltered aside from having an endogenous EmGFP tag knocked in. These cells have normal

Akt phosphorylation (figure 1), and figure S10B shows the EmGFP signal from these cells compared to PIP4K2A$^{EmGFP/EmGFP/EmGFP}$ cells in which all three *PIP4K2A* alleles are endogenously tagged with EmGFP. The background subtracted mean fluorescence intensity in PIP4K2A$^{-/-/EmGFP}$ cells is 68% (95% CI 61% to 75%) of that in PIP4K2A$^{EmGFP/EmGFP/EmGFP}$. This suggests that some compensatory up-regulation of protein production from the undeleted allele in PIP4K2A$^{-/-/EmGFP}$ cells has occurred, and that 68% of wild type PI5P4Kα is sufficient to maintain normal Akt phosphorylation. We can therefore conclude, in round figures, that 70% of wild type PI5P4Kα can sustain normal Akt phosphorylation whilst 20% cannot, but we do not have data to establish more precisely the location of the "tipping point" between these two limits.

Supplementary materials and methods

DT40 tissue culture

Basic culture medium for DT40 cells was formulated according to the protocol in (28). To 500ml of RPMI medium was added 50ml of FBS, 5ml of 200mM L-glutamine, 5ml of chicken serum, and 120µl of 50mM 2-mercaptoethanol. Cells were cultured at 41°C in 5% CO_2 and were typically kept at a density of between $2.5x10^5$ and $4x10^6$ cells per ml of growth medium.

Generation of targeting constructs for targeted transfection of DT40 cells

Targeting constructs were built into an appropriate plasmid vector (typically pBluescript SK+ (Stratagene)). For gene deletions sequences of genomic DNA flanking the region to be deleted (targeting arms) were PCR amplified from DT40 genomic DNA and ligated into the plasmid vector separated by an antibiotic resistance cassette to allow selection of drug-resistant clones. The targeting arms were typically 2kb in length. Following successful transfection and integration into the germline by homologous recombination the antibiotic resistance cassette replaced the region of the gene targeted for deletion. For integration of tags and mutations into the germline a similar strategy was employed, but in this case the targeting arms were manipulated such that successful recombination led to introduction of the new sequence of choice. See below for details of the generation of each targeting construct.

Construct to endogenously tag PIP4K2A with EmGFP at the C-terminus

The construct used to endogenously tag *PIP4K2A* with EmGFP at the C-terminus in DT40 cells was built into the multiple cloning site of pBluescript SK+ (Stratagene). Constructs used for genomic targeting never need to be expressed off their plasmid vector and so pBluescript, a bacterial cloning vector, is suitable for this purpose even though it will ultimately be transfected into eukaryotic cells.

The 2kb 5' homology arm of the *PIP4K2A* tagging construct was PCR amplified from DT40 gDNA using primers 2A5FOR2 and 2A5REV2 and cloned into pBluescript SK+ between KpnI and XbaI sites. An unwanted BamHI site was removed from the 5' arm by site directed mutagenesis using primers SDM5 and SDM6. Next the coding sequence for EmGFP was PCR amplified from the plasmid EmGFP-C1 (Clontech) (primers EGFP_PCR_FOR_1 and EGFP_PCR_REV_1) and cloned into the construct between XbaI and BamHI sites. The 2kb 3' homology arm of the targeting construct was then cloned into the construct between BamHI and NotI sites following PCR amplification from DT40 gDNA (primers 2A3FOR2 and 2A3REV2). Finally either a puromycin resistance cassette or a blasticidin resistance cassette was ligated into the BamHI site of the construct. Having two constructs identical save for the antibiotic resistance conferred allowed sequential targeting of two *PIP4K2A* alleles using different selection antibiotics in order to derive the cell line PIP4K2A$^{wt/EmGFP/EmGFP}$, which is resistant to both puromycin and blasticidin. Note that the antibiotic selection cassettes used contained their own eukaryotic promoters (29). The correct integration of each targeting construct was tested by long-range PCR and sequencing, and the presence of a third *PIP4K2A* allele was verified by the ability to PCR out a wild type region spanning the terminal *PIP4K2A* exon from PIP4K2A$^{wt/EmGFP/EmGFP}$ gDNA. In order to verify the correct expression of the PI5P4Kα– EmGFP fusion protein PIP4K2A$^{wt/EmGFP/EmGFP}$ cells were subjected to immunoprecipitation and western blotting with anti-GFP antibodies. A GFP-containing protein of the correct molecular weight was present in PIP4K2A$^{wt/EmGFP/EmGFP}$ cells but not in WT DT40 cells. Importantly this 70KDa protein was the only GFP-containing species present in the tagged cells. These controls to ensure accurate construct integration and appropriate fusion protein expression were performed for every tagged cell line generated.

Constructs to delete PIP4K2A and PIP4K2B

The 5' arm of the *PIP4K2A* deletion construct was amplified from DT40 gDNA using primers GGPIP4K2A_DEL_5'_FOR_2 and GGPIP4K2A_DEL_5'_REV_2 whilst the 3' arm was amplified using primers GGPIP4K2A_DEL_3'_FOR and GGPIP4K2A_DEL_3'_REV. These arms were ligated into pBluescript SK+ between KpnI and BamHI sites (5' arm) and BamHI and NotI sites (3' arm). A neomycin, puromycin, or blasticidin resistance cassette was then ligated into the BamHI site to generate three constructs that were used sequentially in order to delete all three *PIP4K2A* loci to generate PIP4K2A$^{-/-/-}$ cells.

The primer pairs used to amplify the 5' and 3' arms of the *PIP4K2B* deletion construct were 2b_Ful_Del_PCR_5'_For_1 and 2b_Ful_Del_PCR_5'_Rev_1, and 2b_Ful_Del_PCR_3'_For_1 and 2b_Ful_Del_PCR_3'_Rev_1 respectively. The two *PIP4K2B* loci in DT40 cells were sequentially deleted using puromycin and blasticidin resistance cassettes to derive the cell line PIP4K2B$^{-/-}$.

Endogenous manipulation of the remaining wild type PIP4K2A allele in PIP4K2A$^{-/-/wt}$ cells

PIP4K2A$^{-/-/wt}$ cells, an intermediate on the way to generating PIP4K2A$^{-/-/-}$ cells, formed the starting point for three other cell lines used in this study. Firstly, the wild type allele was endogenously tagged with EmGFP using the targeting construct already described in order to generate PIP4K2A$^{-/-/EmGFP}$ cells. Secondly, the wild type allele was manipulated to render it both kinase dead (10) and EmGFP tagged (PIP4K2A$^{-/-/kinase\ dead\ EmGFP}$ cells). Thirdly, the final allele was manipulated to both confer it with PI4P 5-kinase activity instead of PI5P 4-kinase activity (11) and EmGFP tag it (PIP4K2A$^{-/-/substrate\ switch\ EmGFP}$ cells). In order to make the targeting constructs for the two latter cell lines the 5' arm of the *PIP4K2A* EmGFP-tagging construct already described had to be extended in order to incorporate the region where the necessary mutations were to be inserted. A new 5' arm was therefore generated for this construct using the primers 2a_Kin_Dead_PCR_For_2 and 2A5REV2. After ligating this new 5' arm into the construct the unwanted BamHI site again had to be removed by site-directed mutagenesis using the primers previously specified. This new construct was then subjected to another round of site-directed

mutagenesis in order to introduce either the kinase-dead (10) mutation (D272K, primers 2a_Kin_Dead_SDM_F and 2a_Kin_Dead_SDM_R) or the substrate switch (11) mutation (A370E, primers 2alpha to type 1 SDM FOR and 2alpha to type 1 SDM REV).

Constructs to overexpress PIP4K2A and PIP4K2B

The full length coding sequences for chicken PI5P4Kα and β were not in genome databases at the time of this stage of the project so these were elucidated by 5' RACE from DT40 mRNA. Generation of a full-length *PIP4K2B* cDNA for cloning was very difficult and therefore the overexpression construct for PI5P4Kβ was synthesized commercially (Invitrogen). A construct was made that encoded the PI5P4Kβ protein between HindIII and KpnI sites. The KpnI site then ran immediately into the coding sequence for a C-terminal FLAG-His6-His6 tag followed by a stop codon and a BamHI site. The construct was then ligated into pcDNA3.1 between HindIII and BamHI sites such that the expression of tagged PI5P4Kβ was driven off a CMV promoter with a properly established Kozak consensus sequence.

Synthesis of a full-length cDNA for *PIP4K2A* was much easier and therefore we amplified the coding sequence from this using the primers GGPIP4K2A_OE_FOR and GGPIP4K2A_OE_REV. This coding sequence was then swapped into the PI5P4Kβ overexpression plasmid between HindIII and KpnI sites in order to allow the overexpression of PI5P4Kα appended to a C-terminal FLAG-His6-His6 tag.

In order to derive plasmids to overexpress kinase dead and substrate switched PI5P4Kα the wild type overexpression construct was subjected to site directed mutagenesis using the primers already described.

Construct to knock down PI5P4Kα by stable shRNA

An shRNA sequence was designed against PI5P4Kα. A pair of oligonucleotides to encode this sequence (shRNA_PIP4K2a_FOR and shRNA_PIP4K2a_REV) were then annealed together and ligated into pSuper.neo (Oligoengine). This plasmid drives the expression of the RNA hairpin from an H1 promoter.

Constructs to endogenously tag PIP4K2A and PIP4K2B with the auxin degron at the C-terminus

The coding sequence for osTIR1 was PCR-amplified from the pNHK60 plasmid using the primers PCR-osTIR1-F and R and subcloned into pcDNA3.1 (Invitrogen) between Ecor1 and Kpn1 restriction sites. The DNA sequence encoding for an eight-amino acid flexible linker followed by AtIAA17 tag (degron) were PCR-ampilfied from pNHK60 plasmid using the primers pBAIDII-1-F and R, and subcloned in-frame with the C-terminal purification tag (a FLAG-$(His_6)_2$ tag) using a pBluescript SK+ derivative plasmid (21) to give rise to pBAIDII-1. The complete tag was then excised from pBAIDII-1 by digestion with Sal1 and BamH1 restriction endonucleases, gel-purified, and subcloned into pBAID2 (a pBluescript derivative containing the 3` homology arm to C-terminally tag *PIP4K2B*) to give rise to pBAIDII-2. The 5` homology arm to tag *PIP4K2B* was PCR-amplified from pBAID3 using pBAIDII-3-F and R primers. The resulting DNA fragment was gel-purified, and ligated into pBAIDII-2 between Kpn1 and Sal1 restriction sites to give rise to pBAIDII-3. Selectable marker cassettes were cloned into pBAIDII-3 using the BamH1 site to give rise to pBAIDII-4 plasmid conferring blasticidin resistance and pBAIDII-5 plasmid conferring puromycin resistance. To create the targeting vector to C-terminally tag *PIP4K2A* with degron, the 5` and 3` homology arms to tag *PIP4K2B* in the pBAIDII-3 plasmid were replaced by the 5` and 3` homology arms to tag *PIP4K2A*, which were PCR-amplified from the construct designed to tag *PIP4K2A* with EmGFP at the C-terminus (described above) using the primers PCR-5`ARM-A-AID-F/R and PCR-3`ARM-A-AID-F/R. The resulting plasmid was called pAAIDII-3. Finally, selectable marker cassettes were cloned into pAAIDII-3 using the BamH1 site to generate pAAIDII-4 conferring blasticidin resistance and pAAIDII-5 conferring puromycin resistance.

Endogenous manipulation of the remaining wild type PIP4K2A allele in $PIP4K2A^{degron/degron/wt}$ cells

A new 5`arm had to be generated again as above but this time using the primers PCR-Ext-5`ARM-IIa-F/R. The old 5`arm to tag *PIP4K2A* and the degron sequence were excised from pAAIDII-4 by Kpn1/Xba1 digestion, and the new 5`arm was then ligated into the resulting plasmid backbone so that to insert the new 5`arm in frame with a C-terminal FLAG-$(His_6)_2$ tag. This new construct (called pKDA4C) was then

subjected to site-directed mutagenesis in order to introduce either the kinase-dead mutation (11) (K366-368Q, two rounds of SDM using the primers SDM-IIa-K366-368-F/R-1 and SDM-IIa-K366-368-F/R-2 (the resulting plasmid was called pKDA4M2) or the substrate switch mutation (11) (A370E, two rounds of SDM using the primers SDM-IIa-A370E-F/R-1 (to introduce silent mutations into residues 368 and 369, and the resulting plasmid was called pKDA4C2) and SDM-IIa-A370E-F/R-2 (to introduce the point mutation A370E, which we called pKDA4T1).

Generation of degron tagged DT40 cell lines

PIP4K2B$^{degron/wt}$ cells were generated by transfection of pBAIDII-4 into the DT40 cell line stably expressing osTIR1. The resistance cassette was floxed out by transient transfection of Cre recombinase (as described below). The resulting cell line was then transfected with pBAIDII-5 to generate the PIP4K2B$^{degron/degron}$ cell line. PIP4K2A$^{degron/wt/wt}$ cell line was generated by transfection of pAAIDII-4 into the DT40 cell line stably expressing osTIR1. The resistance cassette was floxed out as previously, and the resulting cell line was then transfected again with pAAIDII-4 to generate the PIP4K2A$^{degron/degron/wt}$ cell line. Finally, pAAIDII-5 was transfected into the latter cell line to generate the PIP4K2A$^{degron/degron/degron}$ cell line.

The PIP4K2A$^{degron/degron/wt}$ cell line was transfected separately with pKDA4C, pKDA4M2, and pKDAT1 plasmids to generate the cell lines PIP4K2A$^{degron/degron/His-FLAG}$, PIP4K2A$^{degron/degron/kinase\ dead}$, and PIP4K2A$^{degron/degron/substrate\ switch}$ respectively.

Targeted transfection of DT40 cells

Targeting constructs were linearized using an appropriate restriction enzyme, recovered by lithium chloride precipitation, and re-dissolved in PBS. DT40 cells in the logarithmic phase of growth were harvested and washed once in growth medium. $1.5x10^7$ cells were then resuspended and mixed with the DNA solution in an electroporation cuvette with a 4mm electrode gap (Biorad). The cells were then electroporated by discharging an exponentially decaying electrical pulse through them (600V, 25μF, Biorad Gene Pulser XCell). The cells were then immediately seeded out in 96 well plates. 24 hours later the appropriate selection antibiotic was added. Cells were incubated for seven days, by which time single drug-resistant colonies were visible. Colonies were picked from wells containing only one colony. After selection

through antibiotic(s) gDNA was harvested in order to perform test PCR and sequencing reactions to check for correct construct integration.

In order to control for off target effects and clonal variability all cell lines were independently derived three times (with the exception of PIP4K2A$^{-/-/\text{kinase dead EmGFP}}$ cells which were independently derived twice) and results confirmed in these independent clones.

Stable non-targeted transfection of DT40 cells

Stable non-targeted transfectants were generated and grown up from single cell clones in exactly the same way as targeted transfectants. Verification of correct expression was by western blotting and qPCR.

Transient transfection of DT40 cells

Transient transfection of DT40 cells was by electroporation using the same parameters as for targeted transfection described above. Following electroporation cells were seeded into growth medium and incubated overnight to allow time for expression from the transfected plasmid. Owing to the low transfection efficiency of DT40 cells transient transfection was only used where the transfected plasmid encoded a fluorochrome-tagged protein as this allowed successfully transfected cells to be identified by their fluorescence.

Quantitative PCR

RNA was harvested from DT40 cells using Trizol (Sigma) / chloroform / isopropanol extraction and used as a template for production of cDNA using Invitrogen SuperScript III primed from random hexamers. qPCR analysis was performed with a StepOne Plus qPCR machine and pre-designed TaqMan primer probe sets from Invitrogen. Gamma actin (*ACTG*) was used as the reference gene and the efficiency of all primer probe sets was determined by serial dilution. The Pfaffl method was used to calculate relative gene expression between cell lines and conditions in order to allow for correction of the differing amplification efficiencies of the primer probe set for each target gene.

Immunoprecipitation of FLAG-(His)$_6$-tagged proteins

Protein extracts were pre-cleared by incubation with 30 µl of agarose beads for 30 minutes at 4°C with gentle agitation. The agarose beads were pelleted by centrifugation at 13000 RPM for 1 minute using a Hettich Mikro 22-R bench-top centrifuge, and the supernatant was transferred into a new tube. IP of degron-tagged proteins was achieved by incubation of the pre-cleared protein extracts with 20µl of ANTI-FLAG® M2 Affinity Gel (Sigma) with constant rotation for 2 hours at 4°C. Beads were then pelleted by centrifugation at 13000 RPM for 30 seconds, washed three times in ice-cold PBS containing 0.1% triton X-100, and then twice in ice-cold PBS (with no detergent). Beads were finally mixed (1:1) with 2X SDS loading buffer and boiled for 5 minutes to elute proteins.

Stimulation of DT40 cells

DT40 cells in logarithmic growth were pelleted, washed twice in PBS, and incubated for one hour in RPMI with only 2% (v/v) FBS added. This served to serum starve the cells. Cells were then harvested, washed again, and resuspended in wash buffer containing either insulin from bovine pancreas (Sigma) at an appropriate concentration (see results), or mouse monoclonal M4 anti-chicken IgM antibody (Abcam) at a final concentration of 2µg/ml. Following stimulation cells were incubated for ten minutes prior to analysis.

Western blotting

Following denaturing SDS-PAGE electrophoresis of cell free extracts protein species were transferred onto an appropriate blotting membrane, which was probed with antibodies as detailed in supplementary materials and methods and detected using chemiluminescent reagents of an appropriate sensitivity.

Lipid kinase assays

These were performed exactly as previously described (30).

Estimation of plasma membrane 3-phosphoinositdes

DT40 cells were transiently transfected with the plasmid EGFP-PH-Akt, which encodes the PH domain of Akt fused to EGFP (13). In order to quantify recruitment of the PH domain of Akt to the plasma membrane the freely available software

ImageJ (31) was used. Regions of interest were drawn both around the plasma membrane and in the cytosol and after background subtraction the mean pixel intensity in the plasma membrane and the cytosol was determined. The ratio of the two values was calculated to give a background corrected plasma membrane to cytoplasm ratio of the PH domain of Akt as a surrogate measurement of plasma membrane 3-phosphoinositides. The absolute value of this ratio is largely meaningless; the power of the technique lies in comparing the ratio between different DT40 cell lines. All cells were analysed blind to cell genotype.

Confocal Microscopy

Images were acquired with a Leica SP5 confocal microscope attached to a Leica DMI6000 inverted microscope stand. For GFP imaging excitation was with the 488nm line of the argon laser and emission was detected between 500 and 550nm. For all imaging the confocal pinhole was set to 1 Airy unit, and an oil immersion 40x 1.25 NA objective lens was used. All imaging was performed on live cells, which were embedded in Matrigel to prevent cell movement and transferred to uncoated glass bottom MatTek dishes. Just prior to imaging full growth medium was removed and replaced with RPMI without phenol red. Imaging was performed at 41°C under 5% CO_2.

Mass Spectrometry Quantification of lipids

This was carried out exactly as in (26) using a QTRAP 4000 (AB Sciex) mass spectrometer and employing the lipid extraction and derivatization method described for cultured cells, with the modification that C17:0/C16:0 PtdIns(3,4,5)P_3/PtdInsP_2 (10ng/ 0.175ng) and C17:0/C16:0 PtdIns (100ng) internal standards were added to primary extracts, and that final samples were dried in a Speedvac concentrator rather than under N_2. Measurements were conducted, in triplicate per experiment, on 2x10e6 cells per sample.

Primers

Primer name	**Primer sequence (5' to 3')**
2A5FOR2	TTA AGG TAC CGA GCT GCT GAG TGA TCC TAA AG
2A5REV2	GCA TTC TAG ACG TCA AGA TGT TGG CAA TAA AG
SDM5	CAA GTA GGG ACA CAT TGC ATC CAA CTG CAG TAG TTC AG
SDM6	CTG AAC TAC TGC AGT TGG ATG CAA TGT GTC CCT ACT TG
EGFP_PCR_FOR_1	TCT TCA TCT AGA ATG GTG AGC AAG GGC GAG GAG
EGFP_PCR_REV_1	CAC CAG GGA TCC TTA CTT GTA CAG CTC GTC CAT GC
2A3FOR2	TAA TGG ATC CCC CCT CAT GTC ACA CCG GAC
2A3REV2	TCA TGC GGC CGC TGC TAC ACA GAC AGA AAG TG
GGPIP4K2A_DEL_5'_FOR_2	TGG TGG TAC CTA GTG GGA GGA CTT GCA AAT GAG G
GGPIP4K2A_DEL_5'_REV_2	ACC AGG ATC CAA TCA GGT GTC AGA CCA CTT CAC G
GGPIP4K2A_DEL_3'_FOR	TAT AGG ATC CTC TGT GGG TAG TGT GCT TAG GTG
GGPIP4K2A_DEL_3'_REV	ATT AGC GGC CGC CTG CCA GTC TGT CCA TCC TCT GA
2b_Ful_Del_PCR_5'_For_1	TCA AGG TAC CTG GAG AGC AGA TTG TCA CAC
2b_Ful_Del_PCR_5'_Rev_1	TGA CGG ATC CCA GAA AGA CAC ACA TAC ACA C
2b_Ful_Del_PCR_3'_For_1	TGC AGG ATC CGC TTC CCT CGT GCT AAG GAT TG
2b_Ful_Del_PCR_3'_Rev_1	TAA TGC GGC CGC GCT ACC TAC ATC CAG CAC AG
2a_Kin_Dead_PCR_For_2	TAA TGG TAC CTT CCC ACT TAG TCC TCC GCT C
2a_Kin_Dead_SDM_F	CTC AGC TAA AAC TCA TGA AAT ACA GCT TGC TAG TTG G
2a_Kin_Dead_SDM_R	CCA ACT AGC AAG CTG TAT TTC ATG AGT TTT AGC TGA G
2alpha to type 1 SDM FOR	GAT GCA AAA AAG AAA GCT GAG CAT GCT GCA AAA ACA GT
2alpha to type 1 SDM REV	ACT GTT TTT GCA GCA TGC TCA GCT TTC TTT TTT

	GCA TC
GGPIP4K2a_OE_FOR	TAA TAT AAG CTT GCT ATG GCG GCC CCC GGC ACC GTC
GGPIP4K2a_OE_REV	GAT AAT GGT ACC CGT CAA GAT GTT GGC AAT AAA GTC
PCR-osTIR1-F	ATA TGA ATT CAT GAC GTA CTT CCC GGA GGA G
PCR-osTIR1-R	ATA TGG TAC CTT ACC ACT AGC AGC AGA ACC GGA G
pBAIDII-1-F	ATA TGT CGA CGG AGC TGG TGC AGG CG
pBAIDII-1-R	ATA TTC TAG AAG CTC TGC TCT TGC ACT T
pBAIDII-3-F	ATA TGG TAC CGT GTG CAG TTT GTC TCA GTC C
pBAIDII-3-R	ATA TGT CGA CTG TCA GGA TAT TGG ACA TA
PCR-5`ARM-A-AID-F	ATA TGG TAC CGA GCT GCT GAG TGA
PCR-5`ARM-A-AID-R	AGT AGT CGA CCG TCA AGA TGT TGG
PCR-3`ARM-A-AID-F	ATA TGG ATC CCC CCT CAT GTC ACA
PCR-3`ARM-A-AID-R	ATA TAT GCG GCC GCT GCT ACA C
PCR-Ext-5`ARM-IIa-F	ATA TGG TAC CGA CCT GCA TGC TAT CAC TAC AT
PCR-Ext-5`ARM-IIa-R	ATG ATC TAG ACG TCA AGA TGT TGG
SDM-IIa-K366-368Q-1-F	ACT CAT TAT GAT GCA CAA CAG AAA GCT GC
SDM-IIa-K366-368Q-1-R	CAT GGG CAG CTT TCT GTT GTG CAT CAT AA
SDM-IIa-K366-368Q-2-F	ATG CAC AAC AAC AAG CTG CCC ATG CTG
SDM-IIa-K366-368Q-2-R	ATG GGC AGC TTG TTG TTG TGC ATC ATA A
SDM-IIa-A370E-1-F	GCA AAA AAG AAG GCC GCC CAT GCT GCA AA
SDM-IIa-A370E-1-R	GCA TGG GCG GCC TTC TTT TTT GCA TCA
SDM-IIa-A370E-2-F	GCA AAA AAG AAG GCC GAG CAT GCT GCA AA
SDM-IIa-A370E-1-R	GCA TGC TCG GCC TTC TTT TTT GCA TCA

Antibodies

Target	**Manufacturer and Product Number**
Akt (total)	Cell Signaling Technology #9272.
Akt (phospho-Ser473)	Cell Signaling Technology #4060.
Akt (phospho-Thr308)	Cell Signaling Technology #13038.

Mouse IgG (H+L) peroxidase conjugated	Vector Laboratories PI-2000.
Rabbit IgG (H+L) peroxidase conjugated	Abcam ab16284.
β-actin	Sigma A2228.
6X His tag	Abcam ab9108.
SGK-1	Abcam ab43606.
phospho-SGK-1 (Ser422)	Santa Cruz Biotechnology SC16745R.
PKCα	BD Biosciences 610108.
phospho-PKCα (Ser657)	Millipore 06822.
p70S6K	Cell Signaling Technology (2708).
phospho-p70S6K (Thr389)	Cell Signaling Technology (9234).

Supplementary references

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28. Buerstedde JM, & Takeda S (Eds.) (2006) Reviews and Protocols in DT40 Research. New York: Springer.
29. Arakawa H, Lodygin D, & Buerstedde JM (2001) Mutant loxP vectors for selectable marker recycle and conditional knock-outs. *BMC biotechnology* 1:7.
30. Clarke JH & Irvine RF (2013) Evolutionarily conserved structural changes in phosphatidylinositol 5-phosphate 4-kinase (PI5P4K) isoforms are responsible for differences in enzyme activity and localization. *Biochem J* 454(1):49-57.
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Supplementary figure legends

Figure S1

A

An example of a DAPI stained DT40 metaphase spread with the macrochromosomes and sex chromosomes identified and re-arranged.

B

Cells were seeded in full growth medium at a density of $2.5x10^5$ viable cells per ml of and their growth observed over the next 48 hours without replenishing the medium. There is no significant difference in the growth rates of any of the cell lines. Means and 95% confidence intervals plotted. n=3 biological replicates.

Figure S2

PIP4K2B knockout cells exhibit off-target down-regulation of PI5P4Kα message and protein.

A

Quantification of *PIP4K2A* and *PIP4K2B* mRNA in *PIP4K2B* null cells ($PIP4K2B^{-/-}$) and in these same cells when they are stably overexpressing PI5P4Kβ. Bars show mean and 95% confidence interval. The *PIP4K2A* mRNA in $PIP4K2B^{-/-}$ cells is reduced to 52% of wild type, 95%CI 47% to 57%. Overexpression of PI5P4Kβ in $PIP4K2B^{-/-}$ cells does not rescue *PIP4K2A* message (mean 53%, 95%CI 39% to 68%).

B

The fluorescence signal from the PI5P4Kα-EmGFP fusion protein when one *PIP4K2A* allele is endogenously tagged with the EmGFP coding sequence in either a *PIP4K2B* wild type background or a *PIP4K2B* null background.

Figure S3

Compromised Akt phosphorylation in $PIP4K2B^{-/-}$ cells is due to off-target downregulation of PI5P4Kα.

A

The phosphorylation status of Akt at Thr308 and Ser473 in *PIP4K2B* null cells, and in the same cells when overexpressing either PI5P4Kβ or PI5P4Kα.

B

The phosphorylation status of Akt at Thr308 and Ser473 in *PIP4K2B* null cells, and in the same cells when overexpressing PI5P4Kα as in figure S3A. In addition the phosphorylation status of Akt is shown when the overexpressed PI5P4Kα is mutated to encode either a kinase dead enzyme or an enzyme with predominantly PI4P 5-kinase activity (see materials and methods).

C

The plasma membrane to cytoplasm ratio of EmGFP-PH-Akt as a 3-phosphoinositide reporter in the cell lines shown in figure S3B. Bars show median and interquartile range. Pooled data from three biological replicates.

Dunn's multiple comparison tests as follows:

DT40 WT versus PIP4K2B$^{-/-}$	P<0.001
PI5P4Kα rescue versus PIP4K2B$^{-/-}$	P<0.001
PI5P4Kα$^{\text{kinase dead}}$ rescue versus PIP4K2B$^{-/-}$	P>0.05
PI5P4Kα$^{\text{substrate switch}}$ rescue versus PIP4K2B$^{-/-}$	P<0.001

Figure S4

Levels of PI5P4Kα and PI5P4Kβ mRNA in *PIP4K2A* null cells (PIP4K2A$^{-/-/-}$) relative to wild type DT40 cells. Bar shows mean and 95% confidence interval.

Figure S5

Analysis of cells in which PI5P4Kα is knocked down by stable shRNA (PIP4K2A$^{\text{shRNA}}$ cells).

A

Levels of PI5P4Kα and PI5P4Kβ mRNA in PIP4K2A$^{\text{shRNA}}$ cells relative to wild type DT40 cells. Bars show mean and 95% confidence interval. n=3 biological replicates.

B

Phosphorylation status of Akt at Thr308 and Ser473 in PIP4K2A^{shRNA} cells. Cells were synchronized in exponential growth prior to analysis. A sample from PIP4K2B$^{-/-}$ cells (see figure S3) is included for comparison.

Figure S6

Further mass spectrometry data from stable knockout cell lines.

A

PIP_3 levels expressed relative to phosphatidylinositol (PI) to correct for cell number. PIP_3 and PI internal standards present in assay. Data from the three cell lines that exhibit normal Akt phosphorylation in figure 2 are combined i.e. WT DT40, PIP4K2A$^{-/-/EmGFP}$ and PIP4K2A$^{-/-/substrate\ switch\ EmGFP}$ cells. Data from the two cell lines that exhibit deficient Akt phosphorylation in figure 1 are combined i.e. PIP4K2A$^{-/-/-}$ and PIP4K2A$^{-/-/kinase\ dead\ EmGFP}$ cells. Data is pooled for 38:4, 38:3 and 36:2 PIP_3. Chart displays means and 95% confidence intervals. The central estimates show that PIP3 levels in cells with deficient Akt phosphorylation are 65% of those in cells with normal Akt phosphorylation, $P<0.0001$, unpaired t-test. Pooled data from 3 replicates.

B

PIP_2 levels expressed relative to PI to correct for cell number in PIP4K2A$^{-/-/-}$ cells. PIP_2 and PI internal standards are present in the assay.

Figure S7

Test of the PI5P4Kβ-degron system. Blots of the PI5P4Kβ-degron fusion protein are anti-poly-His blots of anti-FLAG immunoprecipitates.

A

Endogenous auxin degron-tagged PI5P4Kβ can be acutely removed by auxin addition in a similar manner to degron-tagged PI5P4Kα (see figure 3).

B

When cells are washed and seeded into fresh growth medium the effects of auxin addition are reversible.

Figure S8

PI5P4Kβ was acutely removed from PIP4K2B$^{degron/degron}$ cells by treatment with 500 micromolar auxin for the times shown. The phosphorylation status of Akt at Thr308 and Ser473 was monitored. Cells were synchronized in exponential growth prior to analysis.

Figure S9

Stimulus specificity in the regulation of Akt phosphorylation by PI5P4Kα in PIP4K2A$^{degron/degron/degron}$ cells. A representative blot comparing Akt phosphorylation at the Ser473 site in response to either insulin or M4 stimulation (see materials and methods) in the presence or absence of PI5P4Kα. Treatment with 500 micromolar auxin for one hour. The numbers below the blot are quantification of the p-Ser473-Akt to total Akt ratio by densitometry. Results normalized to the ratio in serum-starved cells.

Figure S10

Representative images from cells in which PI5P4Kα is endogenously tagged with EmGFP.

A

Paired brightfield and confocal images of endogenously tagged PI5P4Kα in PIP4K2A$^{EmGFP/EmGFP/wt}$ cells.

B

Confocal images of the EmGFP signal from PIP4K2A$^{EmGFP/EmGFP/EmGFP}$ cells and PIP4K2A$^{-/-/EmGFP}$ cells. The same illumination and detection settings are used in each case.

Figure S11

The effect of TOR inhibition on the phosphorylation status of mTORC2 targets.

A

The extent of dephosphorylation of Akt at Ser473 upon treatment of exponentially growing DT40 cells with various concentrations the TORC2 inhibitor torin for 60 minutes. DMSO is the vehicle for torin.

B

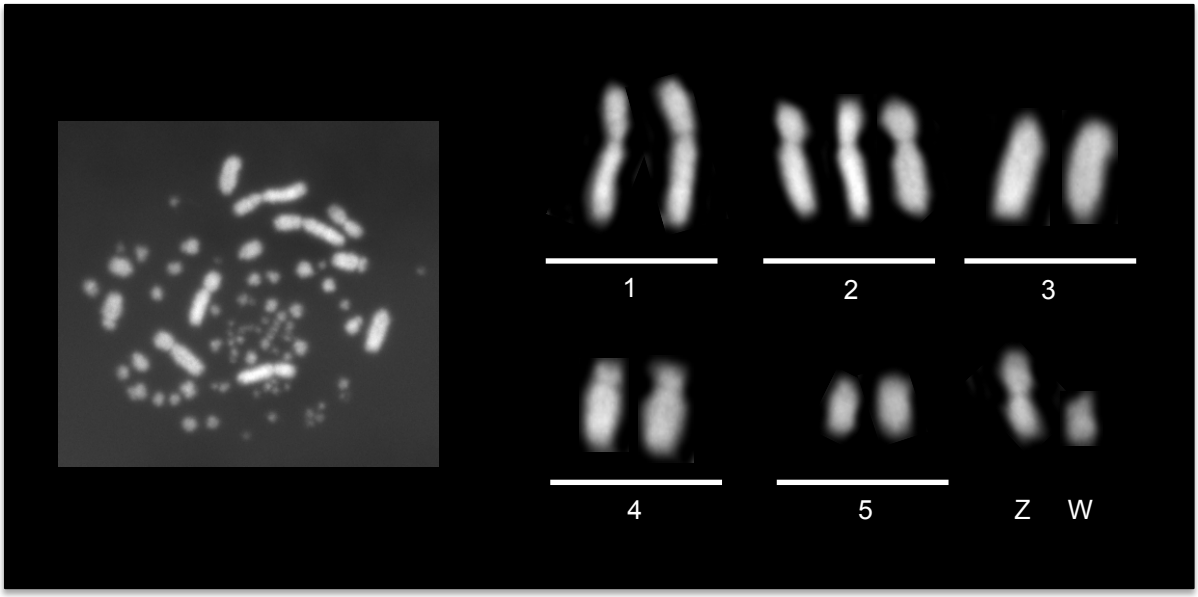
Exponentially growing PIP4K2A$^{degron/degron/degron}$ cells were treated with either auxin or vehicle for 60 minutes, and then the rate of dephosphorylation of Akt at Ser473 was assayed upon inhibiton of TORC2 with torin.

C

Exponentially growing PIP4K2A$^{degron/degron/degron}$ cells were treated with either auxin or vehicle for 60 minutes, and then the rate of dephosphorylation of SGK1 at Ser422 was assayed upon inhibiton of TORC2 with torin.

Figure S1

A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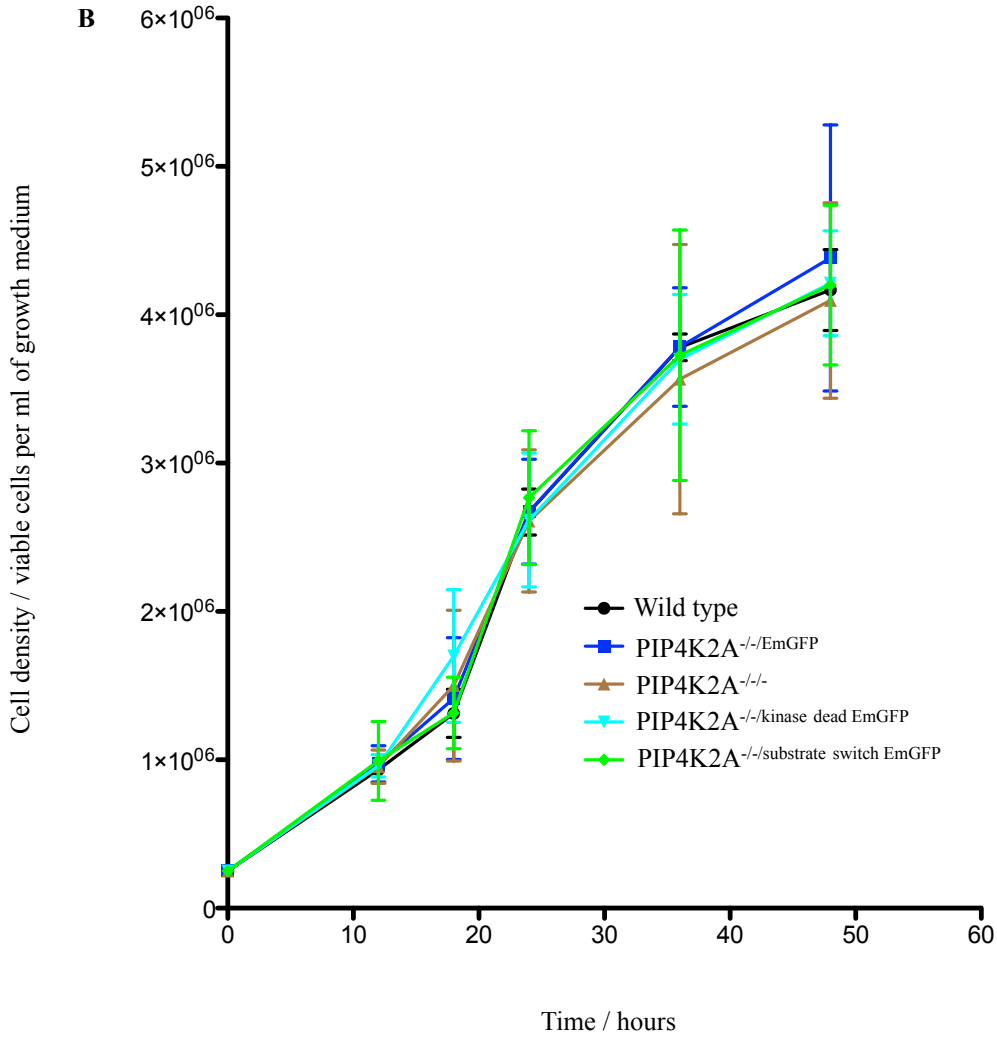
2
3
4
5
Z
W

B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3×10^06
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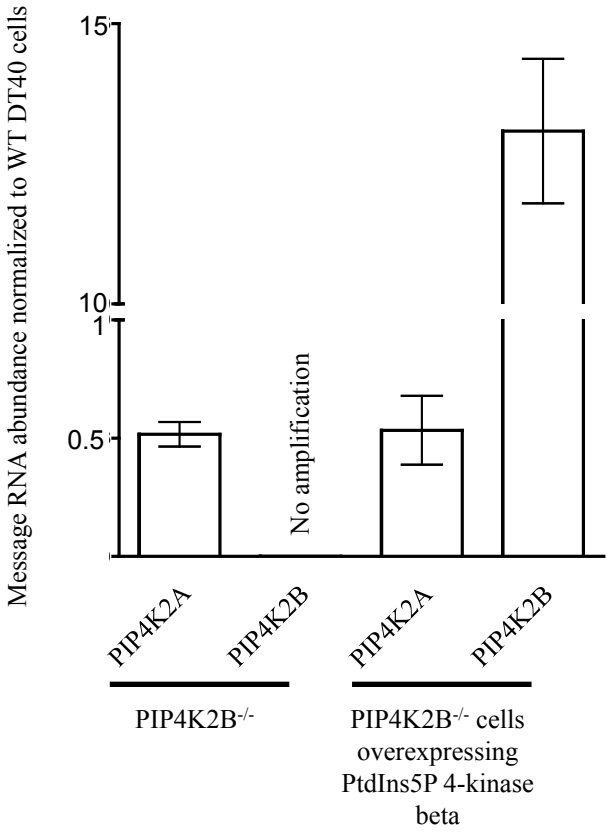
0
0
10
20
30
40
50
60
Cell density / viable cells per ml of growth medium
Time / hours
Wild type
PIP4K2A-/-/EmGFP
PIP4K2A-/-/-
PIP4K2A-/-/kinase dead EmGFP
PIP4K2A-/-/substrate switch EmGFP

Figure S2

A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10
1
0.5
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PIP4K2A
PIP4K2B
PIP4K2A
PIP4K2B
PIP4K2B-/-
PIP4K2B-/- cells overexpressing PtdIns5P 4-kinase beta

B

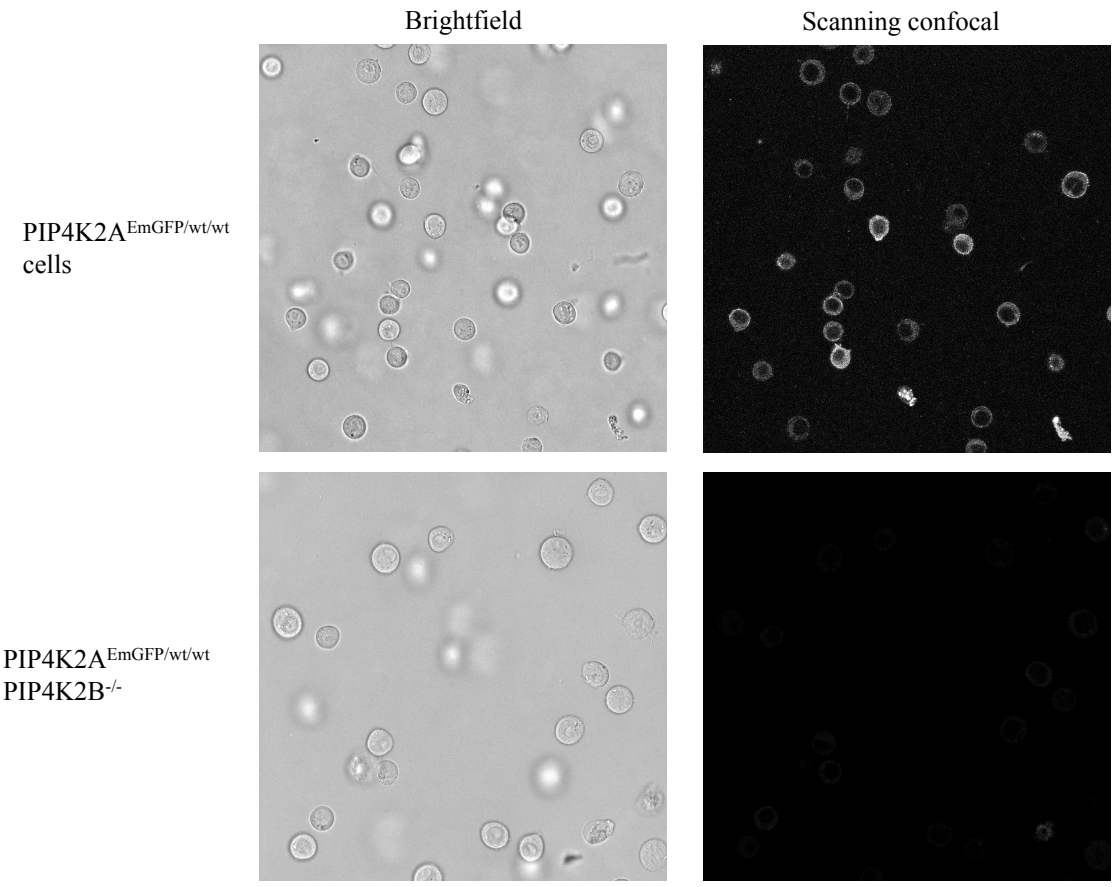
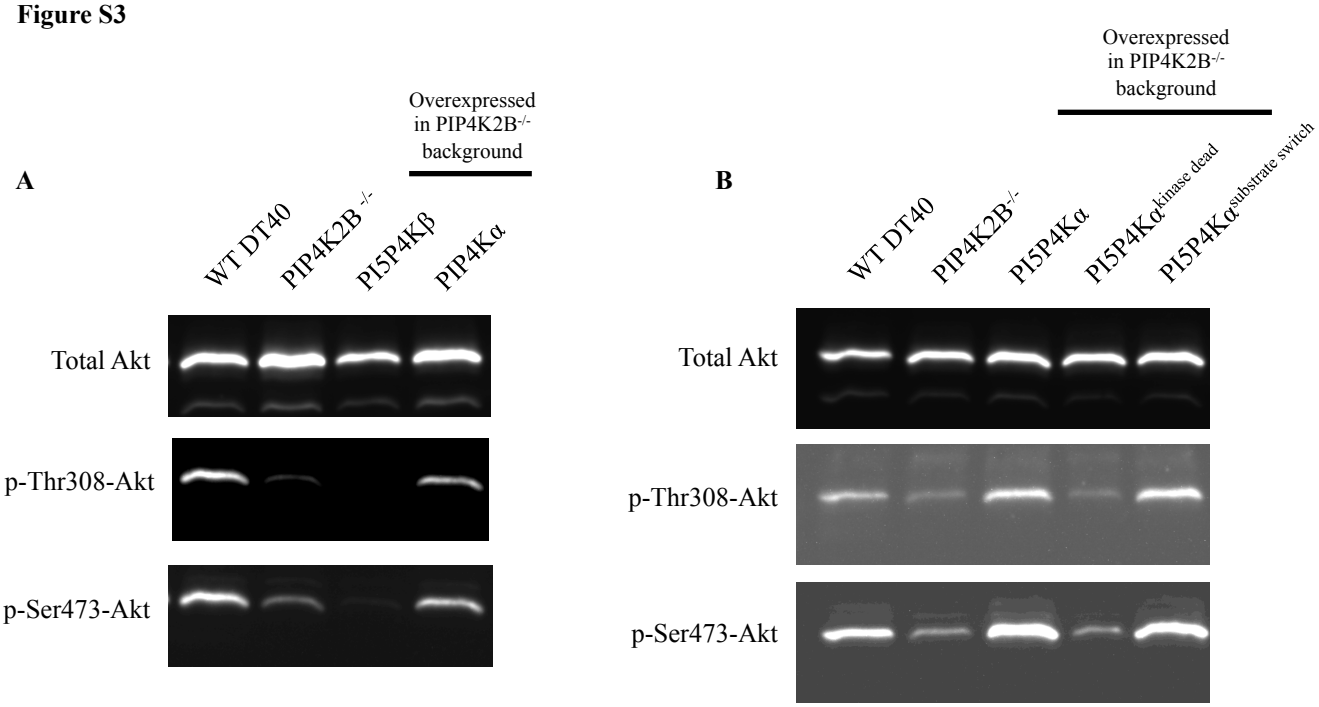

Brightfield
Scanning confocal
PIP4K2AEmGFP/wt/wt cells
PIP4K2AEmGFP/wt/wt PIP4K2B-/-

Figure S3


A
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WT DT40
PIP4K2B -/-
PI5P4Kβ
PIP4Kα
Total Akt
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p-Ser473-Akt
B
Overexpressed in PIP4K2B-/- background
WT DT40
PIP4K2B-/-
PI5P4Kα
PI5P4Kα kinase dead
PI5P4Kα substrate switch
Total Akt
p-Thr308-Akt
p-Ser473-Akt

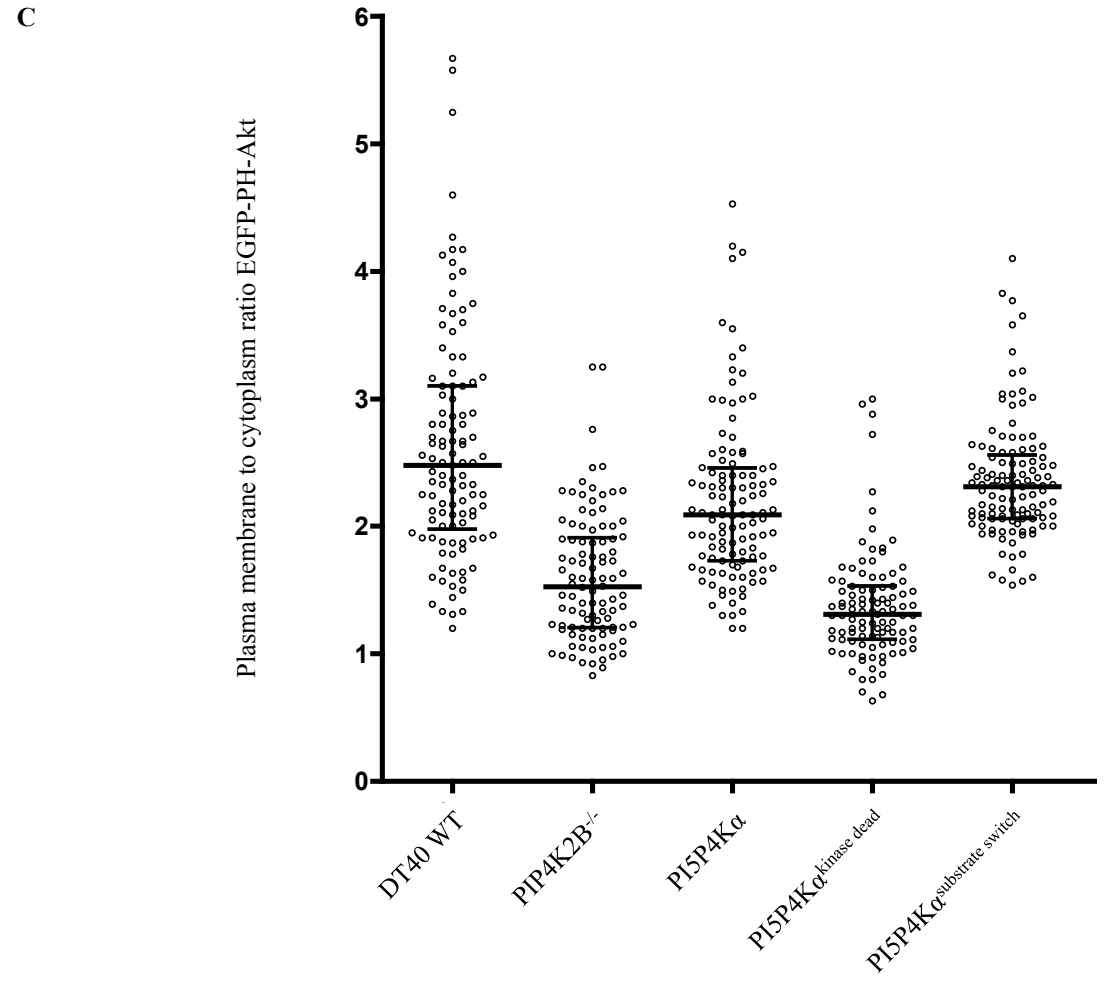

C
Plasma membrane to cytoplasm ratio EGFP-PH-Akt
0
1
2
3
4
5
6
DT40 WT
PIP4K2B-/-
PI5P4Kα
PI5P4Kα kinase dead
PI5P4Kα substrate switch

Figure S4

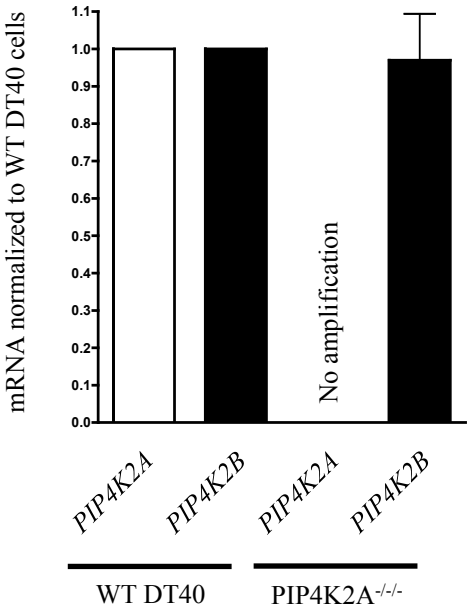
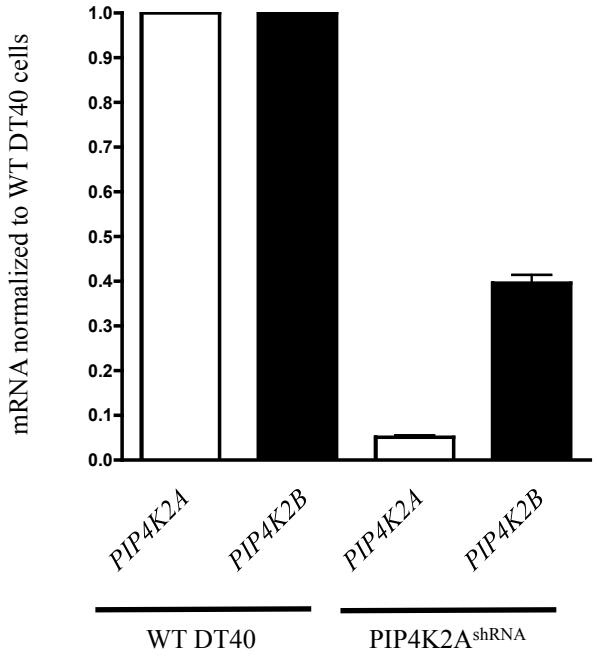

mRNA normalized to WT DT40 cells
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
No amplification
PIP4K2A
PIP4K2B
PIP4K2A
PIP4K2B
WT DT40
PIP4K2A-/-/-

Figure S5

A


mRNA normalized to WT DT40 cells
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
PIP4K2A
PIP4K2B
PIP4K2A
PIP4K2B
WT DT40
PIP4K2A shRNA

B

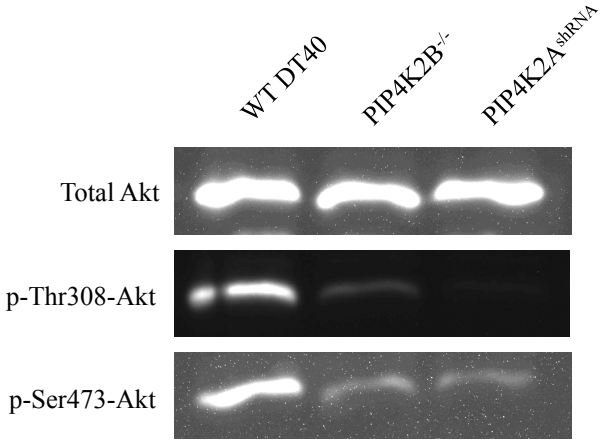
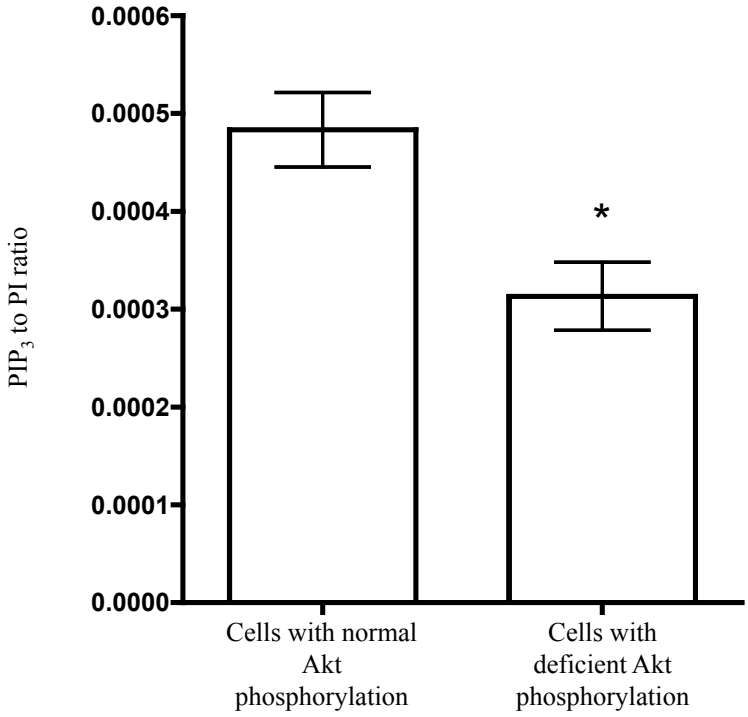

WT DT40
PIP4K2B-/-
PIP4K2A shRNA
Total Akt
p-Thr308-Akt
p-Ser473-Akt

Figure S6

A


0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
PIP3 to PI ratio
*
Cells with normal Akt phosphorylation
Cells with deficient Akt phosphorylation

B

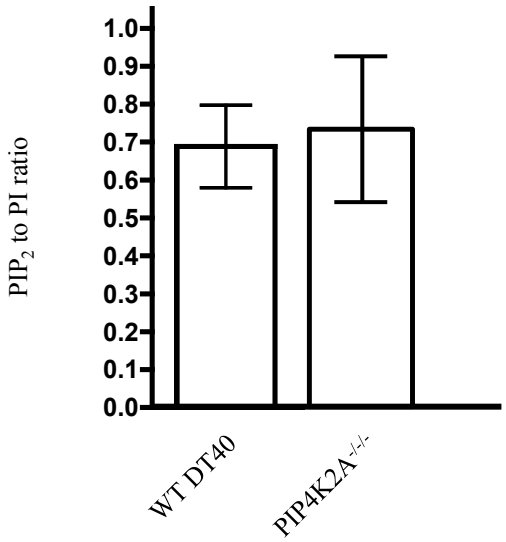
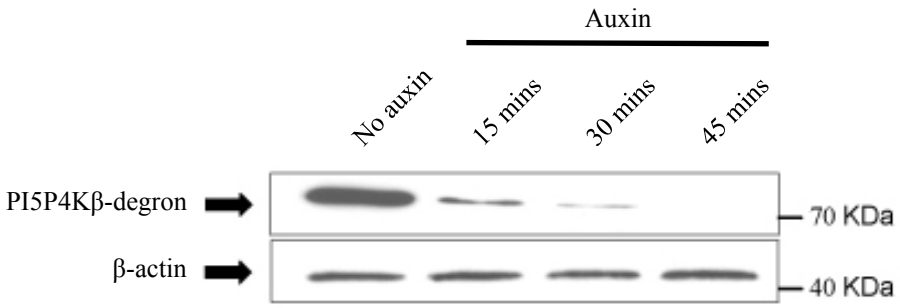

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
PIP2 to PI ratio
WT DT40
PIP4K2A-/-

Figure S7

A


Auxin
No auxin
15 mins
30 mins
45 mins
PI5P4Kβ-degron
β-actin
70 KDa
40 KDa

B

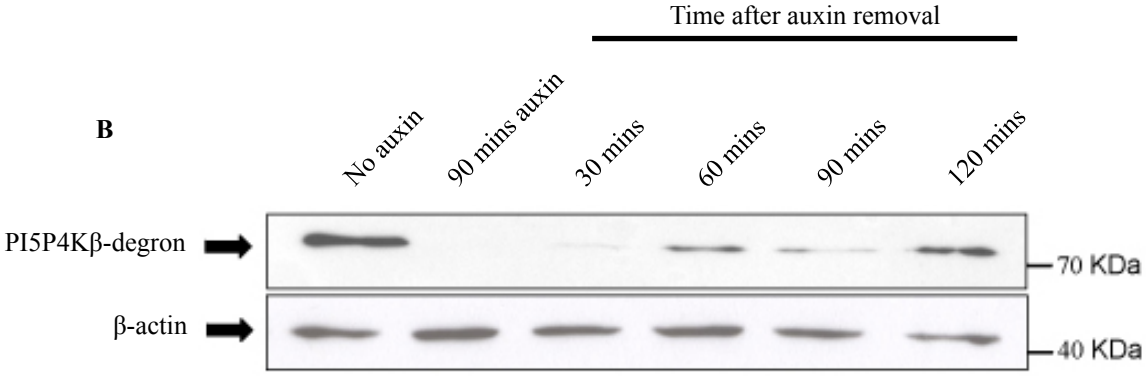

Time after auxin removal
No auxin
90 mins auxin
30 mins
60 mins
90 mins
120 mins
PI5P4Kβ-degron
β-actin
70 KDa
40 KDa

Figure S8

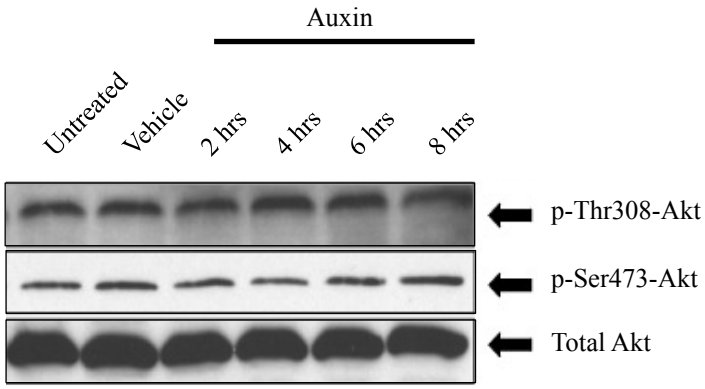
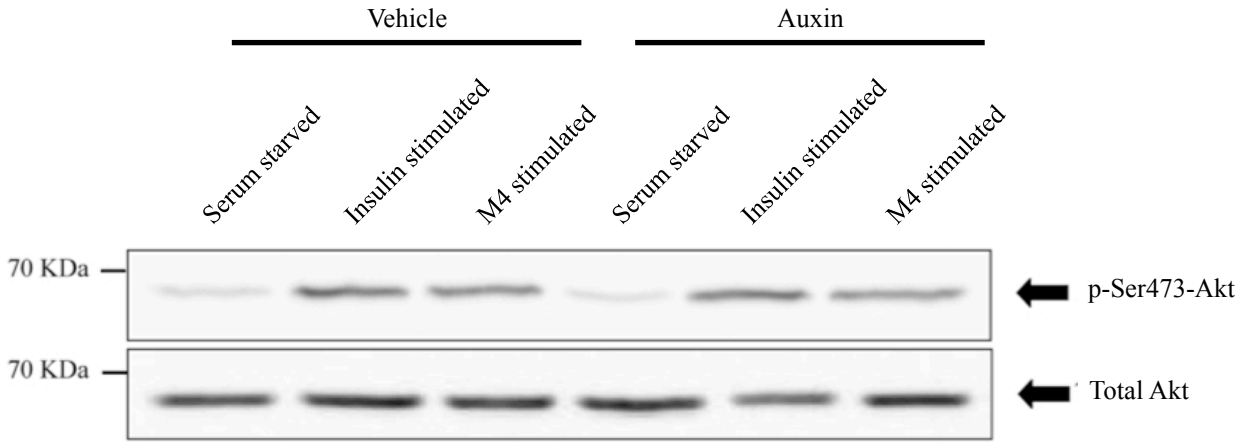

Auxin
Untreated
Vehicle
2 hrs
4 hrs
6 hrs
8 hrs
p-Thr308-Akt
p-Ser473-Akt
Total Akt

Figure S9



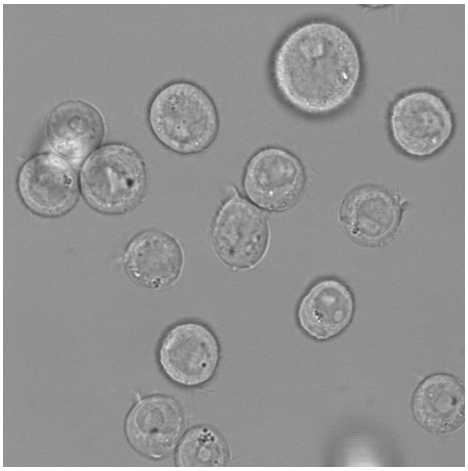
p-Ser473-Akt to total Akt ratio.	1	5.1	5.1	1	11.8	5.1

Figure S10

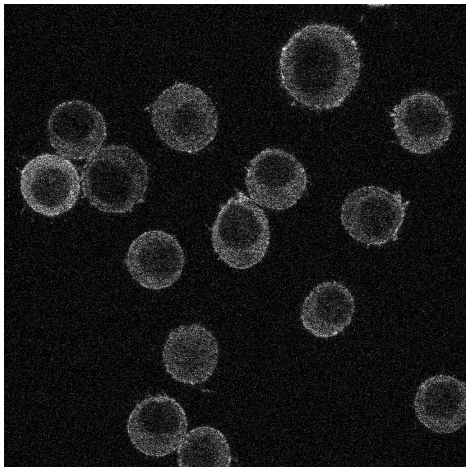
A

PIP4K2A$^{EmGFP/EmGFP/wt}$

Brightfield

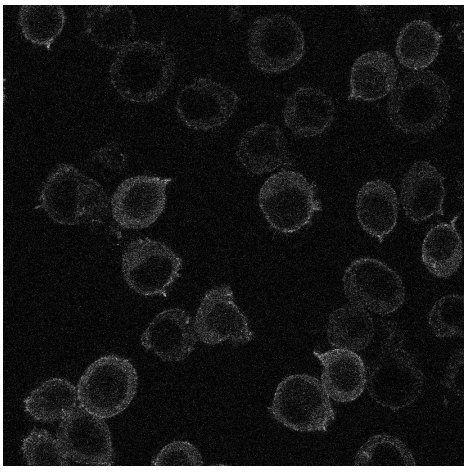


EmGFP



B

PIP4K2A$^{EmGFP/EmGFP/EmGFP}$



PIP4K2A$^{-/-/EmGFP}$

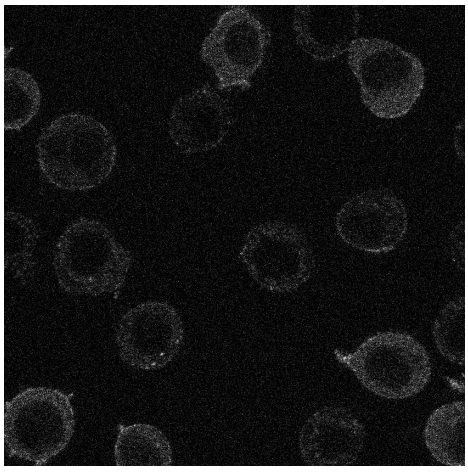
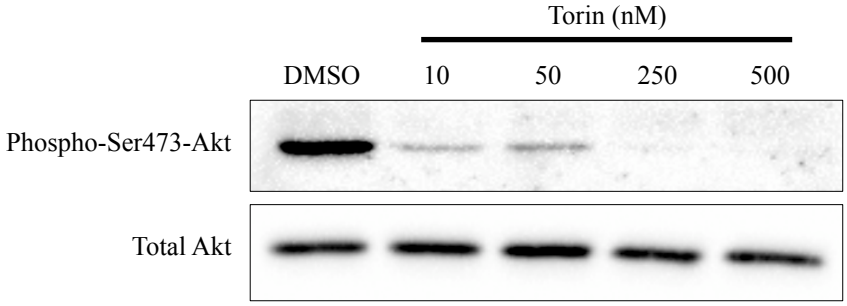
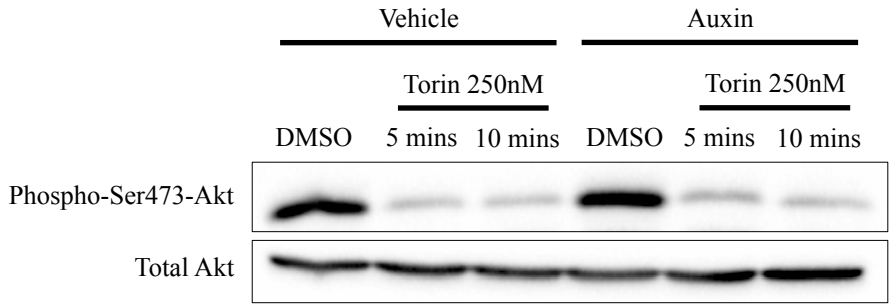


Figure S11

A



B



C