Inositol polyphosphate multikinase is a physiologic PI3-kinase that activates Akt/PKB

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Inositol polyphosphate multikinase (IPMK) is a member of the IP6 kinase family of enzymes but is not primarily associated with the formation of inositol phosphates. IPMK physiologically generates PIP3, as well as water soluble inositol phosphates. IPMK deletion reduces growth factor-elicited Akt signaling and cell proliferation caused uniquely by loss of its PI3-kinase activity. Inhibition of p110 PI3-kinases by wortmannin prevents IPMK phosphorylation and activation. Thus, growth factor stimulation of Akt signaling involves PIP2 generation through the sequential activations of the p110 PI3-kinases and IPMK. As inositol phosphates inhibit Akt signaling, IPMK appears to act as a molecular switch, inhibiting or stimulating Akt via its inositol phosphate kinase or PI3-kinase activities, respectively. Drugs regulating IPMK may have therapeutic relevance in influencing cell proliferation.

Results and Discussion

Because the very early embryonic lethality (E9.5) of conventional IPMK knockouts precludes harvesting tissues, such as fibroblasts (10), we obtained murine embryonic fibroblasts (MEFs) from mice harboring a conditionally targeted IPMK allele (Fig. 1A). Expression of Cre recombinase in these MEFs abolished IPMK expression (Fig. 1A). IPMK is a rate-limiting enzyme in the formation of multiple inositol phosphates. In MEFs lacking IPMK, synthesis of IP3, IP4, and IP5, is abolished, but IP4 formation is reduced about 90% (Fig. 1B), resembling findings of York and colleagues (10) in embryonic stem cells of IPMK knockout mice. To ascertain whether IPMK possesses physiologic PI3K activity in intact cells, we labeled MEFs with [3H]inositol and examined the formation of PIP3 (Fig. 1C). In cells treated with FBS, we observe a 50% decrease in PIP3 generation in IPMK-deleted cells. Thus, IPMK appears to be a physiologic PI3K.

Wortmannin, a potent inhibitor of nearly all known PI3Ks, abolishes PIP3 formation in a wide range of cells (18). It does not, however, inhibit IPMK directly, even at high concentrations (12). Accordingly, we postulated that IPMK is positively regulated by a wortmannin-sensitive PI3K, and that treatment of cells with wortmannin could thereby decrease the formation of PIP3 by IPMK. To test this hypothesis, we isolated IPMK from HEK293T cells treated with 5 or 50 nM wortmannin and monitored PIP3 formation by the purified enzyme (Fig. 1D). The PI3K activity of IPMK is reduced by about 70% at both concentrations. Thus, wortmannin inhibits IPMK in vivo with potency similar to that for p110 inhibition. Given that many protein kinases are activated in response to PIP3 production, we hypothesize that phosphorylation of IPMK by such a kinase is required to switch on the PI3K activity of IPMK in cells. This notion is supported by our observation that dephosphorylation of IPMK with protein phosphatase after purification from untreated HEK293T cells reduces the PI3K activity of the enzyme to a similar extent as wortmannin treatment.

These experiments establish that IPMK is a physiologic PI3K interposed within a PIP3/protein kinase signaling pathway, wherein phosphorylation of IPMK in a wortmannin-sensitive manner regulates the activity of PI3K.
cellular IP5 levels in these cells indicates a 50% loss of IPMK.

If IPMK is a physiologically important PI3K, then it ought to influence Akt signaling. Akt activation requires PI3-dependent phosphorylation at two sites, T308 and S473 (19). However, a distinct mechanism involving phosphorylation of IPMK appears to positively regulate its inositol phosphate kinase activity, as λ protein phosphatase treatment dramatically reduces IPMK’s inositol phosphate kinase activity.

If IPMK is a physiologically important PI3K, then it ought to influence Akt signaling. Akt activation requires PI3-dependent phosphorylation at two sites, T308 and S473 (19). As IPMK appears to be activated by PI3K generated by another PI3K, we wondered whether the phosphorylation of IPMK appears to positively regulate its inositol phosphate kinase activity, as λ protein phosphatase treatment dramatically reduces IPMK’s inositol phosphate kinase activity.

To explore in greater depth the role of IPMK in Akt signaling, we used IPMK-null MEFs (Fig. 2C). Phosphorylation of Akt at both T308 and S473 in response to FBS is reduced by 50% in IPMK-deleted MEFs. IPMK mediates signaling to multiple Akt targets, as levels of phospho-PRAS40, phospho-TSC2, phospho-FoxO1/3A, and phospho-GSK3β are diminished in IPMK-deleted MEFs. The decreased signaling is selective for the Akt pathway, because levels of phospho-Erk1/2, which are not Akt targets, are not altered in the IPMK knockouts.

We also examined Akt activation in response to the growth factors EGF, IGF, and insulin (Fig. S3A). Phospho-Akt-T308 levels in response to these growth factors are substantially reduced in IPMK-deleted MEFs with the greatest reduction being for EGF signaling. We evaluated the time course of EGF-dependent Akt phosphorylation (Fig. 2D and Fig. S3B). In both wild-type and IPMK-deleted MEFs, EGF markedly increases Akt signaling with substantial augmentation evident within one minute and maximal at 3 to 4 min. Akt activation is reduced about 50% in the IPMK-null cells.

Growth factor-dependent Akt phosphorylation and activation requires translocation of Akt from the cytosol to the plasma membrane by virtue of the specific affinity of the Akt plekstrin homology domain for PI3 (23). We investigated this process in IPMK-deleted MEFs (Fig. 2E and Fig. S4A). EGF elicits a 2.4-fold increase in membrane levels of Akt in wild-type MEFs with a 60% reduction in this response evident in IPMK-deleted cells. In both wild-type and IPMK-null cells, the translocation is virtually abolished following wortmannin treatment. We detect substantial amounts of endogenous IPMK in both membrane and cytoplasmic fractions similar to the disposition of Akt, although the relative distribution between these two fractions appears insensitive to growth factors and wortmannin (Fig. S4A). Earlier studies conducted primarily with overexpressed IPMK reported a predominant nuclear localization.
(9, 12). We detect endogenous IPMK in both nuclear and cytoplasmic fractions in MEFs (Fig. S4B).

We wondered whether regulation of Akt by IPMK is because of its PI3K or inositol phosphate kinase activities. To explore this question, we sought a form of IPMK that might possess only one of these two activities. We noted that the Arabidopsis thaliana ortholog of IPMK, atIPK2β, possesses inositol phosphate kinase activity comparable to mouse IPMK but is devoid of PI3K activity (Fig. 3A). In contrast, mutating lysine 129 of mouse IPMK (mIPMK-K129A) virtually abolishes both PI3K and inositol phosphate kinase activities. In IPMK-deleted MEFs, ectopic expression of mIPMK or atIPK2β, but not mIPMK-K129A, restores inositol phosphate production to normal levels (Fig. 3B). We next explored the ability of these various forms of IPMK to rescue the loss of Akt signaling in IPMK-deleted MEFs (Fig. 3C and Fig. S5). As observed previously, in wild-type cells phospho-Akt-T308 levels increase 10-fold 3 to 4 min following treatment with EGF. This activation is reduced 40 to 50% in IPMK-deleted MEFs. Overexpression of wild-type IPMK restores phospho-Akt-T308 to normal levels, but no rescue is evident with mIPMK-K129A or atIPK2β. Thus, although some catalytic activity is required, the inositol phosphate kinase activity of atIPK2β does not suffice to stimulate Akt signaling. Accordingly, we conclude that it is the PI3K activity of IPMK that is predominantly responsible for mediating IPMK’s augmentation of Akt signaling.

These findings establish that the PI3K activity of IPMK physiologically enhances Akt signaling. Akt is a major determinant of cell proliferation. Accordingly, we examined the influence of IPMK on this process. In IPMK-deleted MEFs, the rate of cell proliferation is reduced about 50% (Fig. 4A). In U87MG glioma cells, depletion of IPMK by RNA interference results in a 32% reduction in the rate of proliferation (Fig. 4B). IPMK-depleted U87MG cells also exhibit a reduced rate of anchorage-independent growth relative to control cells (Fig. 4C). Consistent with this finding, overexpression of IPMK causes a 35% increase in the rate of anchorage-independent growth of U87MG cells (Fig. 4D). This process requires the PI3K activity of the enzyme, as a kinase-dead variant and atIPK2β fail to elicit an effect. Thus, IPMK appears to promote cell proliferation in both cancerous and noncancerous cells.

In summary, we established that IPMK is a physiologic PI3K interposed in a PIP3/protein kinase signaling pathway. Thus, depletion of PIP3 by wortmannin markedly reduces IPMK’s PI3K activity by decreasing IPMK phosphorylation, which appears to be important for its catalytic activity. IPMK is a major determinant of Akt signaling, as its deletion leads to a 50% decrease in growth-factor dependent Akt activation. The regulation of Akt by IPMK is selective, as IPMK knockout does not affect the ERK signaling system. IPMK’s regulation of Akt is attributable to its PI3K activity and not its inositol phosphate kinase activity. Finally, the
regulation of Akt signaling by IPMK impacts cell growth, which is markedly diminished with IPMK deletion.

The inositol phosphate kinase and the PI3K activities of IPMK appear to be differentially regulated. For example, in intact cells wortmannin inhibits the PI3K activity but not the inositol phosphate kinase activity. The products of these two activities generally oppose one another in their effects on cellular physiology and the Akt pathway. PIP3 promotes cellular proliferation

![Fig. 3. The PI3K activity of IPMK is required for full activation of Akt in response to EGF.](image)

Fig. 3. The PI3K activity of IPMK is required for full activation of Akt in response to EGF. (A) Comparison of in vitro specific activities of mouse IPMK, mouse IPMK-K129A, and atlpk2β. His-tagged recombinant proteins were purified from Escherichia coli, as previously described for IP6K1 (35). All reactions were monitored over time and specific activities were calculated based on the linear range of the reaction curves. (B) Wild-type IPMK, and atlpk2β were stably expressed in IPMK−/− MEFs. Equal numbers of each cell type were labeled with [3H]myo-inositol. After extraction, inositol phosphates were resolved by HPLC and quantified by scintillation counting. Data represent the sums of cellular IP5, IP6, and IP7, and are means of three independent experiments. Error bars represent SEs. (C) Wild-type IPMK restores EGF-induced Akt phosphorylation in IPMK−/− MEFs, but IPMK-K129A and atlpk2β do not. Equal numbers of the same stable cell lines used in the experiment shown in B were plated, serum-starved overnight, stimulated with EGF, and lysed. Lysates were analyzed for phospho-Akt-T308, total Akt, and myc-IPMK expression by immunoblotting. Data are means of three independent experiments and error bars are SEs. For representative raw data, see Fig. S5.

![Fig. 4. IPMK promotes the proliferation of both MEFs and U87MG glioma cells.](image)

Fig. 4. IPMK promotes the proliferation of both MEFs and U87MG glioma cells. (A) Comparison of proliferation of wild-type and IPMK−/− MEFs. Equal numbers of each cell type were plated, and then harvested and counted at the indicated time intervals. Trypan blue staining indicated that viability was >98% throughout the experiment for all cell lines. Data are means of three independent experiments and error bars represent SEs. Two IPMK−/− cell lines were tested to control for clonal variation. In all panels, P values were calculated using a two-tailed unpaired student’s t test. Statistical comparisons were made between wild-type and IPMK−/− cells at each time point. (B) Depletion of IPMK by RNA interference impairs the proliferation of U87MG cells stably expressing either control or IPMK-targeting siRNAs. Equal numbers of each cell line were plated, allowed to proliferate for 72 h, and analyzed for relative cell number by the MTT assay (Millipore). Data are means of six replicates and the error bars represent the SDs. (C) Depletion of IPMK by RNA interference reduces anchorage-independent growth of U87MG cells. Anchorage-independent growth was assayed using the Cytoselect soft agar colony-formation assay (Cell Biolabs), as recommended by the manufacturer. Data are means of six replicates and error bars represent SEs. (D) The PI3K activity of IPMK promotes anchorage-independent growth of U87MG cells. U87MG cells were transfected with myc vector, myc-hIPMK, a kinase dead variant of myc-hIPMK (KD) or myc-atlpk2β (AT), and analyzed for anchorage-independent growth. Data are means of six replicates and error bars represent SEs. Only wild-type IPMK causes a statistically significant increase in anchorage-independent growth relative to control cells. *P < 0.05, **P < 0.01; no designation indicates P > 0.05.
and survival, largely via activation of Akt. In contrast, a number of reports indicate that higher-order inositol phosphates antagonize proliferation and tumorigenesis and promote apoptosis (24–29). Moreover, several studies indicate that higher inositol phosphates specifically decrease Akt activity (26, 27, 30–32). The differential regulation of PIP3 and inositol phosphate production by IPMK may provide a process enabling cells to switch between Akt activation and inhibition, with corresponding influences upon cellular proliferation and survival (Fig. 5). Recently we showed that IP F generated by IP6 kinase-1 physiologically inhibits Akt by preventing its phosphorylation and activation by PDK1 (30). This finding suggests that IP6K1 and IPMK, both members of the same family, have opposing physiologic activities. Interestingly, IPMK−/− cells have depleted levels of PIP3 and IP7 (Fig. 1), yet still have reduced Akt activity, which suggests that the fraction of Akt activation attributable to IPMK could be greater than what we have observed here. At the very least, it appears that, under normal growth conditions in embryonic fibroblasts, the ability of IPMK to activate Akt is dominant to the inhibition of Akt by IP F. Key to understanding the interplay between these opposing activities will be elucidation of the mechanism whereby IPMK switches between PI3K and inositol phosphate production. It is thus essential to identify the kinases responsible for phosphorylation of IPMK. Many kinases, both proximal and distal, are activated by IP F. Given that IPMK localizes to plasma membranes, we hypothesize that a proximal kinase, such as Akt or PDK1, could be responsible for activating its PI3K activity. However, we cannot rule out a downstream kinase, such as mTOR or S6 kinase. Regarding activation of IPMK’s inositol phosphate kinase activity, it is tempting to speculate that a conventional isoform of PKC is responsible. Given that elevated IP F levels lead to activation of such kinases, phosphorylation and activation of IPMK would coincide with elevated levels of a substrate necessary for higher order inositol phosphate production. We are currently pursuing both pharmacological and genetic approaches to address these possibilities. Finally, these findings may have therapeutic relevance. Depletion of IPMK either by genetic deletion or RNA interference leads to decreased cell growth. Accordingly, drugs that inhibit IPMK might offer therapeutic utility in inhibiting the growth of tumors.

**Materials and Methods**

**Generation and Maintenance of Mice and Cell Lines.** For generation and maintenance of mice and cell lines, see SI Materials and Methods.

**Measurement of Cellular Inositol Phosphates and Phosphoinositides.** For inositol phosphate measurements, MEFs were plated at a density of 2 × 10^4 per well in six-well plates and incubated for 3 d in complete culture medium in the presence of 60 μCi per well [3H]myo-inositol (PerkinElmer). After washing three times with ice-cold PBS, inositol phosphates were extracted into 200 μL ice-cold lysis buffer (0.6 M perchloric acid, 0.2 mg/mL IP F and neutralized with 1 M K2CO3. Extracts were clarified by centrifugation and inositol phosphate species were resolved by anion exchange HPLC, as previously described (12).

For phosphoinositide measurements, isolated glycerophosphoinositides were prepared as in ref. 12 after 72 h of labeling using 20 μCi/mL [3H]inositol (Perkin-Elmer).

**Preparation of Immunoprecipitates for in Vitro Enzyme Activity Assays.** For assays using immunoprecipitated enzyme, HEK-293T cells were transiently transfected with either empty myc vector, myc-WT-mIPMK, or myc-mIPMK-K129A using Polyfect (Qiagen) according to the manufacturer’s recommendations. Thirty-six hours after transfection, cells were treated with either wortmannin or DMSO vehicle. Cells were washed twice in cold PBS and lysed in 40 mM Hepes, pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, protease inhibitor mixtures 1 and 2 (Sigma), and phosphatase inhibitor mixtures 1 and 2 (Sigma). Immunoprecipitations were performed using 1 μg of cell lysates, 0.4 μg of anti-myc antibody (Roche), and 40 μL of protein G Plus/protein A-agarose suspension (Calbiochem). Immunoprecipitates were washed three times in lysis buffer and then once in 1× NEBuffer for protein metallophosphatase (PMP). Immunoprecipitates were then incubated in 1× NEBuffer for PMP supplemented with 1 mM MnCl2, in the presence of lambda protein phosphatase (New England Biolabs) for 45 min at 30 °C. Immunoprecipitates were again washed three times in lysis buffer, and then washed once in 1× IPMK reaction buffer. Reactions were performed as described below. After removing the reaction mixture, immunoprecipitates were washed twice with PBS and then resuspended in 2× NuPAGE LDS Sample Buffer (Invitrogen). HRP-conjugated anti-myc antibody (Roche) was used in immunoblots to confirm the presence of equal amounts of immunoprecipitated enzyme in each assay.

**In Vitro PI3K-Kinase Activity Assays.** PI(4,5)P2 was resuspended via sonication in of 20 mM Hepes (pH 7.4), 1 mM EDTA, and 0.5% deoxycholate. Reactions were performed with immunoprecipitates or with 200 nM His-tagged purified recombinant protein in a total volume of 50 μL containing 10 μL of lipid

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**Fig. 5.** Model describing a role for IPMK in both activation and inhibition of Akt. In response to extracellular stimuli such as growth factors, the p110/p85 PI3Ks produce PIP3 in the plasma membrane, which results in the activation of a host of kinases, including Akt. One such kinase phosphorylates IPMK, activating its PI3K activity and resulting in a feed-forward loop necessary for maximal PIP3 levels and Akt activity. A distinct kinase, perhaps in response to some other specific signal, phosphorylates IPMK on a different site, thus activating its inositol phosphate kinase activity. Inositol phosphates including IP3, IP5, and IP6 have been reported to antagonize Akt signaling.
resuspension, providing a final concentration of 0.03 mg/mL purified synthetic PI(4,5)P2 (Avanti Polar Lipids). Reaction buffer consisted of 20 mM Hepes (pH 7.5), 6 mM MgCl2, and 10 μCi of [γ-32P]ATP (PerkinElmer-NEN; 6,000 mCi/mmol; 1 Ci = 37 GBq) in a carrier of 100 μM unlabeled ATP. Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90 μL of 1 M HCl/methanol (1:1, by volume). Lipids were extracted twice with 100 μL of chloroform/dried, down, and resuspended in chloroform/methanol (2:1, by volume). Lipids were resolved on silica gel 60 TLC plates in a solvent system consisting of water/propanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

In Vitro Inositol Phosphate Kinase Activity Assays. Reactions were carried out with immunoprecipitates or with 500 nM purified His-tagged enzyme, as previously described (12).

Growth Factor Treatments and Immunoblotting. Before growth factor treatment, MEFs were plated at a density of 5 × 105 cells per well in six-well plates. To minimize the impact of differences in growth rates of various cell lines, cells were growth-arrested by overnight serum-starvation 3 h after plating (the minimum time necessary for attachment to the plates). Cells were then stimulated (for 5 min if not otherwise indicated) with 10% FBS, 33 nM inositol 1,4,5-trisphosphate and an inositol pyrophosphate.

Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

To minimize the impact of differences in growth rates of various cell lines, cells were growth-arrested by overnight serum-starvation 3 h after plating (the minimum time necessary for attachment to the plates). Cells were then stimulated (for 5 min if not otherwise indicated) with 10% FBS, 33 nM inositol 1,4,5-trisphosphate and an inositol pyrophosphate.

Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

To minimize the impact of differences in growth rates of various cell lines, cells were growth-arrested by overnight serum-starvation 3 h after plating (the minimum time necessary for attachment to the plates). Cells were then stimulated (for 5 min if not otherwise indicated) with 10% FBS, 33 nM inositol 1,4,5-trisphosphate and an inositol pyrophosphate.

Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

To minimize the impact of differences in growth rates of various cell lines, cells were growth-arrested by overnight serum-starvation 3 h after plating (the minimum time necessary for attachment to the plates). Cells were then stimulated (for 5 min if not otherwise indicated) with 10% FBS, 33 nM inositol 1,4,5-trisphosphate and an inositol pyrophosphate.

Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

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Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

To minimize the impact of differences in growth rates of various cell lines, cells were growth-arrested by overnight serum-starvation 3 h after plating (the minimum time necessary for attachment to the plates). Cells were then stimulated (for 5 min if not otherwise indicated) with 10% FBS, 33 nM inositol 1,4,5-trisphosphate and an inositol pyrophosphate.

Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

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Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

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Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

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Reactions were incubated at 37 °C for 15 min, and stopped by adding the reaction mixture to 90% (v/v) isopropanol/glacial acetic acid (34:65:1, by volume). Alternatively, reactions were stopped by spotting aliquots on nitrocellulose for analysis by the membrane capture method, as described (33). For all in vitro PI3K assays, reactions were monitored as a function of time and activities were calculated based on the linear range.

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SI Materials and Methods

Generation and Maintenance of Floxed Inositol Polyphosphate Multikinase Mice. The gene encoding mouse inositol polyphosphate multikinase (IPMK), Ipmk, is located on chromosome 10 and has six exons. Exon 6 is composed of 4,681 bp, of which the first 609 bp encode the C-terminal region of IPMK (amino acids 194–396), and the remaining 4,062 bp give rise to the 3' UTR of the mRNA. Our strategy was to delete the coding region of exon 6, including the exon 6 splice site. The resulting transcript would lack exon 6 (including the 3' UTR) and therefore be unstable, resulting in a complete knockout. Alternatively, cryptic splicing may occur between exon 5 and the remainder of exon 6, possibly generating a stable mRNA lacking the exon 6 coding sequence, resulting in a catalytically inactive, truncated protein lacking the C-terminal subdomain.

IPMK+/− mice, as well as heterozygous floxed IPMK mice, were generated at Oogene. The targeting construct was based on the sequence of the C57BL/6 strain Ipmk gene (Ensembl gene ID: ENSMUSG00000060733). A loxP site was inserted between exons 5 and 6, 51 bp upstream of the exon 6 splice site. A phosphoglycerate kinase (PGK) Neo cassette flanked by Flp recombinase target (f rt) sequences and another loxP site was inserted 2,476 bp downstream of the stop codon in exon 6. The targeting vector was electroporated into 129SV/J ES cells, and Neomycin-resistant ES cells were microinjected into C57BL/6 blastocysts and implanted into pseudo-pregnant female mice. The resulting chimeric mice were crossed with knockin C57BL/6 mice carrying Cre recombinase driven by a PGK promoter to generate heterozygous mice carrying the IPMK knockout allele and Cre recombinase (IPMK+/−/Cre). These mice were interbred to generate F1 heterozygous mice lacking Cre (IPMK+/−/+). Targeted heterozygous floxed IPMK mice were also crossed with C57BL/6 mice carrying FlPe recombinase driven by the Gt(Rosa)26Sor promoter to generate heterozygous mice carrying the IPMK floxed allele devoid of the Neo cassette. These mice were interbred, and F1 heterozygous mice lacking Flp (IPMK+/−/lox) were subsequently bred to IPMK−/−/lox mice to generate heterozygous mice carrying both the IPMK knockout allele and the IPMK floxed allele (IPMK−/−/lox). These mice were interbred to generate homozygous floxed IPMK mice (IPMK−/−/lox).

All mice were maintained on a 129SV-C57BL/6 mixed background. Animal care and experiments were approved by the Johns Hopkins University Animal Care and Use Committee. Mice were housed in a 12-h light/12-h dark cycle, at an ambient temperature of 22 °C, and fed standard rodent chow.

Genotyping. Mice were genotyped by PCR analysis of genomic DNA from tail biopsies. Genotyping was performed with two sets of primers, one for the knockout allele and another for the floxed allele. The forward primer for the knockout allele P1 (5'-AATTCACCTCTGATGAGCCAGG-3') and the reverse primer P2 (5'-GGCATTACCGAATCTGTCGCACTG-3') yielded a 324-bp product from the knockout allele. The forward primer for the floxed allele P3 (5'-GGGATGCACCTCATGGAAAGGACTTGTGG-3') and the reverse primer P2 yielded a 472-bp product from the floxed allele and a 517-bp product from the wild-type allele.

Cell Lines and Culture Conditions. HEK293T cells and U87MG cells were from American Type Culture Collection. Murine embryonic fibroblasts (MEFs) were harvested from E14 homozygous floxed embryos, as previously reported (1). All cell lines used in this study were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Gemini Bio-products), 1-glutamine (2 mM; Invitrogen), and penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO2 humidified atmosphere. For stably transfected cell lines, blasticidin (4 μg/mL; Invitrogen) was included to maintain transgene expression during culture maintenance.

Floxed MEFs were immortalized by transfection with pS5-Large T (deposited by William Hahn to Addgene) using Polyfect transfection reagent (Qiagen) according to the manufacturer's protocol. After serial-passaging transected cells five times to select against nontransformed cells, IPMK−/−/lox cells were generated by transduction of floxed cells with adenovirus carrying the gene encoding the Cre recombinase (University of Iowa Gene Transfer Vector Core). To enhance transduction efficiency, virus was combined with GeneJammer (Stratagene) transfection agent, as reported (2). After transduction, clonal populations were colony purified and screened for IPMK expression. Two representative IPMK−/−/lox cell lines were chosen for further analysis. Throughout this study, IPMK−/−/+ lines were compared with the parental floxed line (herein referred to as "wild-type"), which is isogenic other than the lack of IPMK expression.

For rescue experiments, IPMK−/− cells were transduced with MMLV. Myc epitope-tagged wild-type mouse IPMK, IPMK-K129A, and atlpk2 cDNAs were subcloned into the pMXs-IREs-Blasticidin retroviral vector (Cell Biosols). The resulting constructs were transduced into the Platinum-E packaging line (Cell Biosols) using Fugene 6, as reported (3). MEFs were incubated for 48 h with viral supernatants and stable transductants were selected with blasticidin.

For RNA interference experiments, constructs were prepared using the BLOCK-it Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen) using pcDNA6.2-GW/EmGPmiR and pcDNA6.2-GW/EmGPmiR-neg control plasmid, per the manufacturer’s recommendations. IPMK targeting inserts were constructed using the following inserts: TGCTGTTCAGGGAGTCTGACTCTGTTTTGGGCGACTGACTGAGTG-CACTTCCCTGAA and CCTGTTCAGGGAAAGTGCATCCTGTTCTTGGCAGACTGACATCTCCCTGAA. Constructs’ knock-down efficacy was tested and confirmed by conformation of myc-hIPMK in 293T cells (Lipofectamine 2000) followed by immunoblotting for myc-tag. Stable, polyclonal U87 lines were generated using ViraPower HiPerform Lentiviral FastTiter Gateway Expression Kit and selection for stable integration.

**Fig. S1.** Targeting strategy for the development of IPMK−/− cells. Exon 6 of the *ipmk* gene was flanked by directly repeated loxP sites to generate a colony of targeted (“floxed”) mice. These mice were overtly identical to wild-type mice, and for the purposes of this publication “wild-type” refers to cells derived from these targeted mice. Upon expression of the Cre recombinase, cells homozygous for the targeted allele delete exon 6, which contains key catalytic residues and the entire 3′ untranslated region. This results in a complete loss of function, as confirmed by metabolic labeling experiments (Fig. 1).

**Fig. S2.** Stable expression of a siRNA targeting IPMK transcripts reduces cellular IP5 levels by 50% in U87MG cells. U87MG cells were stably transfected with plasmids encoding either a control siRNA or a siRNA targeting IPMK. Equal numbers of each cell type were labeled to metabolic equilibrium with [3H]myo-inositol. After extraction, inositol phosphates were resolved by HPLC and quantified. As IPMK is the sole source of IP5 in cells, this experiment confirms significant depletion of IPMK by RNA interference.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>IPMK−/− (Clone 1)</th>
<th>IPMK−/− (Clone 2)</th>
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</thead>
<tbody>
<tr>
<td>EGF</td>
<td>26.2 ± 1.2</td>
<td>9.6 ± 0.5</td>
<td>13.6 ± 1.1</td>
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<tr>
<td>IGF</td>
<td>21.8 ± 6.0</td>
<td>14.3 ± 3.0</td>
<td>16.0 ± 4.3</td>
</tr>
<tr>
<td>Insulin</td>
<td>10.5 ± 2.6</td>
<td>5.7 ± 0.4</td>
<td>6.2 ± 1.4</td>
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</tbody>
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**Fig. S3.** IPMK mediates the activation of Akt in response to multiple growth factors. (A) Phosphorylation of Akt-T308 in response to EGF, IGF, and insulin. Equal numbers of each cell line were plated, serum-starved overnight, stimulated with the indicated growth factor for 5 min, and lysed. Lysates were analyzed for phospho-Akt-T308 and total Akt by immunoblotting. Data are presented as fold-increases in phospho/total Akt relative to untreated cells of the same line. Data are the means of three independent experiments ± SEs. Two IPMK−/− lines were analyzed to control for clonal variation. (B) Time course of EGF-dependent Akt phosphorylation in wild-type and IPMK−/− MEFs. Equal numbers of each cell line were plated, serum-starved overnight, stimulated for 0, 1, 2, 3, 4, or 8 min with EGF, and lysed. Lysates were analyzed for phospho-Akt-T308 or total Akt by immunoblotting.
Fig. S4. Subcellular distribution of IPMK and Akt. (A) IPMK promotes translocation of Akt to the plasma membrane in response to EGF. Equal numbers of wild-type and IPMK−/− MEFs were plated, serum-starved overnight, stimulated with EGF for 3 min, and fractionated into S100 (cytoplasm) and P100 (crude membrane) fractions. Fractions were analyzed for total Akt, caveolin, and GAPDH by immunoblotting. (B) IPMK is abundant in both cytoplasmic and nuclear fractions. Equal numbers of wild-type and IPMK−/− cells were treated as indicated and fractionated into cytoplasmic and nuclear fractions. Fractions were analyzed by immunoblotting for IPMK, tubulin, and HDACII. IPMK−/− cells were included as a control for the specificity of the anti-mIPMK antibody.

Fig. S5. The PI3K activity of IPMK is required for full activation of Akt in response to EGF. Wild-type mouse IPMK, IPMK-K129A, or atIpk2β were stably expressed in IPMK−/− MEFs. Equal numbers of each cell type were plated, serum-starved overnight, stimulated with EGF for 0, 1, 2, 3, 4, or 8 min with EGF, and lysed. Lysates were analyzed by immunoblotting for phospho-Akt-T308, total Akt and myc.