Supporting Information

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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 Ozgene. 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 phosphoglycerine kinase (PGK) Neo cassette flanked by FLP recombinase target (frt) 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 pesudo-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 heteryzygous 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 ($IPMK^{+/flox}$) were subsequently bred to $IPMK^{+/-}$ mice to generate heterozygous mice carrying both the IPMK knockout allele and the IPMK floxed allele ($IPMK^{-/flox}$). These mice were interbred to generate homyzygous floxed IPMK mice ($IPMK^{flox/flox}$).

All mice were maintained on a 129SV-C57BL/6 mixed background. Animal care and experimentations 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′-AATTCACTCTGTAGACCAGG-3′) and the reverse primer P2 (5′-GGCATTCAGGAATCGGTTCTCACTCG-3′) yielded a 324-bp product from the knockout allele. The forward primer for the floxed allele P3 (5′- GGGATGCACTCATGGAAAGGACTTGTG-3′) and the reverse primer P2 yielded a 472-bp product from the floxed allele and a 317-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), L-Glutamine (2 mM; Invitrogen), and penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO_2 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 pSG5-Large T (deposited by William Hahn to Addgene) using Polyfect transfection reagent (Qiagen) according to the manufacturers protocol. After serial-passaging transfected cells five times to select against nontransformed cells, $IPMK^{-/-}$ 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 reagent, as reported (2). After transduction, clonal populations were colony purified and screened for IPMK expression. Two representative $IPMK^{-/-}$ clonal 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 atIpk2β cDNAs were subcloned into the pMXs-IRES-Blasticidin retroviral vector (Cell Bioloabs). The resulting constructs were transfected into the Platinum-E packaging line (Cell Bioloabs) 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/EmGFPmiR and pcDNA6.2-GW/EmGFPmiR-neg control plasmid, per the manufacturer's recommendations. IPMK targeting inserts were constructed using the following inserts: TGCTGTTTCAGGGAAGTCTGACTCTGGTTTTGGCCACTGACTGACCAGAGTCACTTCCCTGAA and CCTGTTTCAGGGAAGTGACTCTGGTCAGTCAGTGGCCAAAACCAGAGTCAGACTTCCCTGAAAC. Constructs' knock-down efficacy was tested and confirmed by contrasfection of myc-hIPMK in 293T cells (Lipofectamine 2000) followed by immunobloting for myc-tag. Stable, polyclonal U87 lines were generated using ViraPower HiPerform Lentiviral FastTiter Gateway Expression Kit and selection for stable integration.

1. Kang BN, et al. (2010) Death-associated protein kinase-mediated cell death modulated by interaction with DANGER. *J Neurosci* 30:93–98.
2. Bosch P, Fouletier-Dilling C, Olmsted-Davis EA, Davis AR, Stice SL (2006) Efficient adenoviral-mediated gene delivery into porcine mesenchymal stem cells. *Mol Reprod Dev* 73:1393–1403.
3. Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007) Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* 2:3081–3089.

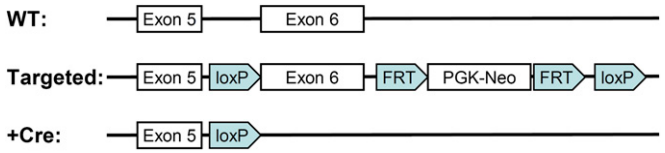

Fig. S1. Targeting strategy for the development of IPMK$^{-/-}$ cells. Exon 6 of the *Ipmk* gene was flanked by directly repeated loxP sites to generate a colony of targeted ("floxed") mice. These mice were overtly identical to wild-type mice, and for the purposes of this publication "wild-type" refers 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).

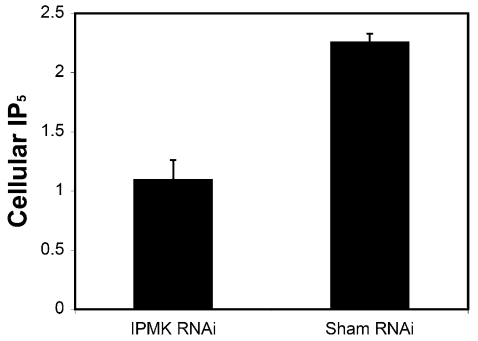

Fig. S2. Stable expression of a siRNA targeting IPMK transcripts reduces cellular IP_5 levels by 50% in U87MG cells. U87MG cells were stably transfected with plasmids encoding either a conrol siRNA or a siRNA targeting IPMK. Equal numbers of each cell type were labeled to metabolic equilibrium with [^{3}H]*myo*-inositol. After extraction, inositol phosphates were resolved by HPLC and quantified. As IPMK is the sole source of IP_5 in cells, this experiment confirms significant depletion of IPMK by RNA interference.

A.

	Wild Type	IPMK$^{-/-}$ (Clone 1)	IPMK$^{-/-}$ (Clone 2)
EGF	26.2 ± 1.2	9.6 ± 0.5	13.6 ± 1.1
IGF	21.8 ± 6.0	14.3 ± 3.0	16.0 ± 4.3
Insulin	10.5 ± 2.6	5.7 ± 0.4	6.2 ± 1.4

B.

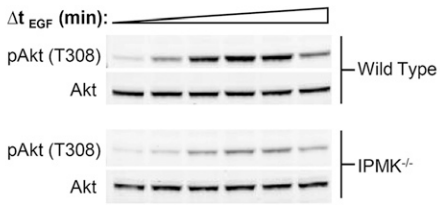

Fig. S3. IPMK mediates the activation of Akt in response to multiple growth factors. (*A*) Phosphorylation of Akt-T308 in response to EGF, IGF, and insulin. Equal numbers of each cell line were plated, serum-starved overnight, stimulated with the indicated growth factor for 5 min, and lysed. Lysates 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.

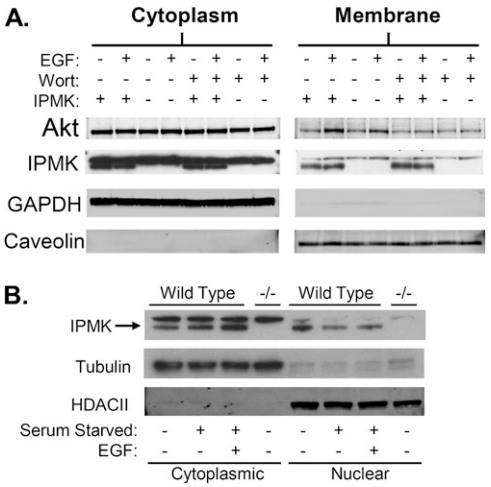

Fig. S4. Subcellular distribution of IPMK and Akt. (*A*) IPMK promotes translocation of Akt to the plasma membrane in response to EGF. Equal numbers of wild-type and IPMK$^{-/-}$ MEFs were plated, serum-starved overnight, stimulated with EGF for 3 min, and fractionated into S100 (cytoplasm) and P100 (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.

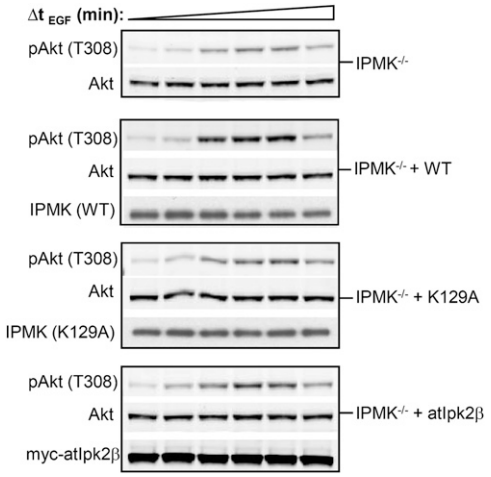

Fig. S5. The PI3K activity of IPMK is required for full activation of Akt in response to EGF. Wild-type mouse IPMK, IPMK-K129A, or atIpk2β were stably expressed in IPMK$^{-/-}$ MEFs. Equal numbers of each cell type were plated, serum-starved overnight, stimulated with EGF for 0, 1, 2, 3, 4, or 8 min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