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Inositol Polyphosphate Multikinase Inhibits Angiogenesis via Inositol Pentakisphosphate-Induced HIF-1α Degradation

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Abstract

Rationale—Inositol polyphosphate multikinase (IPMK) and its major product inositol pentakisphosphate (IP5) regulate a variety of cellular functions, but their role in vascular biology remains unexplored.

Objective—We have investigated the role of IPMK in regulating angiogenesis.

Methods and Results—Deletion of IPMK in fibroblasts induces angiogenesis in both in vitro and in vivo models. IPMK deletion elicits a substantial increase of vascular endothelial growth factor (VEGF), which mediates the regulation of angiogenesis by IPMK. The regulation of VEGF by IPMK requires its catalytic activity. IPMK is predominantly nuclear and regulates gene transcription. However, IPMK does not apparently serve as a transcription factor for VEGF. Hypoxia inducible factor 1α (HIF1α) is a major determinant of angiogenesis and induces VEGF transcription. IPMK deletion elicits a major enrichment of HIF1α protein and thus VEGF. HIF1α is constitutively ubiquitinated by von Hippel-Lindau protein (pVHL) followed by proteasomal degradation under normal conditions. However, HIF1α is not recognized and ubiquitinated by

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DISCLOSURES

None.

pVHL in *IPMK* knock out cells. IP5 reinstates the interaction of HIF1α and pVHL. HIF1α prolyl hydroxylation, which is prerequisite for pVHL recognition, is interrupted in IPMK deleted cells. IP5 promotes HIF1α prolyl hydroxylation and thus pVHL dependent degradation of HIF1α. Deletion of IPMK in mouse brain increases HIF-1α/VEGF levels and vascularization. The increased VEGF in *IPMK* KOs disrupts blood-brain barrier and enhances brain blood vessel permeability.

Conclusions—IPMK, via its product IP5, negatively regulates angiogenesis by inhibiting VEGF expression. IP5 acts by enhancing HIF-1α hydroxylation and thus pVHL dependent degradation of HIF-1α.

Subject Terms

Angiogenesis; Mechanisms; Cell Signaling/Signal Transduction; Basic science research; Physiology

Keywords

IPMK; PHD2; HIF-1α; pVHL; VEGF; angiogenesis; inositol

INTRODUCTION

Inositol polyphosphate multikinase (IPMK, also known as IPK2 and Arg82) is the primary generator of inositol 1,3,4,5,6-pentakisphosphate (IP5) from either of the IP4 isomers (inositol 1,4,5,6-tetrakisphosphate and inositol 1,3,4,5-tetrakisphosphate), though it can also form IP4 from IP3[1,2]. IPMK is essential for embryogenesis, as its deletion results in multiple developmental defects and death at embryonic day 9.5[2]. In *Drosophila*, IPMK is required for imaginal disc development[3]. IPMK is predominantly localized in nuclear compartments[4] and possesses nuclear PI3 kinase activity[5]. A family of inositol phosphates serves multiple functions including the release of calcium by inositol 1, 4, 5-trisphosphate (IP3), while inositol 1,4,5,6-tetrakisphosphate (IP4) acts as an 'intermolecular glue' for the assembly of a histone deacetylase complex[6]. IP5, which is generated from IP4, exerts antineoplastic effects[7,8]. Inositol hexakisphosphate (IP6), which is generated from IP5, has been shown to suppress cancer growth[9]. 5-Diphosphoinositol pentakisphosphate (5-IP7), which is generated from IP6, is required for insulin secretion[10] and cell migration[11,12].

Angiogenesis, the process of new vessel formation, is predominantly induced by vascular endothelial growth factor (VEGF)[13]. The expression of VEGF is primarily stimulated by hypoxia/ischemia. Responses to hypoxia/ischemia are mediated by hypoxia-inducible factors (HIFs), which are heterodimeric transcription factors composed of HIF-α and HIF-β subunits that activate *VEGF* gene expression[14]. Pathological enrichment of HIF-α and VEGF expression occurs in tumors associated with von Hippel-Lindau disease[15,16], ischemic retinal neovascularization[17] and in most advanced human cancers[18–20].

Three isoforms of HIF-α have been identified; the best-characterized isoforms are HIF-1α and HIF-2α[21,22]. HIF-1α is expressed ubiquitously in all cells, whereas HIF-2α and HIF-3α are selectively expressed in certain tissues[23]. HIF-α proteins are constantly degraded under

normal conditions. In normoxic environments, HIF-α proteins are hydroxylated by prolyl hydroxylase domain (PHD) proteins at two proline residues[24–27]. Hydroxylated HIF-α is then bound by pVHL, the substrate recognition subunit of an E3 ubiquitin-ligase[28–30]. Ubiquitinated HIF-α is subject to proteasomal degradation. Under hypoxic conditions, the activity of PHDs is diminished, leading to stabilization of HIF-α proteins, which heterodimerize with HIF-1β to activate transcription of genes involved in angiogenesis and other homeostatic responses to hypoxia[21,23,31].

Three PHDs have been well studied in mammalian cells and shown to utilize oxygen to hydroxylate human HIF-1α at residues P402 and P564[32,33]. Although all three PHDs regulate both HIF-1α and HIF-2α, preferences do exist. PHD2 acts as the major prolyl hydroxylase for HIF-1α, and P564 of HIF-1α is the preferred site for PHD2[34–36]. By contrast, PHD1 and PHD3 predominantly regulate HIF-2α[37]. Tissue deficiency of PHD2 elicits accumulation of HIF-1α[38], while deficiency of PHD1/PHD3 induces upregulation of HIF-2α[39]. Deletion of PHD2 elicits embryonic lethality, whereas mice with deletion of PHD1 and PHD3 develop normally[40]. While oxygen is a critical factor, diverse proteins and other molecules regulate the enzyme activity of PHDs. For example, OS-9 promotes HIF-1α hydroxylation by directly interacting with both PHDs and HIF-1α[41], whereas SSAT2 promotes ubiquitination by directly interacting with both pVHL and HIF-1α[42].

We now report that IP5 generated by IPMK is a major regulator of HIF-1α degradation. It acts by promoting HIF-1α hydroxylation, a prerequisite step for HIF-1α degradation. Deficiency of IPMK elicits enrichment of HIF-1α and VEGF, thereby inducing increased growth of blood vessels.

METHODS

The authors declare that all supporting data are available within the article [and its online supplementary file].

Antibodies and reagents

Anti-VEGF (cat#ABS82), anti-pVHL (cat# MABC14) and anti-NG2 (AB5320) antibodies were purchased from Millipore. Antibodies against PDGF (sc-7878), HIF1α (sc-10790) and ubiquitin (sc-8017) were obtained from Santa Cruz Biotechnology. Antibodies against β-actin (#4970), hydroxy-HIF1α (Pro564) (#3434), PHD2 (# 4835) and PDGF receptor β (#3169) were purchased from Cell Signaling Technology. Antibodies against myc tag (clone 9E10) and flag tag (clone M2) were purchased from Sigma-Aldrich. Anti-CD31 (cat#553370) antibody was from BD Biosciences. Antibody against IPMK raised in rabbit was produced in-house as previous report[5]. Ins(1,3,4,5)P4, Ins(1,4,5,6)P4, IP5, PtdIns(4,5)P2 and PtdIns(3,4,5)P3 were purchased from Cayman Chemical Company. IP6 and Evans blue was purchased from Sigma-Aldrich.

Cell culture

Wild type and IPMK deleted mouse embryonic fibroblasts (MEFs) were cultured in DMEM medium. HEK 293 cells, HEK 293T/17 cells and human primary lung fibroblasts were cultured in DMEM medium. Human umbilical vein endothelial cells (HUVECs) were from

Lonza and cultured in EGM2 medium (Lonza). All cells were maintained at 37°C with 5% CO_2.

Mice

Nestin-Cre/*IPMK* flox/flox mice were generated by breeding *IPMK* flox/flox mice with *Nestin-Cre* mice (The Jackson Laboratory). Mice were backcrossed with C57BL/6 for 10 generations. Athymic nude mice (male, 8 weeks old) were from Charles River Laboratories. All mice were maintained in SPF conditions. All animal experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by Johns Hopkins University Animal Care and Use Committee. 6 weeks old male littermates of *Nestin-Cre*/*IPMK* flox/flox and *IPMK* flox/folx mice were used.

In vitro conditioned media assay

1×10^6 wild type or *IPMK* KO MEFs were cultured for 24 hours; the cell-culture media were filtered (0.22 μm filter) and concentrated (ratio 1:10) by ultracentrifugal filters (10 kDa, Millipore). The concentrated cell-culture media were added to EGM2 medium (ratio 1:10) to suspend HUVECs (1×10^5 /ml). As controls concentrated fresh DMEM was added to EGM2 medium. The HUVECs suspensions were then plated onto Matrigel. Photos were taken 4 hours later[43].

In vivo Matrigel plug assay

A 500 μl Matrigel preparation containing a mixture of 1×10^6 MEFs and 1×10^6 HUVECs was injected subcutaneously in the flank of a nude mouse (male 8 weeks old; 6 mice per group). The plugs were excised 10 days later and stained with hematoxylin and eosin or immunofluorescence staining for MEFs (NG2) and HUVECs (CD31)[43].

Co-culture assay

Wild type or *IPMK* KO MEFs were planted into a cell culture plate for 24 hours and grown to confluence. HUVECs stably expressing DsRed fluorescent protein were then plated on top of the MEFs. The blood vessel-like structures were then formed by HUVECs. Photographs were taken on the 3rd day after co-culture[44].

Plasmid and lentiviral expression in cultured cells

The shRNA and scrambled control plasmids were from Sigma-Aldrich. The myc tagged murine WT IPMK, IPMK K129A, IPMK K129A/S235A and IPMK ΔNLS constructs were cloned to pCDH-EF1-MCS-T2A-copGFP vector (System Biosciences). The myc tagged human WT IPMK, IPMK K146A, IPMK ΔNLS and myc tagged GFP constructs were cloned to pCDH-EF1-MCS-T2A-copGFP vector (System Biosciences). The GFP tagged WT IPMK and IPMKΔNLS were cloned to pLVX-AcGFP1-N1 vector (Clontech). The flag tagged pVHL and HIF-1α genes were cloned to pLVX-AcGFP1-N1 vector (Clontech). The GST tagged GFP and PHD2 were cloned to pCDH-EF1-MCS-T2A-copGFP vector (System Biosciences). Lenti virus were generated by transfection of lenviviral vector together with pMD2.G and psPAX2 to HEK293T/17 cells[44].

RNA isolation and real time PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen), and reverse transcribed to cDNA using SuperScript® III (Invitrogen). Real-time quantitative PCR was performed with the TaqMan® Gene Expression Master Mix and the Step One Plus instrument (Life Technologies). Beta actin was used as loading control.

Immunoprecipitation

The lysis buffer contained 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5% Igepal CA630, 5 mM $MgCl_2$, protease inhibitors and phosphatase inhibitor. Cells were lysed on ice and the lysates were passed 20 times through a 30-gauge needle with syringe. The cell lysates were then centrifuged at 13,000 × g for 10 minutes at 4°C. The supernatants were collected. Protein concentrations were determined by using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and equalized. Equal amount of whole cell lysates were pre-cleared with protein A/G agarose (Santa Cruz Biotechnology) for 1 hour at 4°C. Then the supernatants were added with antibody and incubated overnight at 4°C followed by protein A/G agarose precipitation.

Western blotting

The lysis buffer contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA and 1mM EGTA, protease inhibitors and phosphatase inhibitor. *IPMK* KO MEFs were treated with 10 μM MG132 together with 50 μM of Ins(1,3,4,5)P4, Ins(1,4,5,6)P4, IP5 or IP6 for 4 hours. Cells were lysed on ice and the lysates were sonicated on ice. The cell lysates were then centrifuged at 13,000 × g for 10 minutes at 4°C. The supernatants were collected. Protein concentrations were determined by using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and equalized. Equal amount of whole cell lysates were applied for western blotting.

Enzyme-linked immunosorbent assay (ELISA)

1×10^6 MEFs were cultured for 24 hours, and the cell-culture media were filtered with 0.22 μM filters. The VEGF concentrations in the cell-culture media were calculated using the mouse VEGF ELISA Kit (Life Technology). For blood VEGF, bloods were drawn from euthanized animals. VEGF concentrations in plasma were calculated using the mouse VEGF ELISA Kit (Life Technology).

Subcellular fractionation

The cytosol and nuclear fractions were isolated using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific).

Intracellular inositol phosphate calculation

MEF cells were plated at 50% confluence, 100 μCi [^{3}H]myo-inositol was added to the medium and cultured for 3 days. The inositol phosphates were separated by HPLC[45].

Immunostaining

Animals were perfused and fixed with 4% paraformaldehyde (wt/vol). The brain slices were at cut at 30 μm thickness. Cultured cells were washed with PBS and fixed with 4% paraformaldehyde (wt/vol). Slides were blocked with 10% goat serum (vol/vol) for 10 minutes at room temperature, followed by incubating with primary antibody at 4 °C overnight. Then, fluorescence labeled secondary antibody for 1 hour at room temperature. Cell nucleus was stained with DAPI. Slides were mounted with ProLong Gold Antifade Mountant. Pictures were taken under confocal microscope (Zeiss LSM 700, NIH Award# S10 OD016374).

In vitro hydroxylation assay

GST-tagged PHD2 and flag-tagged HIF-1α (full length) were expressed in *IPMK* KO MEF cells. Cells expressing flag-HIF-1α were exposed to 1% O_2 for 4 hours, then HIF-1α was isolated by flag-tag beads pull down. PHD2 was isolated by glutathione sepharose beads pull down; the GST was removed by PreScission Protease. HIF-1α protein (on beads) was incubated with recombinant PHD2 protein for 1 hour at room temperature. The reaction buffer contains 40 mM Tris-HCl (PH 7.4), 100 mM NaCl, 3 mM $MgCl_2$, 5 mM ascorbate, 1.5 mM $FeCl_2$, 4 mM α-ketoglutarate and 2mg/ml BSA. 20 μM of Ins(1,3,4,5)P4, Ins(1,4,5,6)P4, IP5 or IP6 was added to the reaction. HIF-1α on flag-tag beads was then washed with reaction buffer. The hydroxylation of HIF-1α was analyzed by western blot[46].

Evans blue dye leakage

Evans blue dye (2% in saline) was administered intraperitoneally (80μL) and animals were sacrificed and perfused 2 hours after injection. Photographs of brain tissue were taken immediately after tissue isolation. Evans blue was extracted by homogenizing brain tissue in 50% trichloroacetic acid (wt/vol) solution, and centrifuged at 16,000 g for 20 minutes. The supernatants were diluted with ethanol (1:3), and fluorescence was quantified by using a microplate fluorescence reader. Sample value was determined by using a standard curve of Evans blue (0.1 to 100 ng/ml in the same solvent). Result was shown as nanograms of Evans blue per milligram of tissue[47].

Statistical analysis

Quantitative data are expressed as means with SEM. Data were analyzed by one-way ANOVA or unpaired Student's t test (two-tailed). $P<0.05$ was considered statistically significant. For non-quantitative data, results were representative of at least three independent experiments.

RESULTS

Deletion of IPMK induces angiogenesis

To determine whether IPMK regulates angiogenesis, we assessed the ability of angiogenic factors produced by wild type (WT) or IPMK-deleted mouse embryonic fibroblasts (MEFs) to stimulate tube formation by human umbilical vein endothelial cells (HUVECs), using either MEF conditioned media (Online Figure I A) or MEF co-culture (Online Figure I B).

In both cases, HUVEC tube formation is increased substantially by *IPMK* knockout (KO). The pro-angiogenic influence of IPMK deletion is also evident in intact animals in which mixtures of MEFs and HUVECs are injected subcutaneously into the flanks of mice (Online Figure I C–E). The increased angiogenesis associated with IPMK deletion is reversed by re-expression of IPMK, both in the conditioned media and co-culture assays (Online Figure I F–G). Thus, IPMK suppresses angiogenic processes.

Deletion of IPMK increases VEGF expression

As VEGF is a major determinant of angiogenesis[48], we assessed its levels in IPMK-deleted MEFs. In *IPMK* KO cells, VEGF mRNA and protein levels increase 4-fold and 2-fold, respectively (Figure 1A–B). The cell-culture medium of IPMK deleted MEFs displays almost a 2-fold increase in VEGF levels (Figure 1C). By contrast, re-expressing IPMK in *IPMK* KOs reduces VEGF mRNA and protein levels by about 60 percent (Figure 1D–F).

We explored the possibility that IPMK-associated alterations of VEGF expression mediate the influence of IPMK upon angiogenesis (Figure 1G). Treatment with antibodies to neutralize VEGF abolishes the augmented angiogenesis associated with IPMK deletion, whereas antibody to neutralize platelet-derived growth factor (PDGF) has no effect (Figure 1G and Online Figure I H). Thus, diminished IPMK increases angiogenesis by augmenting VEGF levels.

Regulation of VEGF by IPMK also occurs in HEK293 cells wherein IPMK depletion by shRNA elicits a 6-fold increase of cellular VEGF mRNA and protein levels (Figure 2A–C). In human fibroblasts, depletion of IPMK by shRNA also is associated with increases in VEGF levels (Figure 2D).

Regulation of VEGF by IPMK requires its catalytic activity

Deletion of IPMK depletes intracellular IP5; re-expression of IPMK rescues intracellular IP5 (Online Figure II). In IPMK-deleted MEFs, re-expression of wild type IPMK reduces VEGF levels, whereas catalytically inactive IPMK (K129A) and IPMK (K129A/S235A) are ineffective (Figure 2E). In experiments analyzing cell culture medium, we confirm that the regulation of VEGF by IPMK requires catalytic activity (Figure 2F). Thus, IP5, a major product of IPMK, is critical for regulating VEGF.

IPMK does not serve as a transcription factor for VEGF

IPMK is a nuclear protein[4] and has been reported to regulate gene transcription through kinase dependent and independent mechanisms[49–51]. Deletion of its nuclear localization signal (NLS) prevents nuclear localization of IPMK (Online Figure III A–B), though IPMK lacking a NLS is able to generate IP5 (Online Figure II).

To determine whether IPMK is a transcription factor for VEGF, we overexpressed WT IPMK, IPMK without NLS or kinase dead IPMK in HEK293 cells. Overexpressing WT or mutant IPMK does not decrease VEGF protein levels in HEK293 cells (Figure 2G). Similarly, overexpressing IPMK fails to decrease VEGF mRNA levels in both human

fibroblasts and MEFs (Figure 2H–I). Thus, deletion of IPMK increases VEGF, whereas, overexpressing IPMK does not decrease VEGF levels in WT cells.

Deletion of IPMK increases HIF-1α

HIF-1α is a major determinant of angiogenesis, which acts by regulating *VEGF* transcription[52,53]. However, HIF-1α is constantly degraded under normoxic conditions, as a result of O_2-dependent hydroxylation and ubiquitination[21,23]. Downregulation of HIF-1α hydroxylation and/or ubiquitination blocks its degradation and induces *VEGF* transcription[54,55]. We assessed HIF-1α protein levels in IPMK deleted cells. In MEFs, IPMK deletion leads to substantial increases in HIF-1α protein levels (Figure 3A–D). The gene expression level of HIF-1α (tested by real time PCR), however, does not change in *IPMK* KO cells (Online Figure III C). HIF-2α also regulates *VEGF* transcription and induces angiogenesis[56]. However, HIF-2α is not detectable in WT or *IPMK* KO MEFs used in this study (Figure 3A and C). Knocking down IPMK by shRNA transfection in HEK293 cells elicits increases in HIF-1α protein levels (Figure 3E–F). The augmentation of HIF-1α in IPMK deleted MEFs is diminished by re-expressing WT IPMK but not catalytically-inactive IPMK (IPMK KA or IPMK KA/SA) protein (Figure 3G–H). Overexpressing WT IPMK in HEK293 cells suppresses hypoxia-induced accumulation of HIF-1α and HIF-2α, whereas the kinase-defective IPMK (KA) does not decrease HIF-1α or HIF-2α protein levels (Figure 3I–J). Thus, the features of IPMK required for regulation of HIF-1α are essentially the same as those required for influencing angiogenesis and VEGF. Expression of IPMK ΔNLS also decreases hypoxia-induced HIF-1α and HIF-2α protein levels in HEK293 cells (Figure 3I–J). We examined the expression levels of HIF targeted genes in WT and *IPMK* KO MEFs (Online Figure IV). The gene expression levels of angiopoietin 2 (ANGPT2), angiopoietin-like 4 (ANGPTL4), kit ligand (KitL), egl-9 family hypoxia inducible factor 1 (EGLN1), endoplasmic oxidoreductin-1-like protein (Ero1L), stromal cell-derived factor 1 (SDF1), phosphoglycerate kinase 1 (PGK1), pyruvate dehydrogenase kinase 1 (PDK1), Solute carrier family 2, facilitated glucose transporter member 3 (SLC2A3), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), L-lactate dehydrogenase A chain (LDHA) are increased in the *IPMK* KOs (Online Figure IV).

HIF-1α mediates the regulation of VEGF expression by IPMK

We confirmed that HIF-1α mediates the effect of IPMK upon VEGF expression by depleting HIF-1α via shRNA transfection, which abolishes the upregulation of VEGF levels in *IPMK* KO MEFs (Figure 4A–B). Knocking down HIF-2α by shRNA transfection in IPMK deleted MEFs does not decrease VEGF levels, which is consistent with the lack of HIF-2α expression in these MEFs (Online Figure V A). Further evidence implicating HIF-1α in VEGF regulation by IPMK comes from the ability of 2-methoxyestradiol (2ME2) and PX-478, which are HIF-1α inhibitors[57,58], to prevent the increase in VEGF expression associated with IPMK deletion (Figure 4C–D). Thus, the regulation of VEGF by IPMK is through inhibition of HIF-1α.

IP5 induces HIF-1α degradation

The negative regulation of HIF-1α requires IPMK catalytic activity. Deletion of IPMK abolishes the generation of IP5 and IP6 (Online Figure II). It has been reported that IP6, the

downstream product of IP5, inhibits angiogenesis[59]. To test the possibility that IP6 regulates HIF-1α, we depleted endogenous IP6 production by knocking down IP5K in HEK293 cells (Figure 4E–F). IP5K deletion does not increase HIF-1α (Figure 4E); instead it inhibits hypoxia-induced HIF-1α and HIF-2α expression (Figure 4G–H). Intriguingly, the alteration of HIF-1α expression in IP5K deleted cells is similar to IPMK overexpression, which conceivably produces a large amount of IP5. It is noteworthy that an exceptionally high level of IP5 accumulates in IP5K depleted cells (Figure 4F). Thus, IP5, rather than IP6, negatively regulates HIF-1α expression.

Decreased HIF-1α ubiquitination in IPMK deleted cells

We tested the possibility that IPMK physically associates with HIF-1α. However, we fail to detect binding of these two proteins to each other (Online Figure V B–C). Thus, IPMK does not appear to influence HIF-1α via direct interactions. HDAC1 and HDAC3 stabilize HIF-1α[60] and are also regulated by inositol phosphates[6,61]. In our experiments, depleting HDAC1 and HDAC3 fails to block the increase of VEGF and HIF-1α levels in the *IPMK* KOs, suggesting that these HDACs do not mediate the regulation of HIF-1α by IPMK (Online Figure V D–E).

The ubiquitin E3 ligase pVHL mediates degradation of HIF-1α[15], which prompted us to explore a role for pVHL in the actions of IPMK upon HIF-1α. In WT cells, depletion of pVHL by shRNA elevates HIF-1α levels (Figure 5A–B). In *IPMK* KO cells, however, pVHL depletion fails to augment HIF-1α levels. HIF-1α is elevated in the *IPMK* KO cells to levels similar to cells with pVHL depletion. These findings fit with the possibility that the function of pVHL is disrupted in *IPMK* KO cells.

We directly examined the ubiquitination of HIF-1α in whole cell lysates as well as nuclear extracts (Figure 5C–D). Whereas proteasomal inhibition with MG132 increases the levels of ubiquitinated HIF-1α in WT cells, ubiquitination of HIF-1α is markedly diminished in IPMK deleted MEFs. This implies that function of pVHL is disrupted in *IPMK* KO cells. These findings also fit with the well-supported notion that pVHL is the principal E3 ligase responsible for degradation of HIF-1α[15,24,25].

pVHL shuttles between nucleus and cytoplasm, raising the possibility that IPMK might play a role in such a shuttling[62]. However, our failure to detect any direct binding of pVHL and IPMK does not support such a possibility (Online Figure IV A–B).

Ubiquitination of HIF-1α requires direct binding by pVHL[28]. This binding is lost in *IPMK* KO cells (Figure 5E). We explored whether the products of IPMK activity might be responsible for promoting pVHL/HIF-1α binding. Such binding is reinstated by IP5 but not by IP6, either of the IP4 isomers, PIP2, or PIP3 (Figure 5F–G). The selective effect of IP5 is evident whether we immunoprecipitate pVHL or HIF-1α. The action of IP5 occurs at physiologic concentrations with effects evident at 5 μM and maximal at 20–50 μM (Figure 5H–I). Thus, it appears that generation of IP5 by IPMK is required for pVHL to mediate ubiquitination of HIF-1α. These findings are consistent with our observation that IP5, but not IP6, decreases HIF-1α levels (Figure 4).

IP5 promotes HIF-1α prolyl hydroxylation

It has been reported that IP4 serves as intermolecular glue for HDAC complex assembly[6]. The structural basis of the HIF-1α/pVHL complex precludes the possibility that IP5 serves such a function for HIF-1α/pVHL[29,30]. HIF-1α prolyl hydroxylation is required for the HIF-1α/pVHL interaction[24,25]. Hydroxylated HIF-1α levels are substantially decreased in nuclear extracts from *IPMK* KO MEFs (Figure 6A). The decrease of HIF-1α hydroxylation is further confirmed by immunoprecipitation of HIF-1α from MEFs, in which degradation of hydroxylated HIF-1α is blocked by MG132, a proteasome inhibitor (Figure 6B). Overexpression of IPMK significantly increases HIF-1α hydroxylation (Figure 6C). Further evidence that HIF-1α hydroxylation is regulated by IPMK is that WT but not *IPMK* KO MEFs degrade HIF-1α when the WT form of HIF-1α is overexpressed. Accordingly, HIF-1α accumulation is augmented in *IPMK* KO cells (Figure 6D). However, the difference between HIF-1α protein levels in WT and *IPMK* KO cells is decreased when the prolyl hydroxylation sites of HIF-1α are mutated to alanine (P402A/P564A). Furthermore, overexpression of IPMK decreases hypoxia-induced WT but not mutant HIF-1α (Figure 6E). Thus, IPMK regulates HIF-1α stability by promoting HIF-1α prolyl hydroxylation.

HIF-α prolyl hydroxylation is catalyzed by a family of three prolyl hydroxylase domain (PHD) containing enzymes[32,33]. We performed immunoprecipitation to test whether IPMK physically associates with PHDs. IPMK and PHDs do not pull down each other (Online Figure VI C–D). Thus, IPMK does not directly interact with PHDs.

HIF-1α is mainly hydroxylated by PHD2; however, the accumulation of HIF-1α is not due to alteration of PHD2. Both the gene (EGLN1, which encodes PHD2) (Online Figure IV) and protein (Online Figure VII A) levels are increased in *IPMK* KO cells. Thus, it is possible that the catalytic activity of PHD2 is inhibited in IPMK deleted cells. Because the *EGLN1* gene encoding PHD2 is a HIF target gene[63], the increased PHD2 expression in *IPMK* KO cells is likely due to increased HIF-1α levels. Accordingly, we tested whether HIF-1α prolyl hydroxylation can be rescued by IP5. Treating IPMK deleted cells with IP5 but not isomers of IP4 or IP6 increases HIF-1α hydroxylation (Figure 6F). HIF-1α prolyl hydroxylation is restored by IP5 at physiological concentrations (20–50 μM) (Figure 6G).

We tested whether the increased hydroxylation induced by IP5 is through promoting HIF-1α/PHD2 interaction by immunoprecipitation of PHD2 and HIF-1α from WT and *IPMK* KO MEFs. Equal amounts of PHD2 and HIF-1α are pulled down by each other from WT and *IPMK* KO cells, indicating that IP5 does not affect PHD2/HIF-1α interaction (Online Figure VII B). We performed an in vitro assay to measure the effect of inositol phosphates on the hydroxylation of HIF-1α by PHD2. IP5 alone substantially increases HIF-1α hydroxylation (Figure 6H). Thus, IP5 acts as a specific activator of PHD2 enzyme activity and thereby promotes the O_2-dependent degradation of HIF-1α. Knocking down PHD2 in wild-type preparations increases HIF-1α levels substantially but fails to do this in IPMK deleted cells (Figure 7A). These findings indicate that the regulation of HIF-1α by IPMK is through PHD2.

We tested the possibility that IP5 binds with PHD2. We pulled down PHD2 and detected IP5 via HPLC (Figure 7B). IP5 is pulled down by PHD2 but not GFP, indicating that IP5 binds to PHD2.

Hypoxia decreases IPMK and IP5 levels

IPMK protein levels are dramatically decreased when cells are exposed to hypoxia (Figures 3C and 7C). The decrease of IPMK is evident with 30 minutes of hypoxia exposure (Figure 7D). IP5 level also decline about 40% during hypoxia (Figure 7E). Knocking down HIF-1α or HIF-2α does not prevent hypoxia-induced IPMK degradation (Figure 7F). Blocking new protein synthesis by cycloheximide treatment for 8 hours does not decrease IPMK protein levels (Figure 7G). Thus, IPMK is degraded in hypoxia condition, which does not depend on HIF-1α or HIF-2α.

Deletion of IPMK in mouse brain increases HIF-1α/VEGF levels and vascularization

Because whole body knockout of *IPMK* is lethal, we used Nestin-Cre to knockout *IPMK* in mouse brain and evaluate the in vivo effect of IPMK deletion. In mice with *Nestin-Cre* mediated deletion of *IPMK*, blood levels of VEGF are significantly increased (Figure 8A). In IPMK deleted cerebral cortex, both HIF-1α and VEGF levels are greatly enhanced (Figure 8B). Consistently, the expression of HIF-1α target genes, angiopoietin-2 (ANGPT2)[64] and erythropoietin (EPO)[65] are increased in IPMK KO cerebral cortex (Figure 8C–D). The expression of other HIF targeted genes, such as angiopoietin-like 4 (ANGPTL4), Kit ligand (KitL), egl-9 family hypoxia inducible factor 1 (EGLN1), L-lactate dehydrogenase A chain (LDHA) and phosphoglycerate kinase 1 (PGK1), are also increased in the *IPMK* KO mouse brains (Online Figure VII C). Vascular density is much higher in *IPMK* KO cerebral cortex (Figure 8E–F). Enhanced blood vessel density is also observed in the IPMK deleted cerebellum (Figure 8G–H). Thus, *IPMK* knockout in vivo increases HIF-1α and VEGF expression and stimulates vascularization. Pathologically elevated VEGF levels increase blood vessel permeability and jeopardize blood brain barrier[66,67]. Accordingly, we examined the pericyte coverage of brain blood vessels by staining PDGF receptor β for pericytes and CD31 for endothelial cells (Figure 8I). There is a substantial decrease of pericyte coverage In *IPMK* KOs (Figure 8I–J), indicating that the blood-brain barrier is disrupted. Increased leakage of blood vessels in *IPMK* KOs is evident in brain tissue stained by Evans blue (Figure 8K), which further confirms the disruption of the blood-brain barrier in *IPMK* KOs.

DISCUSSION

Our study establishes that IPMK physiologically inhibits angiogenesis under normoxic conditions by decreasing HIF-1α and VEGF expression (Online Figure VIII). IPMK generates IP5, which promotes HIF-1α degradation by increasing HIF-1α hydroxylation, which is a prerequisite for HIF-1α ubiquitination and degradation[24,25,27]. Thus, depleting IP5 levels by IPMK deletion increases HIF-1α and VEGF expression and induces angiogenesis. HIF-1α is a major determinant of angiogenesis by activating transcription of the *VEGF* gene, which encodes a critical pro-angiogenic growth factor[68]. Abnormal enrichment of HIF-α and VEGF leads to increased vascularization in hemangioblastomas

and renal cell carcinomas in patients with von Hippel-Lindau disease[15,16,54], ischemic retinopathy[17] and many advanced human cancers[18,19]. Deletion of IPMK in mouse brain elicits increased levels of HIF-1α/VEGF and substantial higher blood vessel densities. In addition, increased VEGF disrupts the blood brain barrier and causes brain blood vessel leakage in *IPMK* KOs. Clinically, *IPMK* mutation is associated with a form of intestinal carcinoid tumor[69].

IPMK has been shown to regulate gene transcription in both kinase dependent and independent manners[49–51]. The regulation of *VEGF* transcription by IPMK requires its catalytic activity. IPMK does not directly regulate *VEGF* transcription. Instead, it regulates the stability of HIF-1α, which induces VEGF transcription. IP5, a major product of IPMK, facilitates HIF-1α hydroxylation, ubiquitination and degradation.

IP5 has been reported to inhibit angiogenesis by inhibiting the endothelial cell PI3K/Akt pathway[8]. However, the regulation of HIF-1α and VEGF by IPMK/IP5 is not through inhibition of the PI3K/Akt pathway, because Akt activity is decreased rather than increased in *IPMK* KO cells that lack IP5[70]. In our study, the anti-angiogenic effect of IP5 is through inhibition of VEGF production by fibroblasts or neural cells, rather than endothelial cells, which are the targets of VEGF.

HIF-2α is homologous to HIF-1α and also activates *VEGF* transcription[56]. HIF-2α is selectively expressed in certain tissues, whereas HIF-1α is expressed ubiquitously[23]. HIF-2α is not detectable in the MEFs used in this study. However, the influence of IPMK upon HIF-2α in HEK293 cells is similar to that of HIF-1α.

The regulation of HIF-1α by IPMK differs from other modes of regulation, which require direct protein-protein interactions[41,71]. IPMK does not physically associate with HIF-1α, PHD2 or pVHL. Instead, IP5, a major product of IPMK, mediates HIF-1α prolyl hydroxylation and degradation. IP5 binds with PHD2, thus may act as an allosteric activator.

Overexpressing IPMK increases IP5 levels and decreases hypoxia-induced expression of HIF-1α. Deletion of IP5K also increases IP5 levels and decreases hypoxia-induced HIF-1α. Thus, enhancement of IP5 might be beneficial for the treatment of hypoxia-induced angiogenic diseases. Indeed, IP5 has been shown to inhibit angiogenesis and tumor growth[7,8].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

ANGPT2	angiopoietin 2
ANGPTL4	angiopoietin-like 4
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
EGLN1	egl-9 family hypoxia inducible factor 1
Ero1L	endoplasmic oxidoreductin-1-like protein
HIF	hypoxia-inducible factor
IPMK	inositol polyphosphate multikinase
IP5	inositol pentakisphosphate
KitL	kit ligand
KO	knock out
LDHA	L-lactate dehydrogenase A chain
MEF	mouse embryonic fibroblast
2ME2	2-methoxyestradiol
PDGF	platelet-derived growth factor
PDK1	pyruvate dehydrogenase kinase 1
PGK1	phosphoglycerate kinase 1
PHD	prolyl hydroxylase domain protein
pVHL	von Hippel-Lindau protein
SDF1	stromal cell-derived factor 1
SLC2A3	Solute carrier family 2, facilitated glucose transporter member 3
VEGF	vascular endothelial growth factor
WT	wild-type

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Angiogenesis is induced by vascular endothelial growth factor (VEGF), which is transcriptional activated by hypoxia inducible factor 1α (HIF-1α)
- Under normoxia, HIF-1α, under is constitutively degraded in the proteasome. HIF-1α is hydroxylated by prolyl hydroxylase domain (PHD) proteins, then ubiquitinated by von Hippel-Lindau protein (pVHL). Ubiquitinated HIF-1α is transferred to proteasome for degradation.
- Inositol polyphosphate multikinase (IPMK) generates inositol pentakisphosphate (IP5). IPMK and IP5 regulate numerous cellular functions.

What New Information Does This Article Contribute?

- IPMK via its product IP5 inhibits angiogenesis.
- IP5 promotes PHD-2 mediated HIF-1α hydroxylation, which is prerequisite for HIF-1α degradation.
- Deletion of IPMK/IP5 stabilizes HIF-1α, which increases VEGF production to induce angiogenesis.

Hypoxia inducible factor 1α (HIF-1α) activates the transcription of VEGF, which is a critical proangiogenic growth factor. In this study, we found that HIF-1α degradation is regulated by inositol polyphosphate multikinase (IPMK) acting via its major product inositol pentakisphosphate (IP5). IP5 promotes PHD-2 mediated prolyl hydroxylation of HIF-1α, which is prerequisite for ubiquitination and degradation of HIF-1α. Depletion of IP5 in IPMK mutant cells disrupts HIF-1α prolyl hydroxylation, which blocks HIF-1α ubiquitination/degradation. In IPMK mutant cells, aberrant accumulation of HIF-1α activates VEGF production, which induces angiogenesis. In IPMK mutant animals, substantial higher levels of HIF-1α/VEGF cause abnormal angiogenesis and leaky brain blood vessels. IP5 may elicit beneficial responses in hypoxia-induced angiogenic diseases, such as retinal neovascularization and cancers.

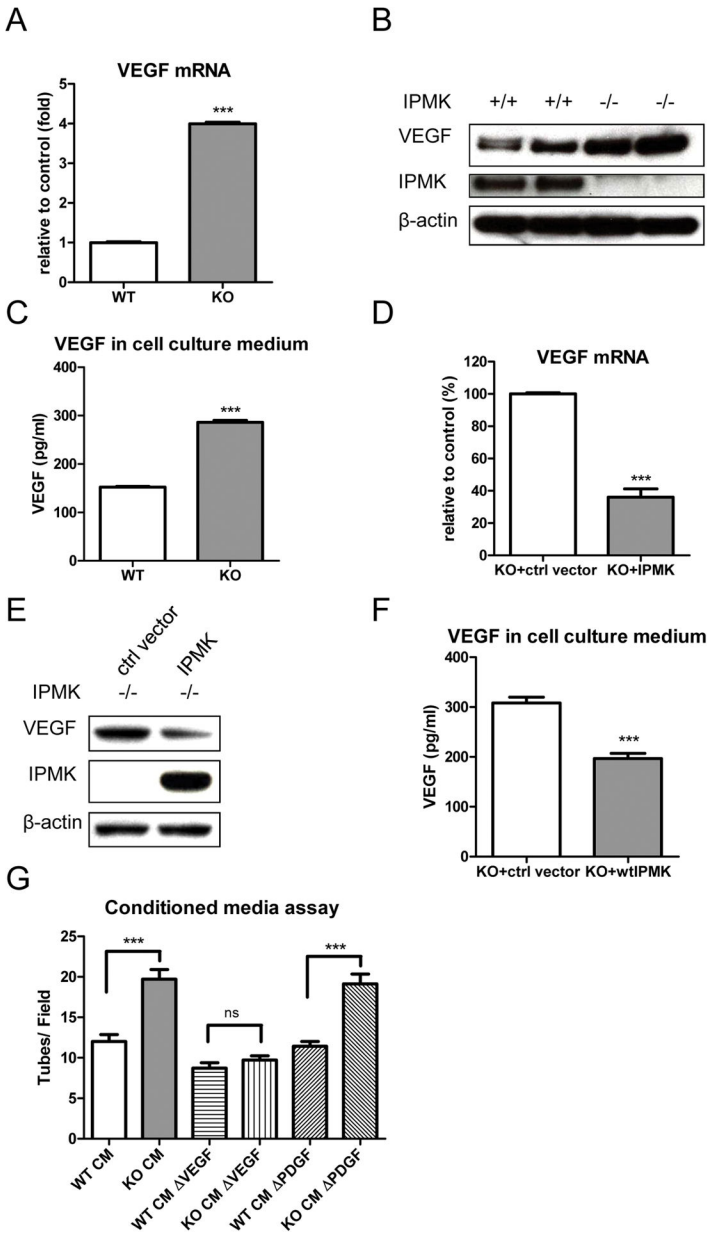

Figure 1. IPMK physiologically inhibits VEGF gene expression

A, The gene expression level of VEGF is significantly increased in IPMK deleted MEFs, analyzed by real time PCR. ***P < 0.001 by unpaired Student's t test (n = 4, mean ± s.e.m.). **B**, Western blot shows VEGF protein level is much higher in *IPMK* KO cells. **C**, VEGF protein level is substantial increased in the cell culture medium of *IPMK* KO MEFs, assessed by ELISA. ***P < 0.001 by unpaired Student's t test (n = 4, mean ± s.e.m.). **D**, Effect of IPMK rescue in *IPMK* KO MEFs on VEGF mRNA levels. ***P < 0.001 by unpaired Student's t test (n = 4, mean ± s.e.m.). **E**, IPMK rescue in *IPMK* KO MEFs reduces the expression of VEGF protein, evaluated by Western blot. **F**, Re-expression of IPMK in IPMK deleted MEFs decreases VEGF protein in cell-culture medium, monitored by ELISA. ***P < 0.001 by unpaired Student's t test (n=4, means±s.e.m.). **G**, Antibody neutralization

of VEGF but not PDGF abolishes the IPMK deletion-induced angiogenesis in the in vitro Matrigel assay. ***P < 0.001 by unpaired Student's t test; ns, no significant difference (n = 4, mean ± s.e.m.).

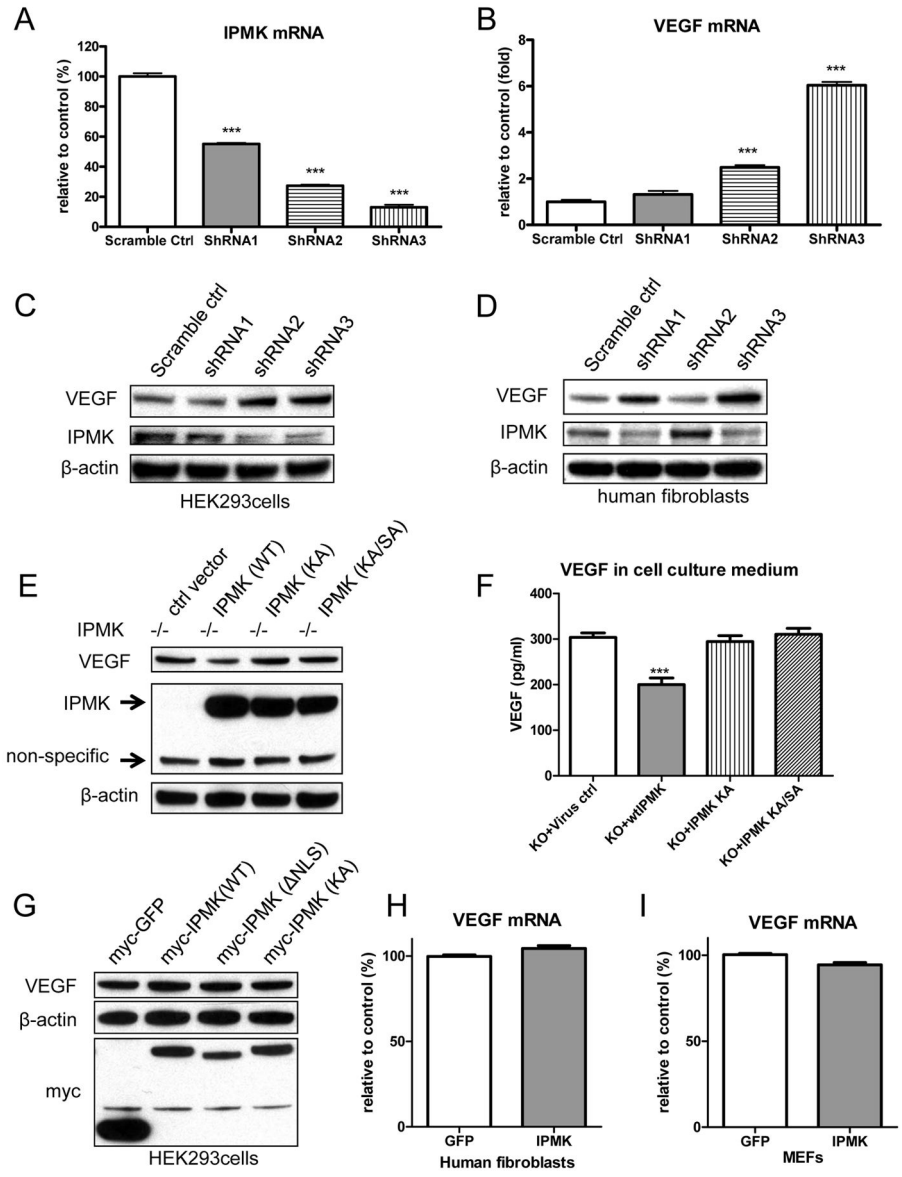

Figure 2. Negative regulation of VEGF expression requires IPMK kinase activity

A, IPMK knockdown in HEK293 cells by shRNA was analyzed by RT-qPCR. ***P < 0.001 by unpaired Student's t test (n = 4, mean ± s.e.m.). **B**, Depletion of IPMK by shRNA increases VEGF mRNA levels in HEK293 cells as determined by RT-qPCR. ***P < 0.001 by unpaired Student's t test (n = 4, mean ± s.e.m.). **C**, Depletion of IPMK by shRNA in HEK293 cells increases VEGF protein levels. **D**, Depletion of IPMK by shRNA in human fibroblasts increases VEGF protein levels. **E**, Re-expression of wild type IPMK but not kinase deficient mutants (IPMK KA or IPMK KA/SA) decreases VEGF protein levels in *IPMK* KO (-/-) cells. **F**, Re-expression of WT IPMK but not catalytically inactive IPMK (IPMK KA or IPMK KA/SA) reduces VEGF protein levels in the cell culture medium, as determined by ELISA. ***P < 0.001 by unpaired Student's t test (n = 4, mean ± s.e.m.). **G**, Overexpressing WT or IPMK lacking nuclear localization signal (IPMK ΔNLS) or kinase

activity (KA) in HEK293 cells does not decrease VEGF protein levels. **H,** Overexpression of IPMK in wild type human fibroblasts does not decrease VEGF mRNA levels. **I**, Overexpression of IPMK in wild type MEFs does not decrease VEGF mRNA levels.

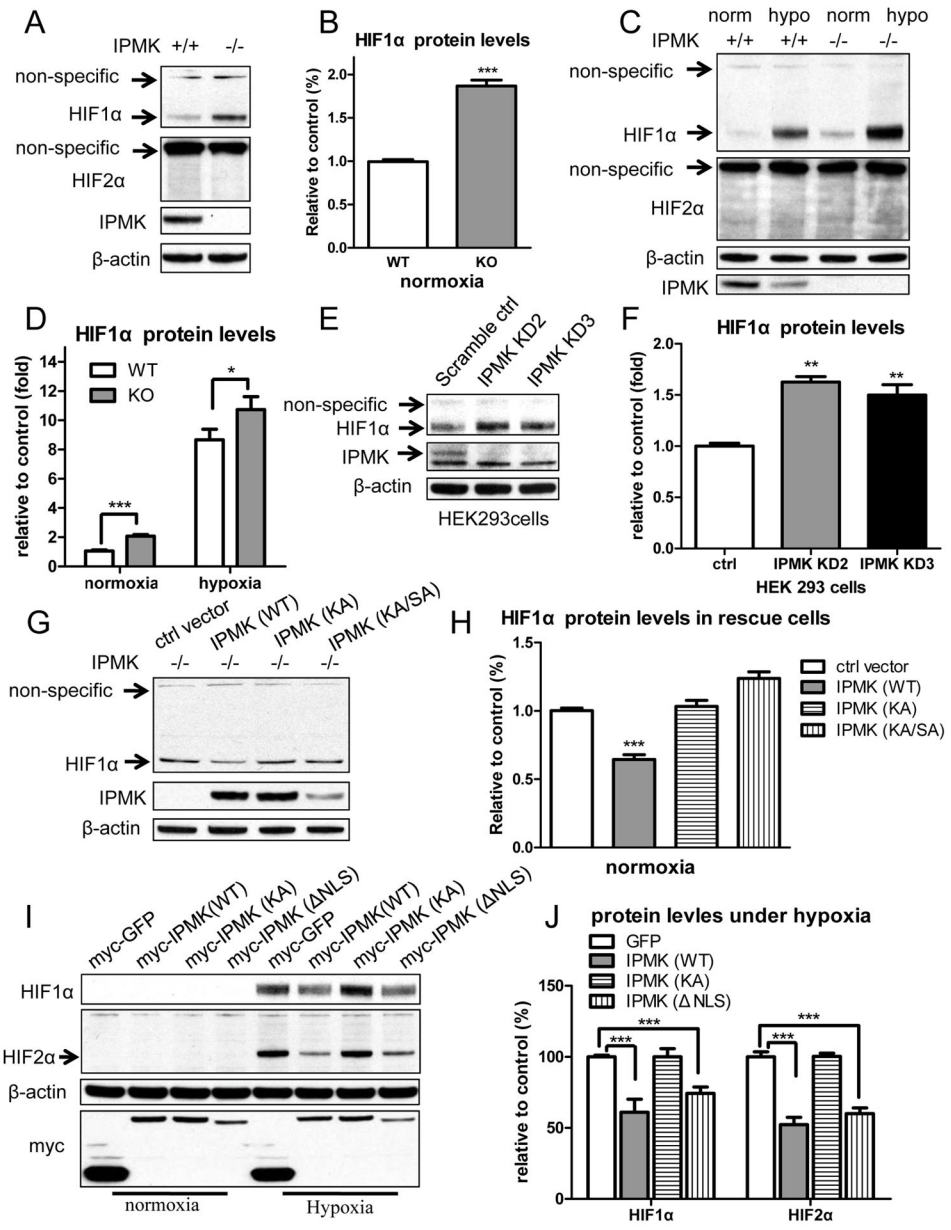

Figure 3. IPMK deletion causes HIF-1α protein accumulation

A and **B**, Western blot shows increased HIF-1α protein levels in *IPMK* KO MEFs under normoxic conditions. ***P < 0.001 by unpaired Student's t test (n = 3, mean ± s.e.m.). **C and D**, Western blot reveals increased HIF-1α levels in *IPMK* KO MEFs both under normoxic (norm) and hypoxic (hypo) conditions. **E and F**, Knockdown of IPMK expression in HEK293 cells increases HIF-1α protein levels. **G** and **H**, Re-expression of wild type IPMK but not kinase deficient mutants (IPMK KA or IPMK KA/SA) in *IPMK* KO MEFs decreases HIF-1α protein levels. ***P < 0.001 by unpaired Student's t test (n = 3, mean ± s.e.m.). **I and J**, Overexpressing WT IPMK or IPMK lacking the nuclear localization signal (ΔNLS), but not kinase deficient IPMK (KA), in HEK293 cells decreases hypoxia-induced HIF-1α and HIF-2α protein levels.

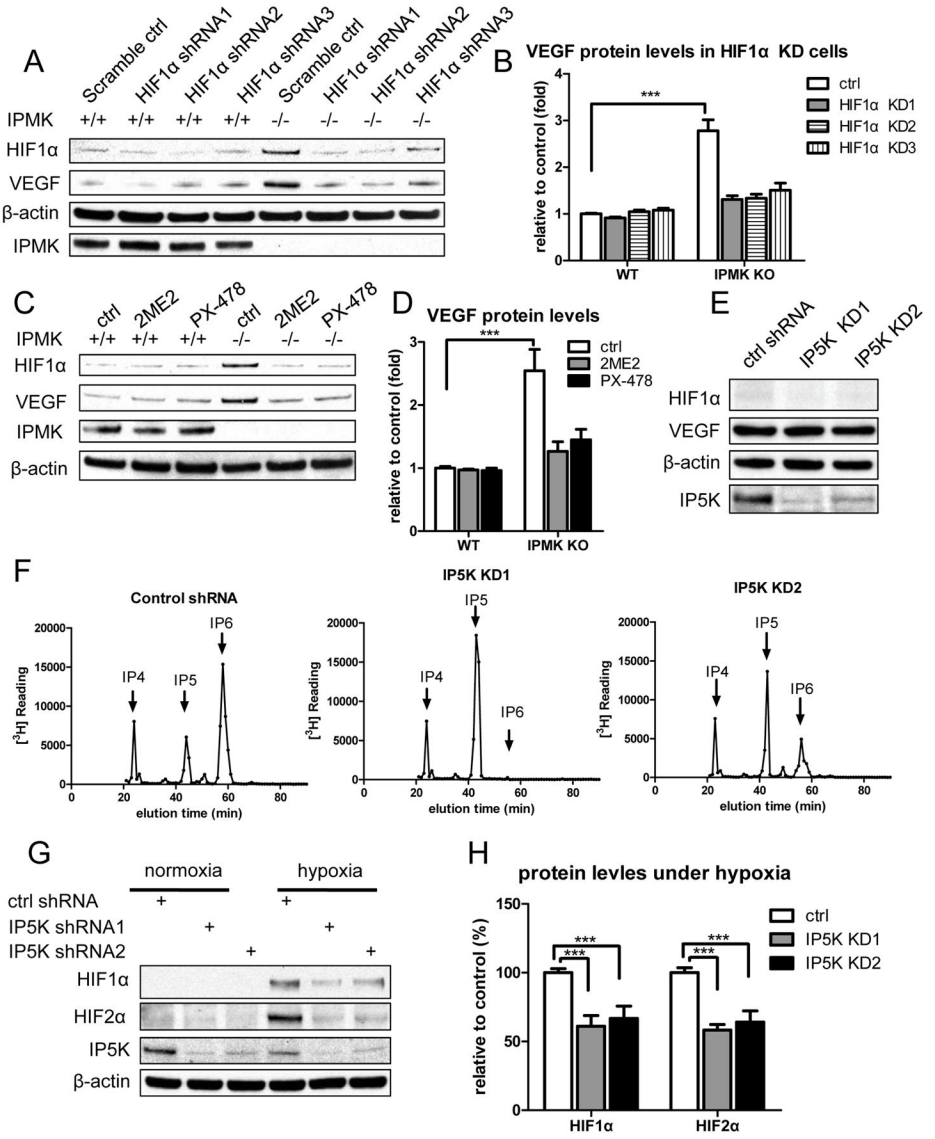

Figure 4. IP5 decreases HIF-1α protein levels

A and B, Depletion of HIF-1α by shRNA abrogates the upregulation of VEGF in *IPMK* KO MEFs. **C and D**, Inhibition of HIF-1α by 10 μM 2-methoxyestradiol (2ME2) or PX-478 abolishes the upregulation of VEGF in *IPMK* KO MEFs. **E**, Knockdown of IP5K expression in HEK293 cells by shRNA does not increase HIF-1α protein levels. **F**, Knockdown of IP5K expression in HEK293 cells decreases IP6 and increases IP5 levels. **G and H**, Depletion of IP5K in HEK293 cells decreases hypoxia-induced HIF-1α and HIF-2α protein levels.

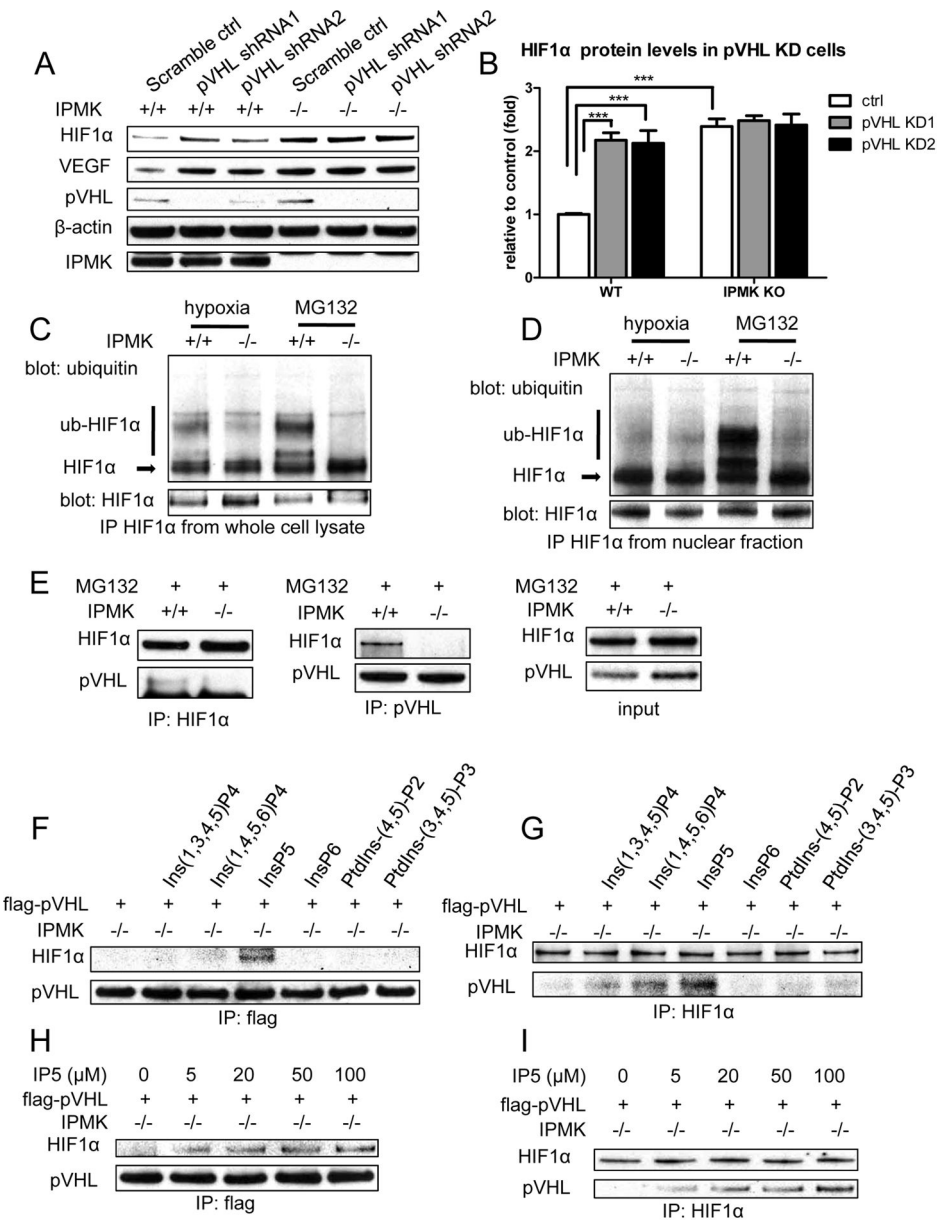

Figure 5. IP5 is critical for HIF-1α ubiquitination

A and B, Depletion of pVHL by shRNA increases HIF-1α protein levels in WT MEFs but fails to augment HIF-1α protein levels in *IPMK* KO cells. **C**, HIF-1α was immunoprecipitated from the lysates of whole cells that were exposed to either hypoxia (1% O_2) or MG132 (10 μM) for 4 hours. Ubiquitination of HIF-1α is markedly diminished in *IPMK* KO MEFs. **D**, HIF-1α was immunoprecipitated from the nuclear fraction of MEFs that were exposed to either hypoxia (1% O_2) or MG132 (10 μM) for 4 hours. HIF-1α ubiquitination is substantially decreased in *IPMK* KO MEFs. **E**, MEFs were treated with 10 μM MG132 for 4 hours; HIF-1α and pVHL were then immunoprecipitated. The physical association of pVHL and HIF-1α is disrupted in *IPMK* KO cells. **F,** Flag-pVHL was immunoprecipitated from lysates of *IPMK* KO cells that were treated with 50 μM Ins(1,3,4,5)P4, Ins(1,4,5,6)P4, IP5, IP6, PtdIns(4,5)P2 or PtdIns(3,4,5)P3. The interaction of

pVHL and HIF-1α is rescued by IP5. **G**, HIF-1α was immunoprecipited from the same lysates described in (**F**). IP5 increases the binding of pVHL with HIF-1α. **H**, Flag-pVHL was immunoprecipitated from *IPMK* KO cell lysates to which were added different concentrations of IP5. IP5 restores the association of HIF-1α with pVHL. **I**, HIF-1α was immunoprecipitated from the same lysates described in (**H**). IP5 restores the association of HIF-1α with pVHL.

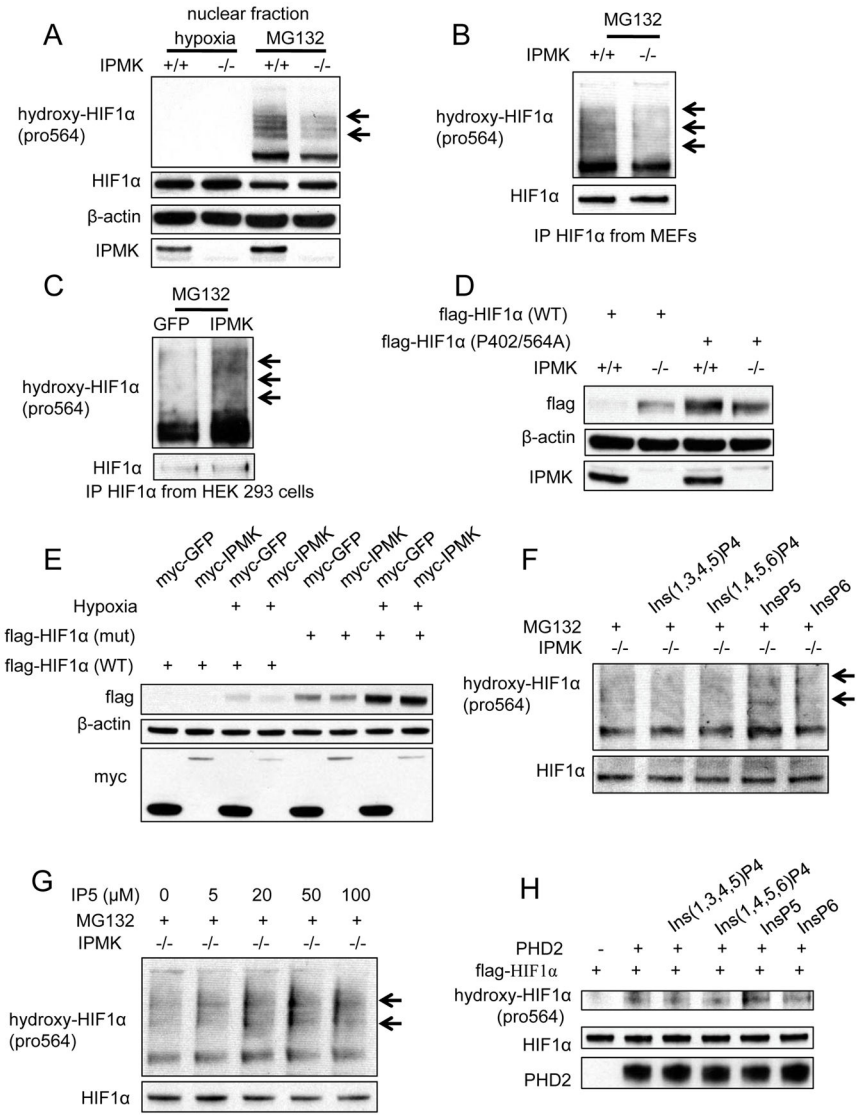

Figure 6. IP5 promotes HIF-1α prolyl hydroxylation

A, WT and *IPMK* KO MEFs were exposed to either hypoxia (1% O_2) or MG132 (10 μM) for 4 hours and nuclear fractions were isolated. IPMK deletion elicits less hydroxylation of HIF-1α at proline-564 (arrows). **B**, WT and *IPMK* KO MEFs were treated with MG132 (10 μM for 4 hours), HIF-1α was then immunoprecipitated and blotted with anti-hydroxy-HIF-1α (Pro-564) antibody. IPMK deletion reveals diminished HIF-1α hydroxylation (arrows). **C**, IPMK or GFP was overexpressed in HEK293 cells and the cells were treated with MG132 (10 μM for 4 hours). HIF-1α was immunoprecipitated and blotted with anti-hydroxy-HIF-1α (pro564) antibody. Overexpression of IPMK increases HIF-1α prolyl hydroxylation (arrows). **D**, WT or hydroxylation site mutant (P402/564A) HIF-1α was overexpressed in MEFs. A substantial amount of WT HIF-1α is accumulated in IPMK deleted but not wild type cells. The difference of HIF-1α protein levels in WT and *IPMK* KO cells is abolished when HIF-1α prolyl hydroxylation sites are mutated. **E**, IPMK or GFP was overexpressed in HEK293 cells, which co-expressed either WT or hydroxylation site

mutant (P402/564A) HIF-1α. The cells were exposed to hypoxia (1% O_2 for 4 hours). Western blot shows overexpressing IPMK decreases levels of hypoxia-induced WT but not mutant HIF-1α. **F**, *IPMK* KO cells were treated with 10 μM MG132 together with 50 μM Ins(1,3,4,5)P4, Ins(1,4,5,6)P4, IP5 or IP6 for 4 hours. Western blot shows IP5 increases HIF-1α hydroxylation at proline-564 (arrows). **G**, *IPMK* KO cells were treated with 10 μM MG132 together with different concentrations of IP5 for 4 hours. Western blot shows IP5 increases HIF-1α hydroxylation (pro564) at 20–50 μM (arrows). **H**, PHD2 and flag-HIF-1α were overexpressed and purified from *IPMK* KO MEFs, then applied to an in vitro hydroxylation assay. Twenty μM of Ins(1,3,4,5)P4, Ins(1,4,5,6)P4, IP5 or IP6 were added to reactions. IP5 substantially increases HIF-1α hydroxylation.

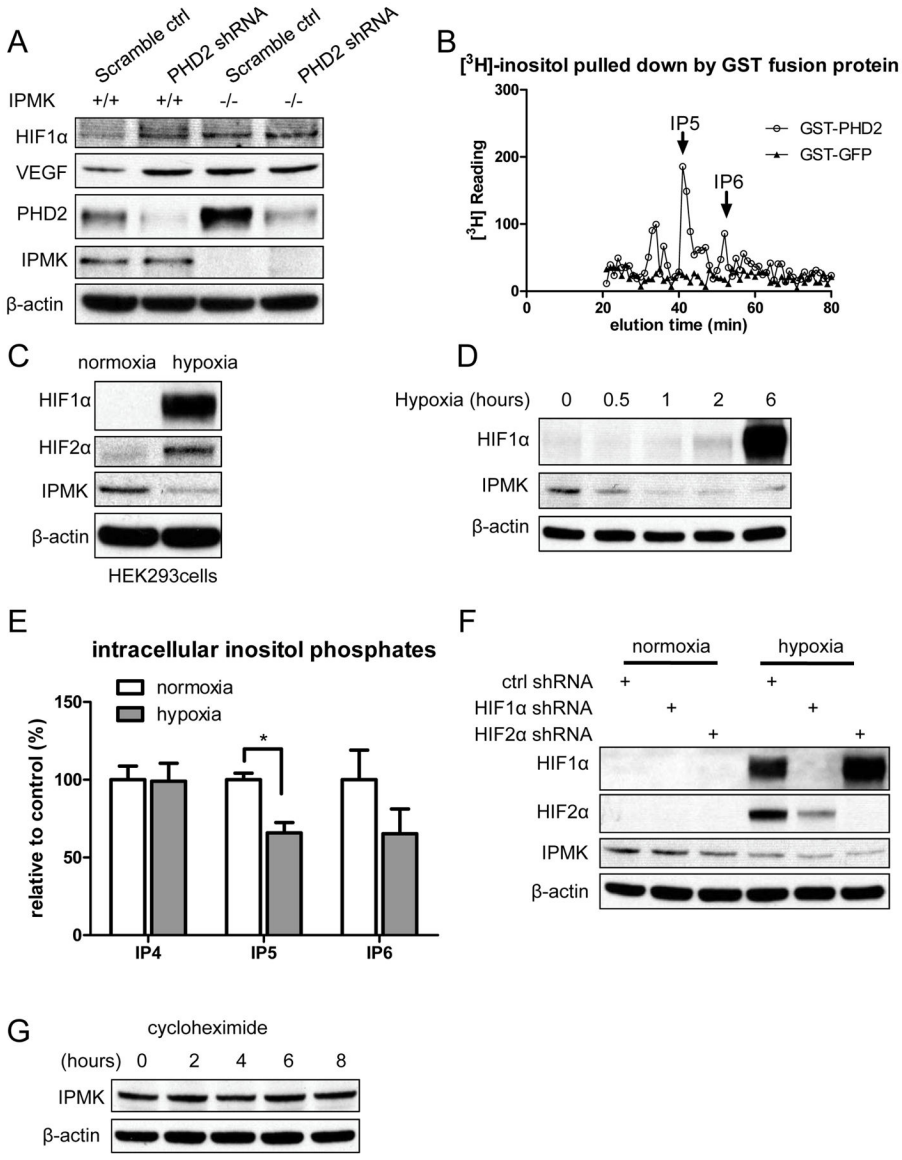

Figure 7. Hypoxia decreases IPMK/IP5 levels

A, PHD2 was knocked down by shRNA transduction in *IPMK* KO and WT MEFs. Knocking down PHD2 increases HIF-1α levels in WT but not *IPMK* KO cells. **B**,GST-PHD2 or GST-GFP was overexpressed in HEK293 cells. The cells were then labeled with [^{3}H]-inositol. PHD2 or GFP was pulled down by glutathione agarose beads. [^{3}H]-inositol phosphates that bind with PHD2 or GFP were detected by HPLC. **C**, HEK293 cells were exposed to hypoxia (1% for 4 hours). Western blot shows a substantial loss of IPMK protein levels in hypoxia treated cells. **D**, HEK293 cells were exposed to hypoxia (1%) for different times. Western blot shows that hypoxia decreases IPMK protein levels. **E**, HEK293 cells were labeled with [^{3}H]-inositol and exposed to hypoxia (1% for 4 hours). The intracellular inositol phosphates were measured by HPLC. Hypoxia treatment significantly reduces intracellular IP5 levels. **F**, HIF-1α or HIF-2α was knocked down in HEK 293 cells. The cells were then exposed to hypoxia (1% for 4 hours). Western blot shows that knocking

down HIF-1α or HIF-2α does not prevent hypoxia-induced IPMK degradation. **G**, HEK 293 cells were treated with cycloheximide for different times. Western blots show that IPMK protein level does not decrease with 8 hours of cycloheximide treatment.

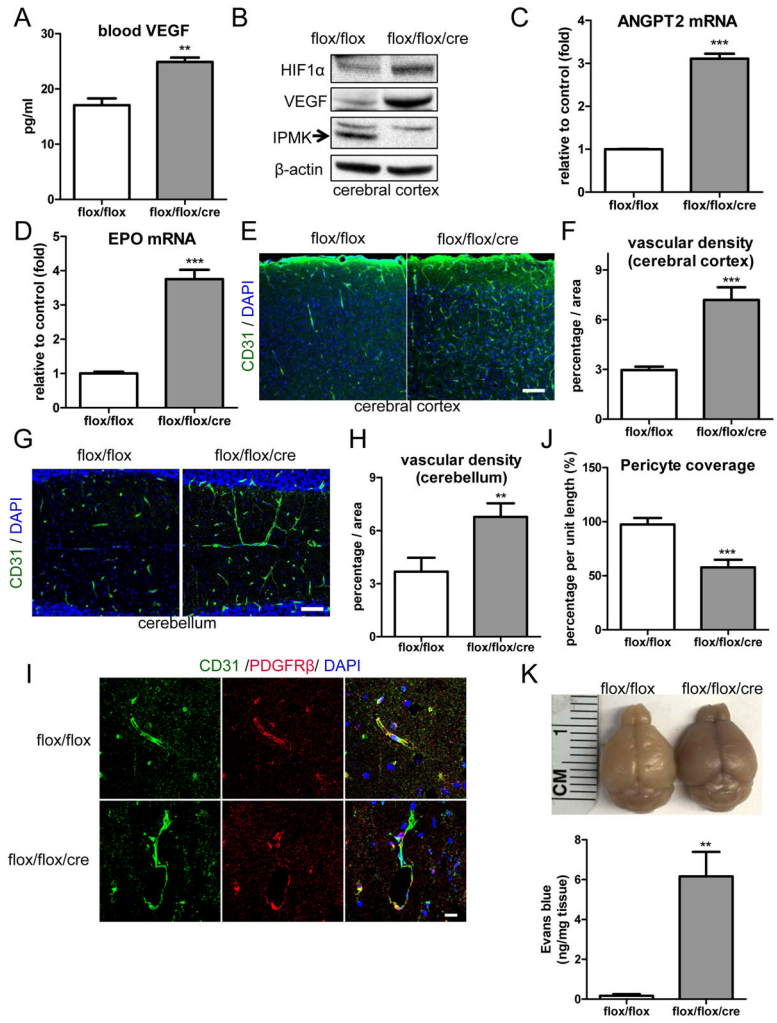

Figure 8. Increased HIF-1α /VEGF expression and blood vessel densities in *IPMK* KO mouse brain

A, A significantly increased amount of VEGF is present in the blood of *IPMK* KO mice compared to controls. ** P<0.01 by unpaired Student's t test (n = 4, male, 6-week old). **B**, HIF-1α and VEGF protein levels are increased in cerebral cortex of *IPMK* KO mice (male, 6-week old). **C**, The gene expression level of angiopoietin 2 (ANGPT2) is substantially increased in *IPMK* KO brains. **D,** The gene expression level of erythropoietin (EPO) is markedly increased in *IPMK* KO brains. **E** and **F**, Immunostaining of blood vessels in cerebral cortex with anti-CD31 antibody. Vascular density increases two-fold in *IPMK* KO mice. Scale bar 100 μm. *** P<0.001 by unpaired Student's t test (n = 4, male, 6-week old). **G** and **H**, Immunostaining of blood vessels in cerebellum with anti-CD31 antibody. Dramatically higher vascular densities are seen in IPMK deleted cerebellum. Scale bar 50μm. Twelve randomly chosen fields (360 × 360 μm) from 4 non-adjacent sections, were analyzed per mouse.** P<0.01 by unpaired Student's t test (n = 4, male, 6-week old). **I** and **J**, Immunostaining of CD31 (blood vessels) and PDGFRβ (pericytes) on *IPMK* KO and WT mouse brain. The pericyte coverage of brain blood vessels is substantially decreased in *IPMK* KO mice. (n=3, male, 6-week old). **K**, Evans blue (2% in saline, 80 μL) was injected

intraperitoneally to *IPMK* KO and WT mice. The animals were euthanized and perfused 2 hours later. *IPMK* KO brains were stained by Evans blue, indicating leaky blood vessels. (n=3, male, 6-week old). Evans Blue was measured in the cortex. ** P<0.01 by unpaired Student's t test.