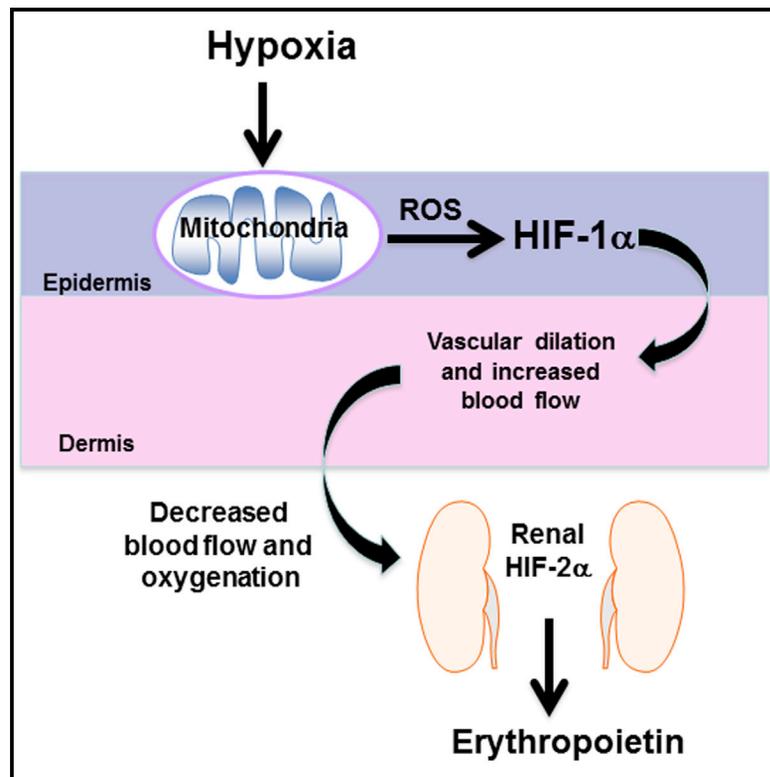


The Mitochondrial Respiratory Chain Is Required for Organismal Adaptation to Hypoxia

Graphical Abstract



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In Brief

Hamanaka et al. show that loss of mitochondrial respiratory activity in the mouse epidermis impairs hypoxic activation of HIF-1. Since epidermal HIF-1 promotes cutaneous vasodilation and blood flow during hypoxia and potentiates the hypoxic response in the internal organs, mice with loss of TFAM in the epidermis are unable to induce renal erythropoietin expression in response to hypoxia.

Highlights

- The respiratory chain is essential for hypoxic activation of HIF in keratinocytes
- Respiratory chain production of ROS is necessary for hypoxic activation of HIF in vitro
- Hypoxic activation of HIF in the epidermis requires the respiratory chain in vivo
- Epidermal respiration is essential for hypoxic expression of renal erythropoietin gene



The Mitochondrial Respiratory Chain Is Required for Organismal Adaptation to Hypoxia

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SUMMARY

Hypoxia-inducible factors (HIFs) are crucial for cellular and organismal adaptation to hypoxia. The mitochondrial respiratory chain is the largest consumer of oxygen in most mammalian cells; however, it is unknown whether the respiratory chain is necessary for *in vivo* activation of HIFs and organismal adaptation to hypoxia. HIF-1 activation in the epidermis has been shown to be a key regulator of the organismal response to hypoxic conditions, including renal production of erythropoietin (Epo). Therefore, we conditionally deleted expression of TFAM in mouse epidermal keratinocytes. TFAM is required for maintenance of the mitochondrial genome, and TFAM-null cells are respiratory deficient. TFAM loss in epidermal keratinocytes reduced epidermal levels of HIF-1 α protein and diminished the hypoxic induction of HIF-dependent transcription in epidermis. Furthermore, epidermal TFAM deficiency impaired hypoxic induction of renal Epo expression. Our results demonstrate that the mitochondrial respiratory chain is essential for *in vivo* HIF activation and organismal adaptation to hypoxia.

INTRODUCTION

The importance of oxygen homeostasis for the survival of eukaryotes has necessitated the evolution of multiple mechanisms by which organisms maintain their oxygen supply. The cellular response to hypoxia is governed largely by a family of transcription factors termed hypoxia-inducible factors (HIFs), which promote the activation of genes involved in glycolysis, angiogenesis, cell-cycle regulation, and survival (Semenza, 2012). Under normoxic conditions, proline residues within the oxygen-dependent degradation domain of HIF- α subunits (HIF-1 α , HIF-2 α , and HIF-3 α) are hydroxylated by a family of 2-oxoglutarate-dependent dioxygenases termed prolyl hydroxylases 1, 2, and 3 (PHD1–3) (Kaelin and Ratcliffe, 2008). This proline-directed hydroxylation targets HIF- α subunits for recognition by a ubiquitin ligase containing the von Hippel-Lindau tumor suppressor protein (VHL). The activity of PHD proteins is inhibited during hypoxia, stabilizing HIF- α subunits, leading to their dimerization

with a common β -subunit (HIF-1 β) and transactivation of HIF target genes.

Mitochondrial metabolism has been implicated in regulating the hydroxylation reaction carried out by PHDs. Mitochondrial generation of tricarboxylic acid (TCA) cycle intermediates, consumption of oxygen, and production of reactive oxygen species (ROS) have been shown to inhibit the hydroxylation of HIFs, resulting in activation of HIF target genes (Bell et al., 2007; Brunelle et al., 2005; Guzy et al., 2005; Hagen et al., 2003; Isaacs et al., 2005; Mansfield et al., 2005; Pan et al., 2007; Selak et al., 2005; Sullivan et al., 2013). Despite evidence for the role of mitochondrial metabolism in regulating the cellular response to hypoxia, it remains to be demonstrated whether this phenomenon, heretofore observed only in cell culture, plays a physiological role in the mammalian systemic response to hypoxia *in vivo*.

Epidermal keratinocytes have been shown to play a critical role in regulating the systemic response to hypoxia (Boutin et al., 2008). The epidermis, which obtains oxygen directly from the atmosphere, responds to reductions in atmospheric oxygen by inducing vasodilation in the underlying dermis in a HIF- and nitric-oxide-dependent manner (Boutin et al., 2008; Minson, 2003; Stücker et al., 2002). This hypoxic vasodilation in the skin results in reduced blood flow to the internal organs, increasing internal hypoxia and promoting renal production of erythropoietin (Epo), a glycoprotein hormone that promotes proliferation and differentiation of erythroid progenitor cells into red blood cells, increasing the oxygen carrying capacity of the blood (Boutin et al., 2008; Bunn et al., 1998).

We recently reported on mice that lack expression of transcription factor A, mitochondrial (TFAM) in epidermal keratinocytes (Hamanaka et al., 2013). TFAM is required for transcription and replication of the mitochondrial genome, and cells lacking TFAM are respiratory deficient (Larsson et al., 1998). Loss of TFAM in epidermal keratinocytes resulted in epidermal barrier function defects and lethality 2 weeks after birth. The epidermal defect was due to a decrease in the production of mitochondrial ROS, which is necessary for Notch-dependent epidermal differentiation (Hamanaka et al., 2013). Here, we show that epidermal keratinocytes derived from these TFAM epidermal knockout (TFAM EpiKO) mice display impaired HIF activation upon exposure to hypoxia. Furthermore, neonatal mice displayed diminished HIF-1 α protein and target gene induction in their epidermis after exposure to hypoxia. This resulted in an impaired hypoxic induction of Epo expression in the kidneys of these mice. Renal Epo expression could be induced in TFAM EpiKO mice by

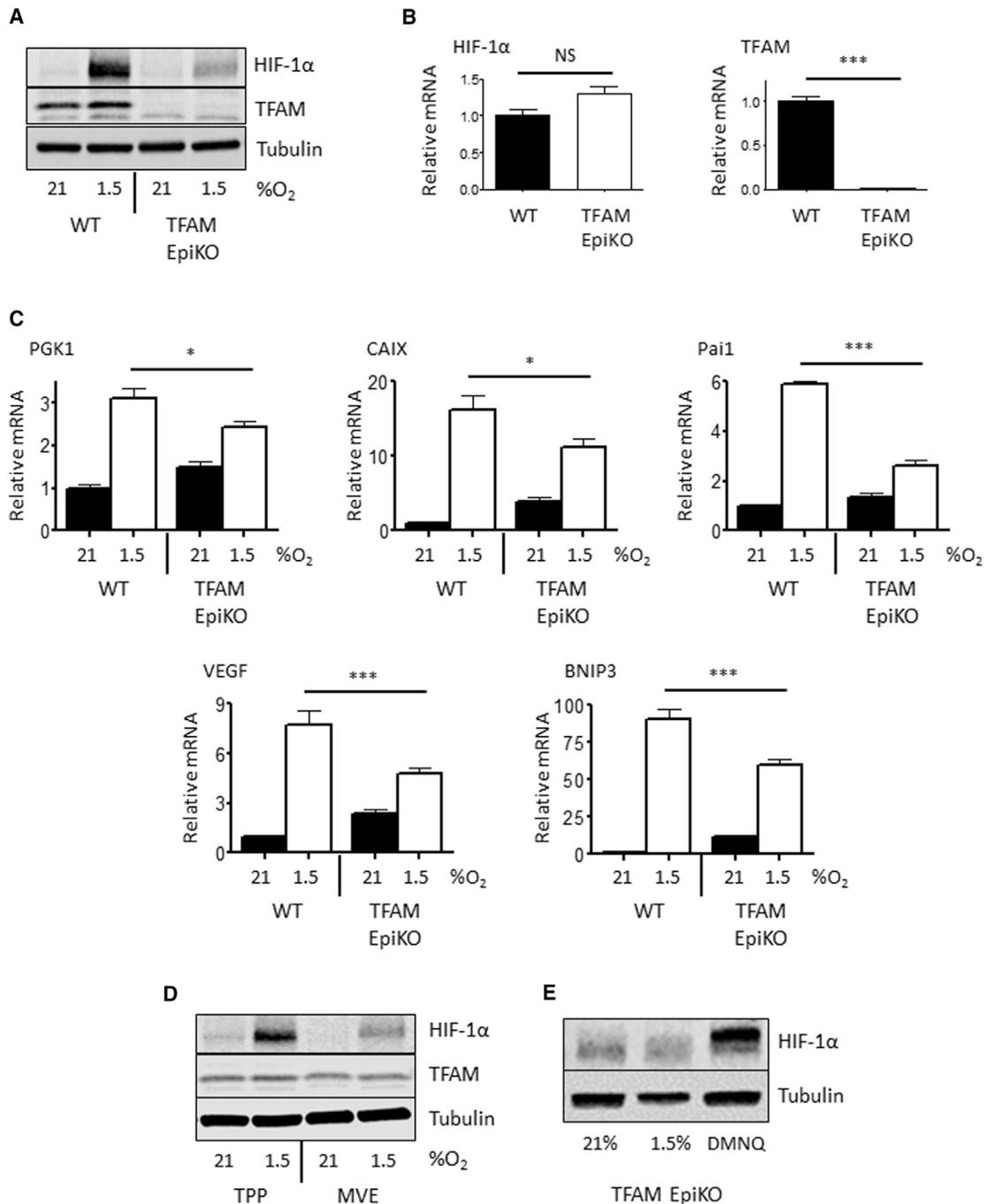


Figure 1. Mitochondrial Generation of ROS Is Required for Hypoxic Induction of HIF-1 α Protein and Target Gene Expression in Mouse Epidermal Keratinocytes

(A) Representative western blot analysis of HIF-1 α and TFAM protein levels in primary mouse keratinocytes isolated from wild-type and TFAM EpiKO mice. Cells were exposed to normoxia (21% O₂) or hypoxia (1.5% O₂) for 4 hr.

(B) Real-time PCR analysis of HIF-1 α and TFAM mRNA expression in primary mouse keratinocytes isolated from wild-type and TFAM EpiKO mice.

(C) Real-time PCR analysis of PGK1, CAIX, Pai1, VEGF, and BNIP3 mRNA expression in primary mouse keratinocytes isolated from wild-type and TFAM EpiKO mice. Cells were exposed to normoxia or hypoxia for 4 hr.

(D) Representative western blot analysis of HIF-1 α protein levels in primary wild-type mouse keratinocytes after exposure to normoxia or hypoxia for 4 hr. Cells were treated with mitochondria-targeted vitamin E (MVE; 1 μ M) or the control compound methyl-triphenylphosphonium (TPP; mitochondria-targeting moiety lacking antioxidant activity).

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pharmacologic promotion of cutaneous vasodilation or by co-deletion of VHL, demonstrating conclusively the link between mitochondria and the HIF degradation machinery *in vivo*.

RESULTS

TFAM EpiKO mice were generated by crossing mice bearing floxed alleles of TFAM to mice that express *Cre* recombinase under the control of the keratin-14 promoter (Dassule et al., 2000). We have previously demonstrated that primary epidermal keratinocytes isolated from TFAM EpiKO mice do not consume oxygen or produce mitochondrial ROS (mROS) (Hamanaka et al., 2013). Consistent with their inability to initiate mitochondria-dependent signaling events, primary epidermal keratinocytes isolated from TFAM EpiKO mice displayed an impaired induction of HIF-1 α protein after exposure to hypoxia (1.5% O₂) (Figure 1A). Hypoxic accumulation of HIF-2 α was similarly inhibited in TFAM EpiKO keratinocytes (Figure S1A). The failure of TFAM EpiKO keratinocytes to induce HIF-1 α was not the result of decreased HIF-1 α mRNA expression (Figure 1B) but was due to the inability of these cells to stabilize HIF-1 α protein upon exposure to hypoxia. This resulted in impaired transcriptional induction of HIF-1 α target genes that regulate metabolism (phosphoglycerate kinase-1; [PGK1] and BCL2/adenovirus E1B 19-kDa interacting protein 3 [BNIP3]), pH balance (carbonic anhydrase 9 [CAIX]), and blood flow (vascular endothelial growth factor [VEGF] and plasminogen activator inhibitor 1 [Pai1]) (Figure 1C). The inability of TFAM-null cells to respond to hypoxia was not due to bioenergetic defects; keratinocytes treated with deferoxamine (DFO), an iron chelator that stabilizes HIF-1 α protein under normoxic conditions (Wang and Semenza, 1993), resulted in HIF-1 α protein accumulation as well as HIF-1 α target gene expression in both wild-type and TFAM EpiKO cells (Figures S1B and S1C).

We have previously demonstrated that mROS are critical signaling regulators of hypoxic HIF activation (Bell et al., 2007; Brunelle et al., 2005; Chandel et al., 2000). Consistent with this, the effects of TFAM knockout on HIF-1 α stabilization were mimicked by the treatment of wild-type keratinocytes with antioxidants such as N-acetylcysteine and mitochondria-targeted vitamin E (Figures 1D and S1D). To determine if elevation of cellular ROS levels could rescue HIF-1 α stabilization in the absence of mROS production, we treated TFAM EpiKO cells with the redox cycling agent 2,3-dimethoxy-4-naphthoquinone (DMNQ). DMNQ has previously been demonstrated to increase cellular ROS levels, leading to HIF-1 α stabilization in transformed cells (Köhl et al., 2006). Treatment with DMNQ induced HIF-1 α protein accumulation in TFAM EpiKO keratinocytes (Figure 1E). Mitochondrial respiratory complex III has been shown to be the major site of mROS production necessary for HIF-1 α protein stabilization during hypoxia (Bell et al., 2007; Orr et al., 2015). Thus, acute knockdown of the complex III subunit Reiske iron sulfur protein (RISP) diminished

HIF-1 α protein stabilization to a similar extent as TFAM knockout (Figure S1E). Collectively, these results demonstrate that TFAM EpiKO keratinocytes exhibit diminished HIF-1 α protein stabilization after exposure to hypoxia due to their inability to produce mROS.

Elimination of TFAM expression in the epidermal keratinocytes of mice leads to premature follicular catagen and development of a hyperplastic interfollicular epidermis (Hamanaka et al., 2013; Figure S2A). TFAM EpiKO mice develop an epidermal barrier defect at postnatal day 14.5 (P14.5) and die soon thereafter (Hamanaka et al., 2013), therefore all *in vivo* experiments on these mice were conducted at day P9.5. Consistent with previous reports (Bedogni et al., 2005; Boutin et al., 2008; Wong et al., 2015), the epidermis of wild-type mice displayed constitutive expression of HIF-1 α protein, even under normoxic conditions (Figure 2A). Reduced levels of HIF-1 α protein were observed in the epidermal cells of TFAM EpiKO mice when compared to wild-type mice. As observed in cultured keratinocytes, the diminished HIF-1 α protein level was not due to a decrease in HIF-1 α mRNA expression in the TFAM EpiKO epidermis as epidermal HIF-1 α mRNA levels in wild-type and TFAM EpiKO mice were similar (Figure 2B).

Despite the constitutive levels of HIF-1 α protein observed in normoxic wild-type mice, exposure of wild-type mice to hypoxia (9% O₂) for 16 hr led to an additional accumulation of HIF-1 α protein in epidermal cells as well as increased expression of HIF-1 α target genes (Figures 2C–2E). Exposure of TFAM EpiKO mice to hypoxia resulted in an attenuated induction of HIF-1 α protein and no increase in the expression of HIF-1 α target genes (Figures 2C–2E).

Activation of the HIF response in epidermal keratinocytes promotes the systemic response to hypoxia by promoting nitric oxide (NO)-dependent vasodilation in the underlying dermis, shunting blood away from the internal organs and causing them to encounter greater levels of hypoxia than they would in the absence of the epidermal response (Boutin et al., 2008). Kidneys respond to this increased level of hypoxia by producing the glycoprotein hormone erythropoietin (Epo), a HIF-2 α target gene (Gruber et al., 2007; Rankin et al., 2007). After exposure to hypoxia for 16 hr, expression of Epo mRNA was highly induced in the kidneys of wild-type mice, an effect that was inhibited in TFAM EpiKO mice (Figure 3A). This led to reduced levels of Epo protein in the serum of TFAM EpiKO mice after exposure to hypoxia (Figure 3B). Hypoxic induction of HIF-1 α target genes such as PGK1, BNIP3, Pai1, CTGF (connective tissue growth factor), and Glut1 (glucose transporter 1) was also inhibited in the kidneys of TFAM EpiKO mice (Figure 3E). As in the epidermis, the renal mRNA expression of both HIF-1 α and HIF-2 α was similar between wild-type and TFAM EpiKO mice (Figure 3C). Furthermore, the levels of renal TFAM expression were not affected in TFAM EpiKO mice, indicating the specificity of the keratin 14-driven *Cre* for epidermal cells (Figure 3D).

(E) Representative western blot analysis of HIF-1 α protein levels in TFAM EpiKO primary mouse keratinocytes after exposure to normoxia, hypoxia, or 2,3-dimethoxy-4-naphthoquinone (DMNQ; 100 μ M) for 4 hr.

Charts represent means \pm SEM. n = 4 independent keratinocyte preparations per genotype. mRNA expression was normalized to RPL19 levels. Significance was determined by (B) Student's t test or (C) one-way ANOVA using Bonferroni's post test.

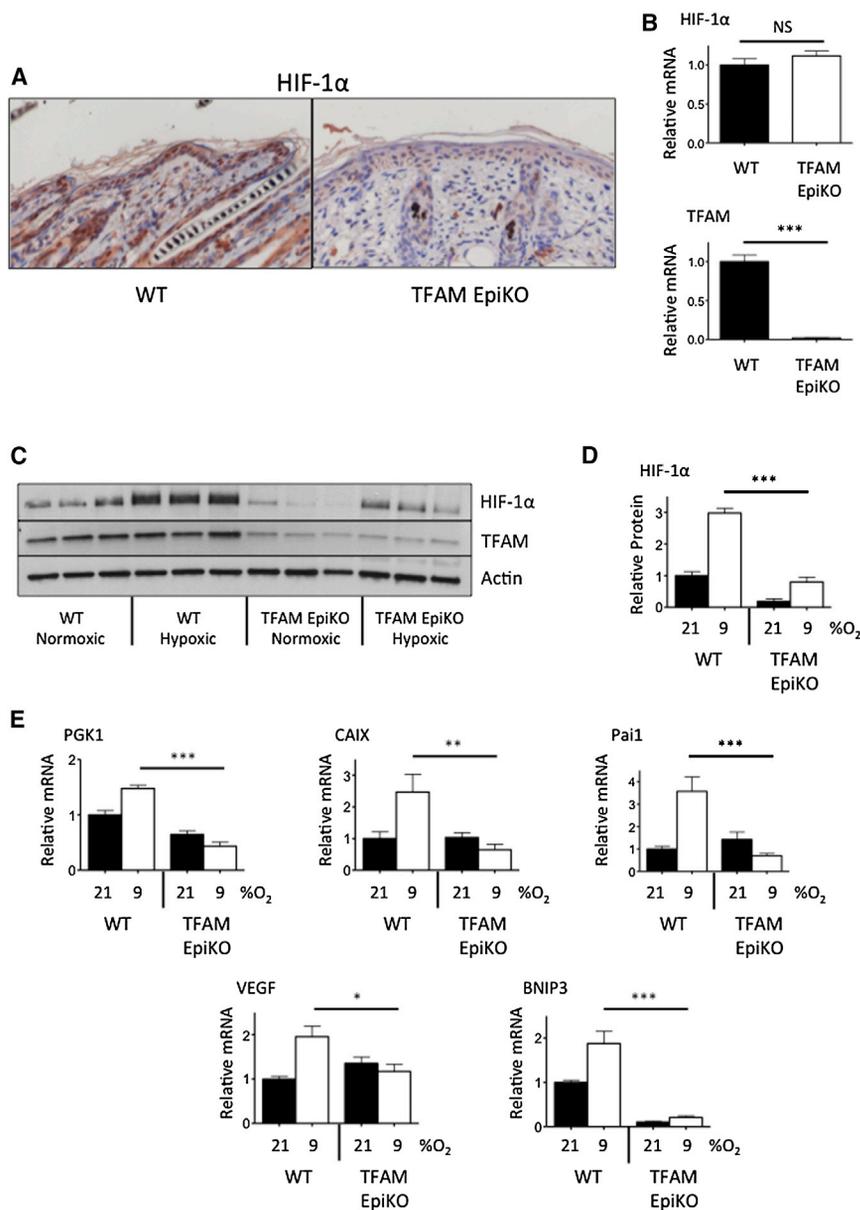


Figure 2. Hypoxic Induction of HIF-1 α Protein and Target Gene Expression Is Inhibited in the Epidermis of TFAM EpiKO Mice

(A) Representative immunohistochemical analysis of HIF-1 α protein expression in the epidermis of wild-type and TFAM EpiKO mice.

(B) Real-time PCR analysis of HIF-1 α and TFAM mRNA expression in the epidermis of wild-type and TFAM EpiKO mice.

(C) Representative western blot analysis of HIF-1 α and TFAM protein levels in epidermal lysates prepared from wild-type and TFAM EpiKO mice. Mice were exposed to normoxia (21% O₂) or hypoxia (9% O₂) for 16 hr.

(D) Densitometric quantification of (C). HIF-1 α protein levels were normalized to β -actin levels.

(E) Real-time PCR analysis of PGK1, CAIX, Pai1, VEGF, and BNIP3 mRNA expression in epidermal lysates prepared from wild-type and TFAM EpiKO mice. Mice were exposed to normoxia or hypoxia for 16 hr.

Charts represent means \pm SEM. n = 5 mice per genotype per condition. mRNA expression was normalized to RPL19 levels. Significance was determined by one way ANOVA using Bonferroni's post test.

ubiquitin ligase, we sought to determine if epidermal deletion of VHL could rescue HIF-1 α activity in the epidermal keratinocytes of TFAM EpiKO mice. To do this, we generated a mouse expressing floxed alleles of both TFAM and VHL and knocked out expression of both genes in the epidermis of mice using the keratin-14 Cre allele. Mice lacking expression of VHL in the epidermis were notable at birth as they were red in color due to increased cutaneous blood flow (Figures 4A and 4B). Both VHL EpiKO and TFAM+VHL EpiKO mice exhibited perinatal mortality, dying by day P5.5. At day P3.5, however, increased levels of HIF target gene mRNA were detected in epidermal lysates from

Pharmacologic induction of cutaneous NO is sufficient to induce renal Epo expression in the absence of hypoxic stimuli (Boutin et al., 2008). We found that the dermal vascular density of TFAM EpiKO mice was similar to that of wild-type mice when assessed using the endothelial marker CD31 (Figure S2B). Thus, we sought to determine whether TFAM EpiKO mice remained sensitive to cutaneous application of mustard seed oil (allyl isothiocyanate), which promotes NO synthesis. Indeed, application of mustard seed oil to the skin of wild-type or TFAM EpiKO mice induced renal Epo mRNA expression to a similar extent (Figure 3F). These results suggest that the impaired hypoxic Epo induction observed in TFAM EpiKO mice is the result of an oxygen sensing defect within the epidermis.

Since mitochondrial metabolism and mROS production regulate HIF activity upstream of recognition by the VHL-containing

both VHL EpiKO and TFAM+VHL EpiKO mice (Figure 4C). Furthermore, epidermal deletion of VHL elevated renal Epo expression to a similar extent in mice either heterozygous or homozygous for the TFAM floxed allele (Figure 4D).

DISCUSSION

At the cellular level, HIFs are crucial for metabolic adaptation to hypoxia (Semenza, 2010). Mitochondria are the primary cellular consumers of oxygen, and mitochondrial generation of TCA cycle intermediates, oxygen consumption, and production of ROS have all been demonstrated to regulate the activity of HIF prolyl hydroxylases (Bell et al., 2007; Brunelle et al., 2005; Guzy et al., 2005; Hagen et al., 2003; Isaacs et al., 2005; Mansfield et al., 2005; Pan et al., 2007; Selak et al., 2005; Sullivan et al., 2013).

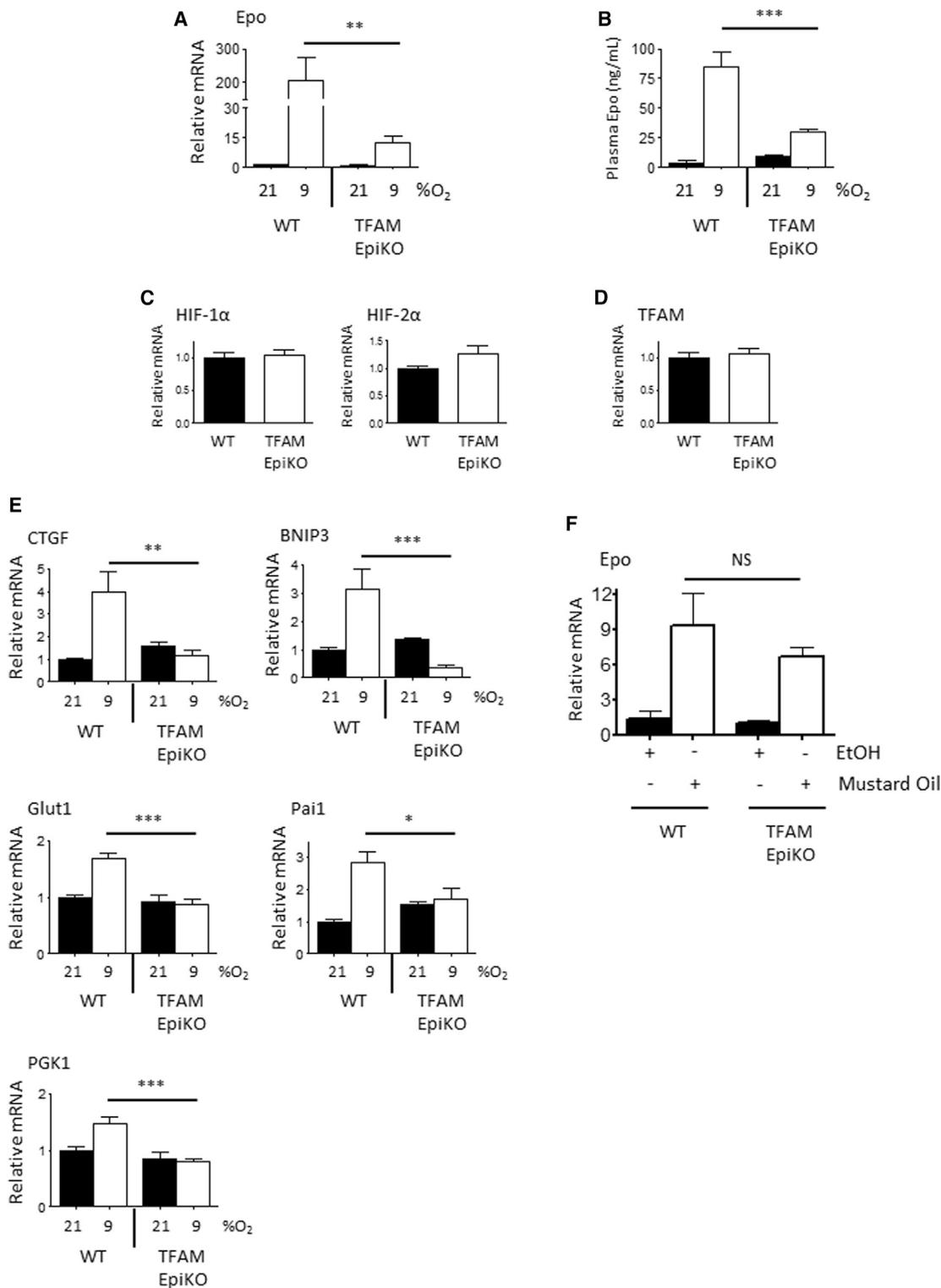


Figure 3. Hypoxic Induction of HIF-1 α Target Genes and Erythropoietin Is Inhibited in the Kidneys of TFAM EpiKO Mice

(A) Real-time PCR analysis of Epo mRNA expression in renal lysates prepared from wild-type and TFAM EpiKO mice. Mice were exposed to normoxia or hypoxia for 16 hr.

(B) Epo protein concentrations in the serum of wild-type and TFAM EpiKO mice as determined by ELISA.

(C and D) Real-time PCR analysis of (C) HIF-1 α , HIF-2 α , or (D) TFAM mRNA expression in the kidneys of wild-type and TFAM EpiKO mice.

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Despite accumulating evidence for mitochondrial regulation of HIF, this requirement had yet to be demonstrated *in vivo*. In this report, we demonstrate that the mitochondrial respiratory chain is an essential regulator of HIF-1 *in vivo*.

Our results demonstrate that although cells that lack TFAM expression are unable to conduct mitochondrial oxidative phosphorylation, they are energetically capable of inducing HIF-1 α protein stabilization when PHD proteins or VHL are inhibited; it is the signaling upstream of the PHD proteins that is inhibited in the absence of TFAM. Thus, inhibition of PHD proteins with DFO or pharmacological elevation of cellular ROS levels with DMNQ was sufficient to induce HIF activity in TFAM EpiKO keratinocytes. In addition, our results support a role for mROS as crucial regulators of hypoxia signaling, as treatment of wild-type keratinocytes with N-acetylcysteine or mitochondria-targeted vitamin E inhibited HIF accumulation to a similar extent as TFAM deletion *in vitro*. To conclusively demonstrate the role that mROS generation plays in promoting HIF activity *in vivo*, it will be critical to develop genetic tools that impair mROS production without simultaneously affecting oxidative phosphorylation.

Our results support previous findings that demonstrate that HIF activation in the epidermis promotes Epo production in the kidney. While it was previously demonstrated that deletion of epidermal HIF-1 α expression impaired the renal response to hypoxia (Boutin et al., 2008), we now demonstrate that deletion of TFAM in epidermal keratinocytes recapitulates the effect of epidermal HIF-1 α deletion. Although deletion of TFAM in epidermal cells inhibited hypoxic sensation and renal induction of Epo, these mice remained sensitive to the effects of cutaneous NO elevation or to co-deletion of epidermal VHL, supporting a crucial role for mitochondrial signaling upstream of VHL. These results demonstrate that mitochondria act as oxygen sensors *in vivo*, promoting HIF activation and adaptation to hypoxic conditions. While it is unclear why deletion of VHL in the epidermis resulted in early mortality, it was recently shown that in addition to regulating hypoxic adaptation, epidermal HIF levels regulate systemic arterial pressure and thermoregulation in mice (Cowburn et al., 2013). Thus, our results add to the increasing evidence for the importance of oxygen sensing and HIF activation within the epidermis for organismal homeostasis.

The importance of mitochondria as regulators of HIF signaling is highlighted by the fact that in a chemical screen of over 600,000 compounds, the 200 most effective inhibitors of hypoxic HIF activation were determined to be mitochondrial inhibitors (Lin et al., 2008). While the toxicities of mitochondrial inhibitors such as rotenone and cyanide are well known, metformin, an inhibitor of complex I of the respiratory chain, is well tolerated in patients receiving it as a diabetes treatment. We recently demonstrated that metformin inhibits mROS generation and hypoxic HIF activation in cancer cells (Wheaton et al., 2014). Recently, Brand and colleagues identified multiple classes of

compounds that inhibit mROS production from respiratory complex III without affecting mitochondrial energy metabolism (Orr et al., 2015). Although the *in vivo* efficacy of these compounds remains to be determined, these selective suppressors of complex III superoxide generation inhibited hypoxic induction of HIF-1 α in cultured cells. Since hypoxia and aberrant HIF levels are associated with human pathologies such as pulmonary hypertension and cancer, it is of great importance to continue studying the mechanisms of mitochondrial HIF regulation.

EXPERIMENTAL PROCEDURES

Mice and Keratinocyte Culture

All animal experiments were approved by Northwestern University's Institutional Animal Care and Use Committee.

TFAM floxed mice (TFAM^{fl/fl}) mice were generated as described previously (Larsson et al., 1998) and were crossed with KRT14-Cre mice (Jackson Laboratory, stock no. 004782) (Dassule et al., 2000). Heterozygous TFAM^{fl/+};K14Cre^{+/-} mice were mated back to TFAM^{fl/fl} mice to obtain control (K14Cre^{-/-}) and TFAM EpiKO mice. To create TFAM+VHL EpiKO mice, TFAM^{fl/fl} mice were crossed with VHL^{fl/fl} mice (Jackson Laboratory, stock no. 004081), and the progeny were mated to produce TFAM^{fl/fl};VHL^{fl/fl} mice. These mice were crossed with KRT14-Cre mice and the heterozygous progeny were crossed back to the TFAM^{fl/fl};VHL^{fl/fl} line.

To obtain primary mouse keratinocytes, pups were skinned at P0.5. Epidermis was separated from dermis by overnight incubation in dispase (5 mg/ml) and disrupted using TrypLE Select (Invitrogen). Cells were cultured in CnT07 media (CELLnTEC) supplemented with 100 μ g/ml uridine and 1 mM sodium pyruvate. Methyl-triphenylphosphonium, N-acetylcysteine, 2,3-dimethoxy-4-naphthoquinone, deferoxamine, and mustard seed oil were purchased from Sigma. Mitochondria-targeted vitamin E was a kind gift of Dr. Balaraman Kalyanaraman (Medical College of Wisconsin).

Human neonatal foreskin keratinocyte cultures were obtained from the Northwestern University Skin Disease Research Center (NU-SDRC) and grown in Medium 153 (Cascade) supplemented with human keratinocyte growth supplement (Thermo Fisher). For knockdown experiments, cells were electroporated with 250 nmol small interfering RNA (siRNA) using a Lonza Nucleofector IIb. siRNAs were purchased from Dharmacon (stock no. D-001810-01 [non-targeting] and L-020100-00-0005 [RISPI]).

Western Blots

Keratinocytes or epidermal preparations were lysed in cell lysis buffer (Cell Signaling) or urea sample buffer, respectively. Urea sample buffer contained 8 M deionized urea, 1% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 0.1% pyronin-Y, and 5% β -mercaptoethanol. Lysates were resolved on 4%–20% Criterion gels (Bio-Rad) and transferred to nitrocellulose. Antibodies used were HIF-1 α (Cayman), TFAM (Gift of Gerald Shadel, Yale University), α -tubulin (Sigma), and β -actin (Sigma).

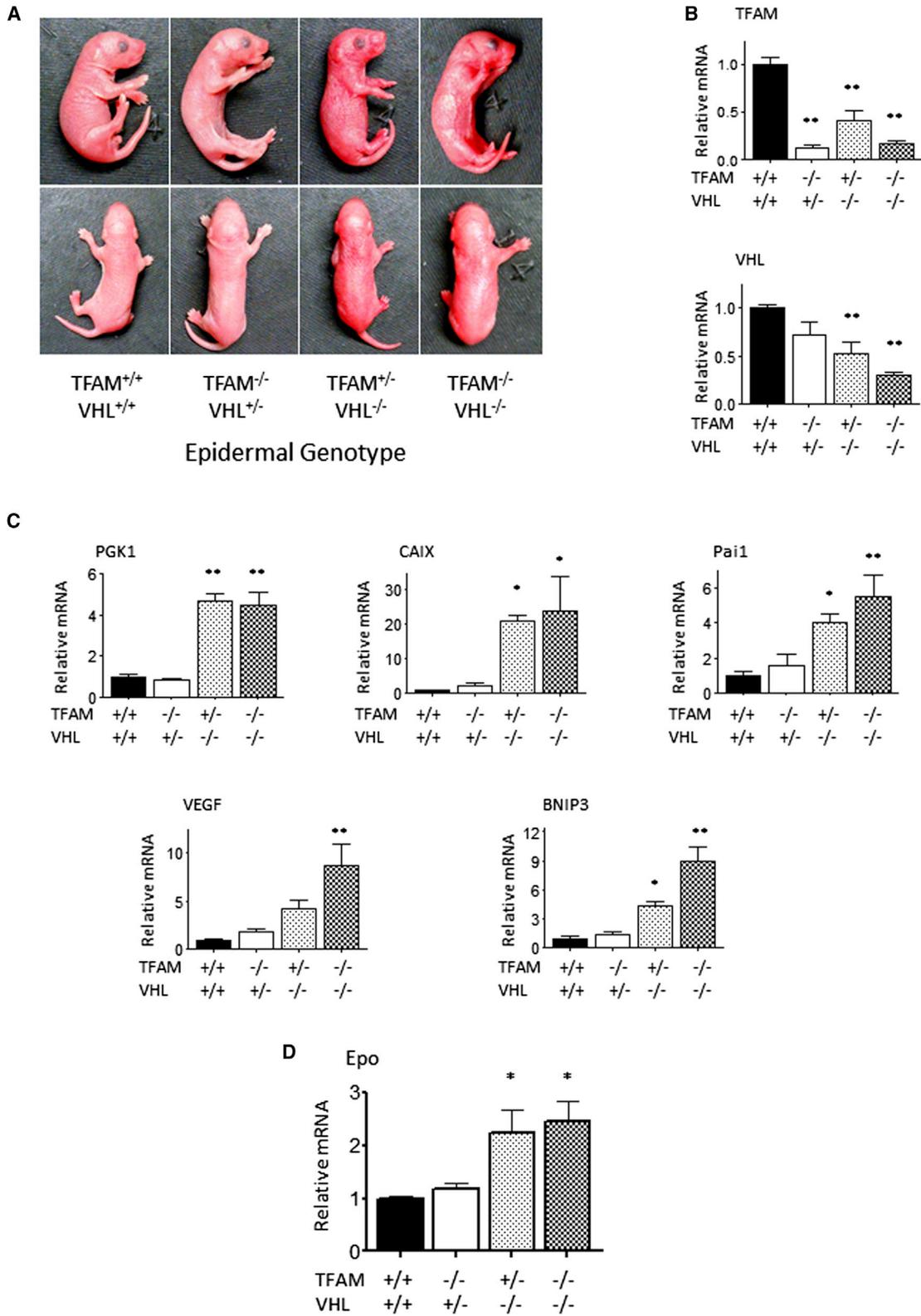
Real-Time RT-PCR

Total RNA was extracted from cells or epidermal preparations using TRIzol reagent (Invitrogen), and 1 μ g was reverse transcribed using RETROscript first-strand synthesis kit (Ambion). Real-time PCR was performed on a Bio-Rad CFX using iQ SYBR green Supermix (Bio-Rad). The following primers were used: TFAM (5'-CCAAAAAGACCTCGTTCAGC-3', 5'-ATGTCTCCGGA TCGTTTAC-3'), VHL (5'-TCCACAGCTACCGAGGTCAT-3', 5'-CGACATTGA GGGATGGCACA -3'), HIF-1 α (5'-GCGAGAACGAGAAGAAAAAGATGA-3',

(E) Real-time PCR analysis of CTGF, BNIP3, GLUT1, PAI1 and PGK1 mRNA expression in the kidneys of wild-type and TFAM EpiKO mice exposed to normoxia (21% O₂) or hypoxia (9% O₂) for 16 hr.

(F) Real-time PCR analysis of Epo mRNA expression in the kidneys of wild-type and TFAM EpiKO mice exposed to an epidermal application of either mustard seed oil (allyl isothiocyanate) or EtOH as vehicle control.

Charts represent means \pm SEM. mRNA expression was normalized to RPL19 levels. (A–E) n = 5, (F) n = 3 mice per genotype per condition. Significance was determined by one-way ANOVA using Bonferroni's post test.



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5'-ACTCTTTGCTTCGCCGAGAT-3'), HIF-2 α (5'-GAACATGGCCCCCGATG AA-3', 5'-AACCCAGTCTTGGCGTTCTC-3'), Epo (5'-AATGGAGGTGGAAGAA CAGGCCAT-3', 5'-CGAAGCAGTGAAGTGAGGCTACGTA-3'), BNIP3 (5'-GAA GCGCACAGCTACTCTCA-3', 5'-TCCAATGTAGATCCCCAAGCC-3'), CAIX (5'-GCGCTAAGCAGCTCCATACT-3', 5'-GCAGGAAGGAAGCCCTCAAT-3'), CTGF (5'-AGAAGTGTGTACGGAGCGTG-3', 5'-GTGCACCATCTTTGGCAG TG-3'), GLUT1 (5'-CGGCCTGACTACTGGCTTTG-3', 5'-GCCAAACACCTG GGCAATAAG-3'), PAI1 (5'-GTAAACGAGAGCGGCACAGT-3', 5'-GAGGAT TGTCTCTGTGCGGT-3'), PGK1 (5'-TCTTGGGAGGCGCTAAAGTT-3', 5'-AAG GCCATTCCACCACCAAT-3'), VEGF (5'-GGCCTCCGAAACCATGAAGT-3', 5'-CTGGGACCACTTGGCATGG-3'), and RPL19 (5'-GAAGGTCAAAGGAA TGTGTTCAA-3', 5'-TTTCGTGCTTCCTTGGTCTTAGA-3').

Epo ELISA

Mouse blood serum was collected using Serum Separator Tubes (BD). Serum Epo levels were determined using the mouse erythropoietin Quantikine ELISA kit (R&D Systems).

Histology and Immunostaining

Excised dorsal epidermal tissues were fixed in 10% buffered formalin and embedded in paraffin. 4- μ m sections were placed on charged slides. For automated HIF-1 α immunohistochemistry, slides were prepared using the bond polymer refine detection kit (Leica Biosystems) and staining was performed using a Leica BOND-MAX fully automated immunohistochemistry machine. Primary HIF-1 α antibody (ab2185; Abcam) was diluted 1:200 in bond primary antibody diluent before use. Imaging was performed using a Zeiss Axioplan 2 microscope and high-resolution AxioCam digital color camera. Image analysis was performed with Zeiss AxioVision software.

For CD31 immunofluorescence, paraffin sections were baked overnight at 50°C and dewaxed, followed by rehydration through Sub-X and graded alcohols, respectively. Sections were blocked in 5% donkey serum followed by overnight incubation in primary antibody (sc-1506; Santa Cruz Biotechnology). A donkey anti-goat Cy3 secondary antibody (Jackson ImmunoResearch Laboratories, 1:250 dilution) was then used, followed by DAPI counterstaining.

Mustard Seed Oil Treatment

Hair was removed from the backs of 9-day-old pups using Nair (Church & Dwight). The mice were rinsed with water and dried. The backs of the mice were then painted with either 100% EtOH or 1% mustard seed oil (allyl isothiocyanate, diluted in 100% EtOH). Solutions were applied every 80 min for 7 hr.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.03.044>.

AUTHOR CONTRIBUTIONS

R.B.H. and S.E.W. performed mouse experiments and gene expression analysis. R.B.H. and C.R.R. performed western blot analysis. R.B.H. and N.S.C. wrote the manuscript.

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Figure 4. Deletion of VHL Is Sufficient to Induce HIF-1 α Target Gene Expression in the Epidermis of TFAM EpiKO Mice

(A) Representative images of wild-type (KRT14-Cre⁻), TFAM EpiKO, VHL EpiKO, and TFAM+VHL EpiKO mice.

(B and C) Real-time PCR analysis of (B) TFAM and VHL and (C) PGK1, CAIX, Pai1, VEGF, and BNIP3 mRNA expression in the epidermis of wild-type (KRT14-Cre⁻), TFAM EpiKO, VHL EpiKO, and TFAM+VHL EpiKO mice.

(D) Real-time PCR analysis of Epo mRNA expression in the kidneys of wild-type (KRT14-Cre⁻), TFAM EpiKO, VHL EpiKO, and TFAM+VHL EpiKO mice.

Charts represent means \pm SEM. mRNA expression was normalized to RPL19 levels. n = 5 mice per genotype. Significance was determined by one-way ANOVA using Dunnett's post test.

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