Glucocorticoids promote Von Hippel Lindau degradation and Hif-1α stabilization

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Glucocorticoid (GC) and hypoxic transcriptional responses play a central role in tissue homeostasis and regulate the cellular response to stress and inflammation, highlighting the potential for cross-talk between these two signaling pathways. We present results from an unbiased in vivo chemical screen in zebrafish that identifies GCs as activators of hypoxia-inducible factors (HIFs) in the liver. GCs activated transcription of hypoxia response element (HRE) reporters in a glucocorticoid receptor (GR)-dependent manner. Importantly, GCs activated HIF transcriptional responses in a zebrafish mutant line harboring a point mutation in the GR DNA-binding domain, suggesting a nontranscriptional route for GR to activate HIF signaling. We noted that GCs increase the transcription of several key regulators of glucose metabolism that contain HREs, suggesting a role for GC/HIF cross-talk in regulating glucose homeostasis. Importantly, we show that GCs stabilize HIF protein in intact human liver tissue and isolated hepatocytes. We find that GCs limit the expression of Von Hippel Lindau protein (pVHL), a negative regulator of HIF, and that treatment with the c-src inhibitor PP2 rescued this effect, suggesting a role for GCs in promoting c-src-mediated proteasomal degradation of pVHL. Our data support a model for GCs to stabilize HIF through activation of c-src and subsequent destabilization of pVHL.

Significance

An in vivo chemical screen in zebrafish identified glucocorticoids (GCs) as activators of hypoxia-inducible factor transcriptional responses in the liver. This cross-talk is conserved in human liver and requires glucocorticoid receptor signaling but not DNA binding. In human liver cells, GCs down-regulate Von Hippel Lindau expression at a posttranscriptional level most likely through c-src-mediated proteasomal degradation. Since the liver is an important regulator of blood glucose and hypoxia-inducible factors regulate gluconeogenesis/glycogen synthesis, cross-talk between these transcriptional regulators may be essential to control glucose metabolism in the liver. This identified, conserved, noncanonical pathway may have wider physiological significance in health and disease.


The authors declare no conflict of interest.

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Glucocorticoids (GCs) are steroid hormones secreted from the adrenal glands that regulate carbohydrate, lipid, and protein metabolism. GCs are widely used as anti-inflammatory agents for treating pathological conditions where hypoxia plays a role in disease progression such as rheumatoid arthritis and chronic obstructive pulmonary disease. GCs and hypoxia pathways have a close interplay in physiology and disease (1–3); however, recent studies report conflicting results on the cross-talk between GC action and hypoxia (4, 5). Hypoxia-inducible factors (HIFs) are oxygen-sensitive transcriptional complexes constituted by α- and β-subunits that activate diverse pathways regulating cellular glucose and lipid metabolism and proliferation (6, 7). Under normoxic conditions, the HIF-1α transcriptional subunit is recognized by prolyl hydroxylases and targeted for degradation via the Von Hippel Lindau (VHL)-mediated ubiquitin proteasome pathway; however, under hypoxic conditions HIF-1α is stabilized and translocates to the nucleus to exert its transcriptional activity. HIFs play a central role in many disease processes and provide a therapeutic target for treating pathological conditions including cancer, ischemia, stroke, inflammation, and chronic anemia (8–11). Screens to identify agents that stabilize HIFs have identified numerous agents, with the majority acting either via iron chelation or as 2-oxoglutarate analogs (12). In vitro HIF-reporter screening methods, although extremely valuable, do not provide physiological information and may overlook tissue-specific activators that require a physiological context.

To identify regulators of the HIF pathway, we developed several HIF-reporter zebrafish lines (13) and completed an unbiased chemical screen. GCs activated HIF-associated transcriptional responses most prominently in the liver. Importantly, we translate these observations to human tissue and show that GCs stabilize HIF in primary human hepatocytes and intact liver slices. Our data support a model where GCs act via a transcriptional-independent mechanism by activating c-src to repress Von Hippel Lindau (pVHL) expression and stabilize HIF protein under normoxic conditions. Our study identifies a role for GCs to stabilize HIF and to regulate liver glucose metabolism.

Results

GCs Activate Hypoxic Signaling. Prolyl hydroxylase 3 (PHD3) transcription is regulated by HIFs, and we used our zebrafish phd3:eGFP HIF-reporter line to screen a chemical library (Dataset S1) for HIF activators. Dimethylxaloylglycerine (DMOG), a well-established activator of HIF signaling, was used as a positive control (14). Of the 41 initial hits, several GCs activated the reporter to comparable levels as DMOG (60 μM) (Fig. 1L). We confirmed that two GR agonists, betamethasone 17,21-dipropionate (BME) and dexamethasone (DEX), activated the reporter (Fig. 1B and C).

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An in vivo chemical screen in zebrafish identified glucocorticoids (GCs) as activators of hypoxia-inducible factor transcriptional responses in the liver. This cross-talk is conserved in human liver and requires glucocorticoid receptor signaling but not DNA binding. In human liver cells, GCs down-regulate Von Hippel Lindau expression at a posttranscriptional level most likely through c-src-mediated proteasomal degradation. Since the liver is an important regulator of blood glucose and hypoxia-inducible factors regulate gluconeogenesis/glycogen synthesis, cross-talk between these transcriptional regulators may be essential to control glucose metabolism in the liver. This identified, conserved, noncanonical pathway may have wider physiological significance in health and disease.
noted increased phd3-GFP expression mainly in the liver (Fig. 1B), suggesting that activation may be due to nonspecific stress as result of drug metabolism. To address this potential issue, reporter embryos were treated with GCs in the presence or absence of the GR antagonist RU-486 that GC-augmented BME activation of phd3:eGFP. Maximum pixel intensity in embryos as grouped by injection showing mean ± SEM (n = 14). Significance was calculated using Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison test for treatments against DMSO (P < 0.05; **P < 0.01; ***P < 0.001).

To confirm that GCs directly activate HIF-transcriptional activity, we created a HIF reporter zebrafish expressing tandem copies of a hypoxia response element (HRE) driving eGFP expression, named Tg(4xhre-tata:eGFP)\textsuperscript{+}\textsuperscript{a}. Reporter activity is decreased in Hif-1β morphants and increased by expression of a dominant active form of HIF-1α mRNA or DMOG treatment, confirming that the transgene responds to modulators of the HIF-1-signaling pathway (Fig. 2 and Fig. S2). To examine the influence of GCs, the offspring obtained from a Tg(4xhre-tata:eGFP)\textsuperscript{+}\textsuperscript{a} carrier were exposed to increasing concentrations of DEX for 24 h. Treated embryos showed a dose-dependent activation of the 4xhre-tata transgene, confirming that GCs activate HIF transcriptional responses under normoxic conditions (Fig. 3 and Fig. S3A). To independently assess whether GCs affect HIF-1 signaling and the glycolytic pathway, we analyzed the expression of three known hypoxia-dependent regulators of glucose metabolism in zebrafish (19) using whole-mount in situ hybridization (WISH); as expected, pckb3, ldha, and galt1 were up-regulated after DEX treatment as a consequence of HIF-1 activation (Fig. S3B).

**GC Activation of HIF Is Independent of GR DNA Binding.** GCs regulate gene transcription through binding and activating cytoplasmic GR, through nuclear translocation, and through direct binding to GC response elements (GRE) (20). To analyze the mechanism

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**Fig. 1.** Identification of glucocorticoids as HIF activators. (A) Retest of hits from the Spectrum screen for activators of the HIF response in phd3:eGFP zebrafish, where compounds showing an average GFP level of >44 are shown. Averaged maximum pixel intensity and SEM are shown for between five and nine embryos. Dark gray: controls; black: GC-like compounds; light gray: non-GC hits. AMG, amcinonide; BME, betamethasone; CBP, clobetasol propionate; CPO, ciclopirox olate; DEX, dexamethasone; DFL, 8,2-dimethoxyflavone; FLC, fluorocortisone; HAL, halcinonide; HCB, hydrocortisone butyrate; ICA, icariin; NIN, 7-nitroindazole; PTE, ptaeroxylin; TAA, triamcinolone acetonide; TAD, triamcinolone diacetate. (B) BME activates phd3:eGFP in an RU-486-dependent manner; representative images show the activation specific to the liver in BME-treated embryos and reduced GFP expression following BME+RU-486 cotreatment. (Magnification: 16×) (C) Quantitation of phd3:eGFP response. DMSO was added to 1% vol/vol in all experiments except Control (C), DMOG to 60 μM, and RU-486 to 5 μM as indicated (n ≥ 9). (D) BME induction of hypoxia-regulated genes and GC target genes, where values above bars indicate average fold change (FC) and triplicate samples of 20 embryos were used. For the hypoxia-induced genes, FC induction in phd3 mutants vs. wild type is given as a comparison, where FC is calculated using ΔΔCT ± SEM. (E) HIF-1β-MO abrogates BME activation of phd3:eGFP. Maximum pixel intensity in embryos as grouped by injection showing mean ± SEM (n = 14). Significance was calculated using Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison test for treatments against DMSO (P < 0.05; **P < 0.01; ***P < 0.001).

**Fig. 2.** Generation of 4xhre-tata HIF-reporter lines. (A) Schematic representation of the Tol-2 vector used to generate the Tg(4xhre-tata:eGFP)\textsuperscript{+}\textsuperscript{a} line. The construct consists of a 98-bp fragment encoding four HREs (in red) from the murine lactate dehydrogenase followed by a TATA minimal promoter (in boldface), EGFP (in green), and the poly(A) signal. (B–D) Representative image of Tg(4xhre-tata:eGFP)\textsuperscript{+}\textsuperscript{a} embryos at 48 hpf injected with a control MO (B) or the HIF-1β-MO MO (C). (Magnification: 5×) Down-regulation of Hif1α significantly decreases transgene activity as reported by the integrated density analysis of fluorescence (D). (E–G) HIF-1α dominant active (DA) mRNAs injected in Tg(4xhre-tata:eGFP)\textsuperscript{+}\textsuperscript{a} embryos increase transgene expression (F) compared with control embryos (E). (Magnification: 5×) (H–J) Treatment from 72 hpf to 96 hpf with 100 μM DMOG significantly increases 4xhre-tata transgene activity (I) with respect to the control embryos (H). (Magnification: 4×) (J) G, and A average values of fluorescence integrated density calculated for treated embryos and controls. Values represent the mean ± SEM (***P < 0.001; **P < 0.01; *P < 0.05). A.U., arbitrary units.
confirmed by measuring endogenous CRISPR-induced null mutant of the GR gene (Fig. S3). Dependency of HIF transcriptional activation was observed in a B activity in controls but had minimal effect on the 24 h, and reporter activity was measured. DEX increased endogenous HIF target genes (Fig. S3). We also studied the zebrafish grs357 mutant (22) that lacks DNA-binding function and abrogates GR transcriptional activity (Fig. 4A). Interestingly, DEX activated the Tg(4xhre-tata:eGFP)m21 reporter in a gr−/− mutant background (Fig. 4B), suggesting that a non-transcriptional mechanism underlies GC activation of HIF signaling. To independently assess the role of the GR DNA-binding domain in GC/HIF cross-talk, offspring from two grs357 homozygous carriers (72 hpf) were incubated with DEX or BME, and phd3 mRNA levels were analyzed by WISH (Fig. 4C–E). DEX and BME activated phd3 transcription in both wild-type and grs357 mutant embryos, confirming that HIF transcriptional activity was preserved.

**GCs Activate HIF Signaling in Human Hepocytes.** To verify whether the GC/HIF cross-talk observed in zebrafish is relevant to humans, we focused our efforts on human liver-derived cells. Culturing hepatocyte-derived Huh-7 cells under low oxygen stabilized HIF-1α at comparable levels to prednisolone treatment (Fig. 5A and B). To assess transcriptional responses, Huh-7 cells were transfected with a hre-luciferase (hre-luc) reporter and treated with prednisolone and dexamethasone that activated the reporter (Fig. 5C). Furthermore, prednisolone induced mRNA levels of three endogenous HIF target genes (VEGF, PDK, and GLUT1) to a similar degree as low-oxygen treatment, thus paralleling the in vivo results observed in zebrafish embryos (Fig. 5D). Furthermore, GC activation of HRE activity was sensitive to the GR antagonist RU-486 (Fig. 5D), demonstrating GR dependency. Next we screened a panel of hypoxia-regulated target genes in Huh-7 cells and found that 29/38 HIF target genes were induced by prednisolone, whereas only 3 of 38 were induced by cortisone (Dataset S2).

To confirm that the response is not simply a result of using immortalized Huh-7 cells, we showed that prednisolone activated the HRE reporter in primary human hepatocytes (Fig. 5E). To ascertain whether GCs increase HIF expression and associated transcriptional activity in an authentic liver microenvironment, we treated human liver slices with prednisolone or culture under low oxygen (3%) for 16 h and imaged nuclear HIF-1α (Fig. 6A and B) and quantified HIF target gene mRNA levels (Fig. 6 B and C). Both prednisolone and low-oxygen treatments stabilized HIF-1α (Fig. 6A and B) and increased HIF target gene expression (Fig. 6 B and C). Together, these data demonstrate that, as in the zebrafish, GCs can activate HIF signaling in the human liver.

To determine how GCs activate HIF signaling, we investigated the effect of GCs on pVHL expression, a crucial negative regulator of HIFα protein. We showed that GCs reduce pVHL expression in Huh-7 cells (Fig. 6D). This is unlikely to be mediated by a transcriptional mechanism since VHL mRNA levels were not affected (Fig. S4A). pVHL is degraded by the proteasome (Fig. 6D) following posttranslational modification by kinases such as c-src and CKII (24). Indeed, we found that inhibiting c-src activity with PP2 (25) rescued the inhibitory effects of GC on VHL expression (Fig. 6E), blocked HIF-1α induction (Fig. 6F), and normalized HIF target gene expression.

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target gene expression (Fig. S4B), suggesting a mechanism for GCs to stabilize HIFs via degrading VHL.

Discussion

The Spectrum Collection (Microsource Discovery Systems) includes compounds that modulate a wide variety of pathways; however, only GCs showed a consistent and robust HIF activation. It is surprising that GCs were not previously identified as HIF modifiers in cell-based chemical screens. We can only speculate what the apparent liver specificity of the GC effect may have contributed. Thus, in vivo chemical screens, providing a variety of cell types in a physiological context, are a useful expansion compared with more classical screening approaches, even with “well-trodden” pathways like HIF.

We confirmed that GCs promote HRE-dependent HIF transcriptional activity in fish and human liver cells. Surprisingly, fish expressing a GR mutant lacking a functional DNA-binding domain (DBD) still activated the *phd3* promoter and 4xhre reporter models after treatment with DEX. However, complete knockdown of GR and a newly generated truncated GR mutant abrogated the ability of DEX to stabilize HIF. The GR is essential for survival as demonstrated by the report that 90% of GR KO mice die soon after birth (26) and only 10% of GR KO zebrafish survive, with a reduced fitness. Of note, both mouse and zebrafish homozygous mutants of the GR-DBD survive with minor defects (22, 27), suggesting essential roles for GRE-independent GR activities, as exemplified by our study.

Since GCs are reported to activate c-src kinase (28, 29), which is known to phosphorylate and target pVHL for protesosomal degradation (24), we hypothesized a role for GCs in stabilizing HIF via c-src degradation of VHL. Consistent with our results, RU-486 inhibited such nongenomic effects of GR (30). Our in vitro studies confirm that GCs destabilize VHL protein expression, and this was rescued by cotreating cells with the c-src inhibitor PP2 (Fig. 6E). As VHL is a negative regulator of HIF, this results in HIF expression under normoxic conditions.

Although we see activation of *phd3* and 4xhre promoters and other hypoxia response genes by GCs, GCs do not fully replicate a hypoxic response. We find increased expression of many HIF target genes (Dataset S2), but some exceptions were noted such as Enolase1 and Carbonic anhydrase 9, which were downregulated by prednisolone. A direct interaction between ligand-activated GR and HIF proteins cannot be excluded, as Kodama et al. (5) have suggested that GR binds the HIF dimer. However, a simpler explanation is that, in addition to stabilizing HIF protein, GR binds a subset of promoters to regulate transcription. A complex interplay is suggested by other reports: for example, while HIF up-regulates VEGF expression and promotes angiogenesis, GCs are generally angiostatic (31) and negatively regulate VEGF expression (32–34). Similarly, during high altitude sickness, GC and HIF may have apparently opposing effects (35, 36). Therefore, we propose that the interplay that we have identified is likely to be tissue- and possibly context-specific.

We found that GC agonists increased mRNA levels of the classical HIF target *erythropoietin* both in cell culture (Dataset S2) and in zebrafish embryos and adult zebrafish livers (Fig. S5). Our data are consistent with reports showing a synergistic effect of GCs and HIFs in hematopoiesis (37, 38), and it will be interesting to investigate whether this cooperativity is GR-DBD-dependent. GCs received their name because they promote blood glucose levels as well as glucogenogenesis and glycogen storage in the liver and provide an acute response to stress (39). HIF signaling has a profound impact on cellular metabolism and induces glucogenogenesis and glycogen storage (40, 41). We suggest that GCs modulate VHL and HIF may contribute to their ability to modulate blood glucose and glycogen storage. In addition, HIF has been shown to impact lipid metabolism in the liver, where its (in)appropriate activation reduces beta oxidation and increases lipid storage capacity leading to steatosis (42, 43). It is worth noting that long-term treatment with GCs was reported to increase...

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**Fig. 5.** Glucocorticoids stabilize HIF in human cell lines. (A) Mock or prednisolone-treated (100 nM) Huh-7 cells were stained for HIF-1α expression (green) and counterstained with DAPI (gray); images show nuclear localization of HIF-1α. (Magnification: 260×) (B) HIF-1α expression in hypoxic (3% O2) and prednisolone (Pred)-treated Huh-7 cells. (C) Huh-7 cells were transfected with a HRE luciferase reporter and 24 h later treated with increasing doses of various steroids (100 nM) for 16 h. GC agonists activated the HRE reporter while 17OH did not. (D) Huh-7 cells treated with Pred/RU-486 (100 nM) for 24 h, lysed, and RNA-purified to quantify HIF transcriptional targets. Data represents fold change over control sample (Dataset S2). (E) Huh-7 cells and primary human hepatocytes (PHH) were transfected with a HRE luciferase reporter and 24 h later treated with Pred (100 nM) and/or RU-486 (100 nM) for 16 h, and HRE luciferase activity was measured. ***p < 0.001; ns, not significant (ANOVA). Data are represented as mean ± SD.

**Fig. 6.** Glucocorticoids stabilize HIF in human liver slices. (A) HIF-1α expression in human liver slices treated with prednisolone or cultured under low oxygen (3%) for 24 h and counterstained with DAPI. Both prednisolone and hypoxic treatments induce nuclear HIF-1α expression. Images are representative of five donor livers. (Magnification: 55×) (B) HIF target gene *PKD1* and *VEGF* mRNA levels in human liver slices from three donors treated with Pred/RU-486 for 24 h. (C) VHL expression in Huh-7 cells treated with prednisolone (Pred) or dexamethasone (Des) in presence or absence of proteasome inhibitor MG-132 at 10 μM. (D) VHL expression in Huh-7 cells treated with prednisolone (Pred) or dexamethasone (Des) in presence or absence of c-src inhibitor PP2 at 5 μM and probed for VHL (E) or HIF-1α (F). All samples were stained for actin as a loading control. ***p < 0.001; ns, not significant (ANOVA). Data are represented as mean ± SD.
steatosis (44). Activation of the HIF pathway via cross-talk with the GR may explain how GC excess regulates hepatic fat metabolism and the steatotic phenotype. Our experiments show that GCs predominantly stabilize HIF in the liver and may activate only a subset of HIF targets, which could be addressed by studying tissue-specific knockout models. Based on the elegant experiments of Rankin et al. (43), we would predict that liver-specific deletion of HIF-2α should protect mice from GC-induced steatosis and may reduce the effect of GCs on blood glucose levels.

It is becoming clear that HIF signaling is regulated not only by low oxygen, but also by several other inflammatory mediators (45–47). Our data support GC as a further key modulator of HIF signaling to add to this growing list. It is interesting to note that many common GC and HIF gene targets are implicated in regulating hepatic metabolism. This is potentially important, as GCs are secreted in a rhythmic circadian fashion, where circulating GC levels reach a maximum to coincide with the onset of the active phase—morning in humans, evening in mice. Since metabolic processes in the liver are coupled to the circadian clock, our data provide an additional level of control, where GC activation of the HIF pathway helps to ensure that the changing metabolic demands throughout the day are met. Our data suggest a role for GC-driven circadian components in other aspects of HIF signaling.

**Material and Methods**

**Zebrafish Strains.** vlh mutant and Tgphd3:eGFPi144 (13) fish were maintained in a mixed Tufpel long fin London wild-type (TULWT) background. The Tg(bxCRE-HSV.U2D:eGFP)116 line is a GC pathway reporter (23). The zebrafish GR mutant line gfrα-2 was incised to create homozygotes (22). To obtain wild-type embryos, LFTs were incised. To obtain homoygous vlh mutants, vlhs1171/vlhs1171; phd3:eGFP1164/eGFP1164 fish were incised. Embryos were incubated at 28 °C in E3 (5 mM NaCl, 0.17 mM KC, 0.33 mM CaCl2, 0.33 mM MgCl2, pH 7.2) containing methylene blue (Sigma-Aldrich) at 0.0001%. The generation of Tg(dh4tere-tata: eGFPp Tri) and Tg(dh4tere-tata: Cherry, cm02/eGFPp2 reporters and GR CRISPR mutation is described in **SI Text**.

**Drug Treatment of Embryos.** The Spectrum Collection (Microsource Discovery Systems) of 2,000 compounds was used; tested compounds and detailed methods can be found in **SI Text** and Dataset S1.

**Hypoxic Target Gene Activation in Zebrafish.** Three biological replicates of 10 phd3:eGFP1164 embryos were treated for 8, 12, 24, 36, and 48 h starting from 72 hpf. Embryos were treated with 10 μM betamethasone 17,21 dipropanoate and 60 μM DMOG in 1% DMSO, with untreated and 1% DMSO controls. The shB2171/shB2172, phd3:eGFP1164/eGFP1164 mutants were used as positive biological controls. Primers of 48-hpf Tg(dh4tere-tata:eGFPp2 embryos were incubated with or without 10 μM DEX and/or 0.1% Na deoxycholate/0.1% SDS) containing protease and phosphatase inhibitors (Roche). Lysates were clarified by centrifugation, and protein concentration was determined using the BCA assay (Thermo Fisher Scientific). Protein lysates (20 μg) were used for Western blotting.

**Real-time PCR Analysis.** The thermal cycler qPCR System was used to perform real-time PCR analysis. The cycling conditions were the following: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min, and 60 °C for 1 min. Fold change was calculated using the ΔΔCT method.

**Ex Vivo Liver Slices.** Liver tissue samples were collected from local National Health Service research ethics committee approval (Walsall LREC 04/Q2708/40) and with written informed consent. Donor liver tissue surplus to transplantation requirements were collected from the Queen Elizabeth Hospital. Liver slices were incised from the tissue immediately upon receipt using a Krumdieck Superfrost Tissue Slicer (Alabama R&D; 51). Briefly, the core was placed into the slice assembly under aseptic conditions, and 240-μm sections were collected and immediately transferred into Williams E media/1% L-glutamine/0.5% insulin. An albumin ELISA (Bethyl Laboratories) was used to monitor the viability of the slices. Samples from five donors were serum-starved followed by incubation under hypoxia (3% O2 or normoxia (20% O2) for 24 h. Liver slices were treated with prednisolone (100 nM) for 24 h before fixing for detection of HIF-1α by confocal microscopy.

**Confocal Microscopy on Cells.** Cells were grown on 13-mm borosilicate glass coverslips at a density of 4 × 10^4/well for 24 h and serum-starved for 5 h before treating with prednisolone (100 nM). Cells or liver slices were fixed in 3% paraformaldehyde for 25 min at room temperature (RT) and permeabilized with 0.1% TX-100 for 10 min. Cells were incubated with anti-HIF-1α for 1 h at RT, unbound antibody was removed by washing, and bound antibody was detected with Alexa-488 secondary antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Life Technologies). Cells were imaged using a Zeiss Meta Head confocal microscope with a 63× water immersion objective.

**Western Blotting.** Cells were harvested in lysis buffer (PBS/1% Triton-X100/0.1% Na deoxycholate/0.1% SDS) containing protease and phosphatase inhibitors (Roche). Lysates were clarified by centrifugation, and protein concentration was determined using a Bradford Protein Assay Reagent (Pierce). Protein lysates (20 μg) were added to sample running buffer (30% glycerol/6% SDS/0.02% bromophenol blue/10% β-mercaptoethanol/0.2 M Tris HCl; pH 6.8) and separated by SDS/PAGE. Separated proteins were transferred to PVDF membranes and incubated with primary antibodies before detection with HRP-conjugated secondary antibody and enhanced chemiluminescence (Geneflow) using a PKI imaging system (Syngene).

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