| **Title**  | Regulation of IL-1β-induced NF-κB by hydroxylases links key hypoxic and inflammatory signaling pathways |
| **Authors(s)** | Scholz, Carsten C.; Cavadas, Miguel A. S.; Tambuwala, Murtaza M.; Hams, Emily; Rodriguez, Javier; Kriegsheim, Alexander von; Cotter, Philip; Bruning, Ulrike; Fallon, Padraic G.; Cheong, Alex; Cummins, Eoin P.; Taylor, Cormac T. |
| **Publication date** | 2013-10 |
| **Publication information** | Proceedings of the National Academy of Sciences of the United States of America, 110 (46): 18490-18495 |
| **Publisher** | National Academy of Sciences |
| **Item record/more information** | http://hdl.handle.net/10197/5487 |
| **Publisher's version (DOI)** | 10.1073/pnas.1309718110 |
Regulation of IL-1β-induced NF-κB by hydroxylases links key hypoxic and inflammatory signaling pathways.

Carsten C. Scholz, 1,2 Miguel A.S. Cavadas 3,4 Murtaza M. Tambuwala, 4 Emily Hams, 1 Javier Rodriguez-Martinez, 1 Alexander Von Kriegsheim, 1 Philip Cotter, 2 Ulrike Bruning, 4 Padraic G. Fallon, 1,3 Alex Cheong, 2,3 Eoin P. Cummins* & 1,2,3 Cormac T. Taylor*.

1 Systems Biology Ireland & 2 School of Medicine and Medical Science & 3 The Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland & 4 Institute of Molecular Medicine, Trinity College Dublin, Dublin 8, Ireland.

*These authors contributed equally to this study.

Running title: Hydroxylases regulate IL-1β signaling.

Keywords: Hydroxylase, Hypoxia, IL-1β, Inflammation, NF-κB.

Classification: Major category: Biological sciences

Minor category: Biochemistry

Corresponding author: Prof. Cormac T. Taylor
UCD Conway Institute
University College Dublin
Belfield,
Dublin 4
Ireland.
E-mail: cormac.taylor@ucd.ie
Tel: +353 1-716-673
Fax: +353 1-716-6701
ABSTRACT

Hypoxia is a prominent feature of chronically inflamed tissues. Oxygen-sensing hydroxylases control transcriptional adaptation to hypoxia through the regulation of Hypoxia-Inducible Factor (HIF) and nuclear factor κB (NF-κB), both of which can regulate the inflammatory response. Furthermore, pharmacologic hydroxylase inhibitors reduce inflammation in multiple animal models. However, the underlying mechanism(s) linking hydroxylase activity to inflammatory signaling remain unclear. IL-1β, a major pro-inflammatory cytokine which regulates NF-κB is associated with multiple inflammatory pathologies. We demonstrate that a combination of PHD1 and FIH hydroxylase isoforms regulates IL-1β–induced NF-κB at the level of (or downstream of) the TRAF6 complex. Multiple proteins of the distal IL-1β-signaling pathway are subject to hydroxylation and form complexes with either PHD1 or FIH. Thus, we hypothesize that hydroxylases regulate IL-1β signaling and subsequent inflammatory gene expression. Furthermore, hydroxylase inhibition represents a new approach to the inhibition of IL-1β-dependent inflammatory signaling.
SIGNIFICANCE STATEMENT

Oxygen-sensing hydroxylases are a family of enzymes which control the cellular adaptive response to hypoxia. Hydroxylase inhibitors reduce inflammation \textit{in vivo}, however the anti-inflammatory mechanism of action remains unclear. IL-1\(\beta\) is a cytokine which potently promotes inflammation through activation of the transcription factor NF-\(\kappa\)B. Here, we demonstrate that hydroxylase inhibition leads to a suppression of IL-1\(\beta\)-induced NF-\(\kappa\)B activity and provide insight into the underlying mechanism involved. This work develops our understanding of how hydroxylase inhibition regulates IL-1\(\beta\)-induced inflammation and sheds light on our understanding of the association between hypoxic and inflammatory signaling pathways underscoring the potential use of hydroxylase inhibitors for the treatment of inflammatory disease.
INTRODUCTION

Hypoxia occurs when the demand for oxygen necessary to satisfy metabolic requirements exceeds the vascular supply. While it is well established that tissue hypoxia is a feature of a range of physiologic and pathophysiologic states including fetal development, exercise, tumor growth and ischemia, it has recently become appreciated that hypoxia is also a prominent feature in inflammatory pathologies including rheumatoid arthritis and inflammatory bowel disease (1, 2). Furthermore, hypoxia profoundly impacts upon important inflammatory processes including the regulation of neutrophil survival, macrophage survival and differentiation, T-cell differentiation and dendritic cell function (3).

A key mediator of the immunological and inflammatory sequelae of hypoxia is the hypoxia-inducible factor (HIF). HIF is suppressed in the presence of oxygen through the activity of a family of evolutionarily conserved hydroxylases, of which there are three prolyl hydroxylases (PHD1, PHD2 and PHD3; also called EGLN2, EGLN1 and EGLN3, respectively) and a single asparaginyl hydroxylase termed Factor Inhibiting HIF (FIH). PHDs control the degradation of HIF through proline hydroxylation with a dominant role for PHD2 while FIH-dependent asparagine hydroxylation is involved in fine-tuning HIF activity by regulating interactions with CBP/p300 (4). HIF has been identified as a key regulator of inflammation and immunity (5, 6) although whether its activation is ultimately pro- or anti-inflammatory in vivo is likely context specific. However, the net effect of pharmacologic activation of this pathway through inhibition of hydroxylases in vivo is anti-inflammatory. The complex role of HIF and
inflammation and its potential as a therapeutic target have been recently reviewed (1-3, 7).

NF-κB, a key regulator of inflammation is another hypoxia-responsive transcription factor (8). The same hydroxylases which confer hypoxic sensitivity upon HIF have been reported to be responsible for the hypoxic sensitivity of NF-κB (9). While PHDs have been implicated in the regulation of NF-κB, the functional site(s) of proline hydroxylation in the pathway have yet to be identified (9-11). Conversely, FIH-dependent asparagine hydroxylation sites on a number of key proteins in the NF-κB pathway have been identified, however, the functional impact of this remains unclear (12, 13). Little is known about whether hydroxylases regulate NF-κB in the stimulated state as occurs during active inflammation. However, the role of hydroxylases in inflammation is evidenced by the profoundly protective effect of pharmacologic hydroxylase inhibition in models of colitis, ischemia/reperfusion, infection and sepsis (14, 15). The mechanism underpinning this anti-inflammatory effect of hydroxylase inhibition is the topic of the current study.

IL-1β is secreted from multiple cell types and is associated with a range of inflammatory, metabolic and infectious diseases (16). Upon binding of IL-1β to its cognate receptor, a signaling cascade is initiated which signals via TRAF6 and the IKK complex resulting in the activation of NF-κB, a master regulator of inflammatory gene expression (17, 18). IL-1β plays a key role in septic shock, rheumatoid arthritis, inflammatory bowel disease and type II diabetes and is thus a major therapeutic target (16). Here, we investigated the regulation of IL-1β-induced NF-κB activity by hydroxylases with an aim to identifying new therapeutic targets in the control of IL-1β-induced inflammation.
RESULTS

Hydroxylase inhibition attenuates IL-1β-induced NF-κB activity in vitro and in vivo.

We have previously shown that hypoxia elevates basal NF-κB activity both in vitro and in vivo (19). In contrast to the regulation of basal NF-κB activity, in cells stimulated with IL-1β (but not TNFα), hypoxia inhibits stimulated NF-κB activity (Fig. S1A, B). Because, hydroxylases are key oxygen-sensing enzymes in cells, we investigated the impact of hydroxylase inhibition on IL-1β-induced NF-κB activity in vivo. IL-1β treatment led to an increase in NF-κB activity in transgenic NF-κB-luciferase reporter mice in a manner that was significantly attenuated in animals that had been pre-treated with the pan-hydroxylase inhibitor Dimethylloxallyl Glycine (DMOG) (Fig. 1A, B). Ex-vivo measurement of luciferase activity in tissues revealed that the majority of IL-1β-induced NF-κB activity was in the liver and that this was strongly attenuated in mice pre-treated with DMOG (Fig. S1C, D).

In order to gain mechanistic insight, we next investigated the effects of hydroxylase inhibition on IL-1β-induced NF-κB activity in cultured cells. HeLa cells were exposed to DMOG (which inhibits both PHDs and FIH) or the PHD-selective inhibitor JNJ-42041935 (JNJ1935) (Fig. S2) (20) prior to stimulation with IL-1β. Consistent with our in vivo experiments, pre-treatment of HeLa cells with DMOG reduced IL-1β-induced NF-κB activity in a time- and dose-dependent manner and over a range of IL-1β concentrations (Fig. 1C, D). However, JNJ1935 was without effect on IL-1β-induced NF-κB activity, leading us to hypothesize that inhibition of both prolyl and asparaginyl hydroxylation may be required. Supporting this hypothesis, graded hypoxia causes gradual reduction in IL-1β-induced NF-κB activity
with maximal inhibition occurring at 1 % O₂. This is consistent with PHD inhibition at higher oxygen levels and inhibition of both PHDs and FIH at lower oxygen levels (Fig. 1E) (21).

**PHD1 and FIH regulate IL-1β-induced NF-κB activity in a combinatorial manner.**

Four isoforms of HIF hydroxylases (PHD1, 2, 3 and FIH) have been described to confer hypoxic sensitivity on the HIF transcriptional pathway. We developed our investigation into the relative role of each of these isoforms alone and in combination in the hydroxylase-dependent regulation of IL-1β-induced NF-κB. siRNA-mediated RNA Interference allowed us to specifically and potently inhibit expression of PHD1, PHD2, PHD3 or FIH either individually or in combination (Fig. S3A-F). In a high-throughput screening assay, single knockdowns of FIH and PHD1 but not PHD2 or PHD3 reduced IL-1β-induced NF-κB activity (Fig. S3G-J). Furthermore, in time course studies both PHD1 and FIH knockdown significantly reduced IL-1β-induced NF-κB activity (Fig. S3K, L). Notably however, the combinatorial knockdown of PHD1 and FIH was at least additive (Fig. 2A). Conversely, the overexpression of the combination of FIH and PHD1 (Fig. S4) significantly enhanced IL-1β-induced NF-κB activity (Fig. 2B). These data led us to hypothesize that it is a combination of inhibiting both PHD1 and FIH, which is primarily responsible for the inhibitory effects of hydroxylase inhibitors on IL-1β-induced NF-κB activity.

**Combinatorial PHD1 and FIH knockdown attenuates IL-1β-induced NF-κB dependent gene expression.**

We next investigated the impact of combinatorial knockdown of PHD1 and FIH on the expression of IκBα (a known NF-κB target gene) in response to stimulation with
Cells exposed to IL-1β demonstrated an acute and transient decrease in IκBα expression which is associated with the activation of NF-κB and which was followed by a rebound expression as a result of transcriptional upregulation by NF-κB (22). Simultaneous knockdown of FIH and PHD1 reduced the recovery of IκBα indicating that the combinatorial knockdown of PHD1 and FIH inhibits IL-1β-induced NF-κB-dependent gene expression (Fig. 2C, D).

_Hydroxylase inhibition reduces TRAF6-dependent NF-κB activity._

We next investigated possible site(s) on the IL-1β pathway at which hydroxylases may regulate NF-κB. First, we investigated whether proteins upstream of the TRAF6 complex (Fig. S5A) could be targets for hydroxylation responsible for the regulation of IL-1β-induced NF-κB signaling. To do this, we bypassed the IL-1β receptor and associated proteins by activating NF-κB directly through overexpression of TRAF6 (23). DMOG strongly inhibited TRAF6-induced NF-κB activity (Fig. 3A) indicating that the functional hydroxylation occurs at the level of the TRAF6 complex or downstream of it. Importantly, while IL-1β and DMOG increased HIF expression as previously described (24), the abundance of components of the TRAF6 complex were not altered (Fig. 3B). Investigating the impact of DMOG on the formation of the functional E2 ubiquitin conjugating enzyme of TRAF6, we found that the interaction between UBC13 and UEV1A (the two components of the E2 complex) overexpressed in HeLa cells was not affected by treatment with DMOG (Fig. S5B).

_PHD1 and FIH physically interact with proteins of the IL-1β signaling pathway._

Having demonstrated that hydroxylase inhibitors regulate IL-1β-induced NF-κB signaling, we next investigated possible substrates for hydroxylation in the IL-1β
pathway. To do this we used an unbiased mass spectrometry-based approach to identify proteins which co-immunoprecipitate with individual hydroxylase isoforms. UEV1A and OTUB1, two proteins associated with the TRAF6 complex (18, 25), were found to be associated with PHD1 and FIH respectively but not with PHD2 or PHD3 (Table 1 and Dataset S1). UBC13 is described to be a further, central component of the complex which interacts with both UEV1A and OTUB1 (26). In order to investigate this in our system, a pull down of UBC13 was performed. Consistent with previous reports, we found that UBC13 interacted with both UEV1A and OTUB1 (Table 1). These data indicate that a complex containing UBC13, UEV1A, OTUB1, PHD1 and FIH exists in the IL-1β signaling pathway.

Downstream of the TRAF6 complex, we found that IkBβ, an ankyrin-repeat domain (ARD) containing protein, was associated with FIH. This is consistent with previous reports that ARD-containing proteins (including other IkB family members) are substrates for hydroxylation by FIH (12). We further found that interaction between FIH and OTUB1, IkBβ and previously identified substrates was promoted in cells treated with DMOG (Fig. S6 and Dataset 1). As UEV1A is cytosolic and PHD1 has previously been described as predominantly nuclear (27), we investigated the cellular distribution of PHD1. We found that endogenous PHD1 is expressed in both nuclear and cytosolic compartments in the HeLa cell culture model utilized here (Fig. S3M).

In summary, several proteins of the IL-1β signaling pathway form complexes with either PHD1 or FIH.

*Hydroxylation of proteins in the IL-1β signaling pathway.*

We next investigated whether proteins associated with IL-1β signaling were possible substrates for hydroxylation. For the unbiased identification of hydroxylated proteins
of the IL-1β pathway, we searched a large-scale qualitative dataset (28) downloaded from the Trance repository against a human database including M, P, W, Y, D, N, H-oxidations as variable modifications (Table S1 and Dataset S1) for proteins included in the IL-1β pathway (18). MS/MS analysis of this HeLa cell proteome demonstrated that UEV1A (shown to co-immunoprecipitate with PHD1; Table 1) was hydroxylated on two adjacent proline residues (proline 153 and 154 in UEV1A isoform 1). These proline residues are conserved over 5 different UEV1A isoforms (Fig. 4A, D, E and Fig. S7). Furthermore, UBC13, the protein forming the functional E2 conjugating enzyme with UEV1A was also shown to be hydroxylated on two different proline residues (Table S1) although no interaction with a PHD had been identified. OTUB1 (which was found to interact with FIH; Table 1) showed 5 hydroxylations on amino acid residues identified to be specifically targeted by FIH (N, D, H) (Table S1) (29, 30). Additionally, we found evidence for prolyl hydroxylation of OTUB1 in this dataset, although, similar to UBC13, no direct interaction with a PHD was detected (Table S1). However, UBC13 interacts strongly with UEV1A (Table 1) (25) and UEV1A interacts with PHD1 (Table 1). Similarly, OTUB1 forms a complex with UBC13 and UEV1A (Table 1) (26, 31). The potential spatial proximity of both UBC13 as well as OTUB1 to PHD1 could therefore explain the observed prolyl hydroxylations. IκBβ, which, as well as OTUB1, specifically co-immunoprecipitated with FIH, was found to be hydroxylated on one aspartate residue (Table S1).

In addition to those listed above, a number of other proteins involved in the IL-1β pathway were also found to be hydroxylated (Table S1).

FLAG-UBC13 pull down followed by Mass Spectrometry-based analysis confirmed hydroxylations on proline 19 and 21 (Table S1). Furthermore, the analysis identified
multiple sites for hydroxylation on UBC13 including proline 59 (Fig. 4B). MS/MS analysis of immunoprecipitated FLAG-HA-OTUB1 confirmed asparaginyl hydroxylation on N22 in isoform 1 (Otubain-1) (Fig. 4C and Table S1). In addition, we identified a hydroxylation of Y26 which is likely to be a non-enzymatic oxidation (Fig. 4C).

Overall, we have demonstrated that PHD1 and FIH play an important role in modulating IL-1β-induced NF-κB activity. A number of proteins in the IL-1β signaling pathway were found to be associated with hydroxylases. Furthermore, peptides from these (and other) IL-1β signaling proteins are found in the hydroxylated state. Importantly, although demonstration of association with hydroxylases and the detection of hydroxylated peptides indicate potential sites of action within this pathway, they do not definitively prove that enzymatic hydroxylation has taken place. Indeed, it is likely that non-enzymatic oxidation of proteins also occurs. Future studies will be aimed at deciphering spurious oxidations from enzymatic hydroxylations and identifying which hydroxylation(s) is/are functionally associated with altered IL-1β-induced NF-κB activity. Based on this data, we propose that hydroxylation is a key post-translational modification in the IL-1β pathway. This may have important implications for the use of hydroxylase inhibitors in a number of inflammatory disorders.
DISCUSSION

Hypoxia is a microenvironmental feature in chronically inflamed tissues due to increased metabolic activity and disrupted perfusion leading to increased oxygen demand and decreased oxygen supply at the inflamed site (1). Furthermore, hypoxia-sensitive pathways such as HIF and NF-κB, which are under the control of oxygen-sensing hydroxylases, drive pro-inflammatory responses in macrophages, T-cells, dendritic cells and neutrophils (1-3). Based on these observations, it would be predicted that pharmacologic hydroxylase inhibition in vivo would promote inflammation. However, a number of recent studies have somewhat paradoxically demonstrated a profoundly anti-inflammatory effect of hydroxylase inhibition in multiple models of acute and chronic inflammation (10, 11, 15, 32). In the current study, we investigated the underlying mechanism(s) of anti-inflammatory action of hydroxylase inhibition with an aim to develop our understanding of the role of hydroxylases in regulating inflammatory signaling pathways and the potential for hydroxylase inhibitors as anti-inflammatory therapeutics. In contrast to its effect on basal NF-κB activity we found that hydroxylase inhibition strongly reduced IL-1β-induced NF-κB activity in a manner which was dependent upon the combinatorial blockade of both PHD1 and FIH. This is consistent with data demonstrating activation of basal NF-κB but inhibition of LPS-induced NF-κB in models of sepsis (15). The regulation of a hypoxia-sensitive pathway by combinatorial activity of a prolyl hydroxylase together with FIH has been previously reported in HIF signaling where inhibition of PHD2 and FIH leads to optimal HIF-dependent transcriptional activity (4). We describe a comparable combinatorial role for a prolyl hydroxylase and an
asparaginyl hydroxylase (in this case PHD1 and FIH) conferring optimal oxygen sensitivity upon the IL-1β signaling pathway.

The mechanism underpinning the beneficial impact of hydroxylase inhibition on complex inflammatory pathways in vivo remains incompletely understood (11, 15, 32). A role for IL-1β in the pathogenesis of IBD has been implicated as it is increased in the diseased tissue and amplifies NF-κB activity leading to an increase of the secretion of inflammatory mediators, the recruitment of inflammatory cells and the secretion of enzymes such as matrix metalloproteinases (33, 34). Therefore, the down-regulation of IL-1β-induced NF-κB activity reported likely plays a role in the beneficial effects of hydroxylase inhibitors in models of IBD (14, 35).

Previous reports have demonstrated that basal and TNFα-induced NF-κB activity which is TRAF6-independent is increased with hydroxylase inhibition. Furthermore, the non-canonical NF-κB signaling pathway which is also TRAF6-independent is unaffected by hydroxylase inhibition (36). However, LPS-induced NF-κB activity which is TRAF6-dependent is down-regulated (11, 15, 32, 36, 37). We found that similar to LPS, IL-1β-induced NF-κB activity is down-regulated by hydroxylase inhibition. Additionally, TRAF6-induced NF-κB activity was also down-regulated in the presence of DMOG. Therefore, it appears that the effects of hydroxylase inhibition on NF-κB signaling is dependent upon the stimulus employed. Overall these data indicate that the effect of hydroxylase inhibition might be a general effect for pathways using TRAF6 as a major part of their signaling cascade.

We addressed the identification of putative functional hydroxylation sites in the IL-1β pathway, which may account for the effects of hydroxylases on IL-1β-signaling.
Using immunoprecipitation combined with mass spectrometric analysis, we identify that UEV1A and OTUB1 are associated with PHD1 and FIH respectively leading us to hypothesize that these may be sites of functional hydroxylation. Furthermore, we found that UEV1A and OTUB1 are also associated with UBC13 leading us to the hypothesis that PHD1, FIH, UEV1A, OTUB1 and UBC13 are part of one multi-protein complex in the IL-1β pathway. Investigating whether UEV1A is indeed a target for PHD1 we showed that hydroxylation occurred on two different proline residues with UBC13 and OTUB1 also being prolyl-hydroxylated. Additionally, we also demonstrated that OTUB1 was hydroxylated on residues, which are specific for FIH-dependent hydroxylation (N, D, H) (29, 30). UEV1A and UBC13 together form the functional E2 conjugating enzyme for TRAF6 while OTUB1 was reported to negatively regulate TRAF6 ubiquitin ligase activity (25, 26). Furthermore, it has previously been reported that this complex signals through the TRAF6 ubiquitin ligase activity.

Downstream of the TRAF6 complex we found IκBβ to interact with FIH and to be hydroxylated on an aspartate residue. Moreover, we showed that several proteins of the IL-1β signaling pathway downstream of the TRAF6 complex are hydroxylated. Overall, these results demonstrate that hydroxylation occurs on multiple proteins in the IL-1β signaling pathway and PHD1 and FIH are the main hydroxylase isoforms that regulate IL-1β-induced NF-κB activity.

The inhibition of IL-1β-induced NF-κB represents a new mechanism by which hydroxylase inhibitors are anti-inflammatory in multiple models of IL-1β-driven inflammation. However, it is likely that other mechanisms also contribute to the protective effects of these drugs \textit{in vivo}, such as expression of HIF-dependent
epithelial barrier protective genes or hydroxylase-dependent regulation of intestinal epithelial cell death by apoptosis in inflammatory bowel disease (1). Further general mechanisms implicated in the anti-inflammatory activity of hydroxylase inhibitors, which may complement the inhibition of IL-1β signaling include the regulation of regulatory T-cell abundance (via FoxP3) and the promotion of adenosine signaling (38, 39).

In summary, pharmacologic hydroxylase inhibition represents a new approach to anti-inflammatory therapy. In the current manuscript we provide evidence that a key aspect of the mechanism of anti-inflammatory action of hydroxylase inhibition is via suppression of IL-1β-induced NF-κB-dependent gene expression. Developing our understanding of the crosstalk, which exists between oxygen-sensing and inflammatory pathways will promote our understanding of how the microenvironment contributes to the development of inflammation and allow the development of new approaches to its control.
MATERIALS AND METHODS

Cell Culture and Transfection

Unless otherwise indicated HeLa cells were used for cell culture experiments under standard conditions.

For both the transfection of plasmids and siRNA treatment Lipofectamine® 2000 reagent (Invitrogen) was used according to manufacturer’s instruction. The plasmids coding for FLAG-UBC13 and FLAG-HA-OTUB1 were obtained from Addgene (FLAG-UBC13: plasmid 12460) (40) (FLAG-HA-OTUB1: plasmid 22551) (41).

Western Blot analysis

Protein concentration of whole cell, cytosolic and nuclear extracts were determined by Bio-Rad DC™ protein assay and equal protein amounts were resolved by SDS PAGE. Western blot analysis was performed as previously described (32). The membranes were blocked in 5 % milk in 1x TBST (5 mM Tris Base, 15 mM NaCl, 0.3 % Tween 20)) for 1 h at room temperature and subsequently probed with the antibodies of interest.

In vivo imaging

Female transgenic mice expressing a transgene encoding Firefly luciferase under the control of a concatamer of NF-κB response elements (Caliper LS) were injected intravenously with 10 µg/kg recombinant murine IL-1β (R&D Systems) in sterile PBS 4 h before imaging. Mice pre-treated with 8 mg/mouse DMOG were injected intraperitoneally 24 h before IL-1β treatment. Institutional animal research ethics committee approval was obtained.
**Luciferase reporter assays**

HeLa cells were transiently transfected with a plasmid coding for either a Firefly-derived or a Gaussia-derived luciferase under the control of NF-κB-responsive elements (NRE) (termed “NF-κB Gaussia/Firefly luciferase reporter assay”) or HIF-responsive elements (HRE) (termed “HIF Gaussia/Firefly luciferase reporter assay”) (dependent on the experiment).

**Immunoprecipitation**

Immunoprecipitations were performed 24 h post-transfection and after the specific treatment(s) needed for the individual experiment. Lysates were incubated with specific antibodies covalently linked to agarose beads and the (co-)immunoprecipitants were analyzed either with Western Blot or Mass Spectrometry.

**Mass spectrometric analysis of interacting proteins**

In order to detect proteins that interact with either PHD1, 2, 3, FIH or UBC13, immunoprecipitated samples were subjected to in-solution trypsin digest and mass spectrometric identification on a Q-Exactive mass spectrometer (Thermo Scientific). Relative protein concentration and identifications was determined by label-free quantification using the MaxQuant package.

**Mass spectrometric analysis of hydroxylated proteins**

We identified the UEV1A hydroxylation by searching a large-scale qualitative dataset (28) downloaded from the Trance repository against a human database including M, P, W, Y, D, N, H-oxidations as possible modifications. This data originated from HeLa cells that were grown in normoxic conditions, lysed and proteins were digested.
with LysC, Trypsin or GluC. HCD MS/MS Spectra were searched with the MaxQuant version 1.2 and 1.3.

UBC13 and OTUB1 hydroxylations were detected in immunoprecipitated FLAG-UBC13 or FLAG-HA-OTUB1 samples using a Q-Exactive mass spectrometer (Thermo Scientific) and searching with the MaxQuant with M, P, W, Y, D, N, H-oxidations as possible modifications. For the identification of isoform-specific hydroxylations, peptide-sequences containing the hydroxylated amino acid were crosschecked with the Uniprot database (http://www.uniprot.org/).

Statistical analysis

For the analysis of statistical significance of experiments with more than two different data sets to compare to each other One-Way ANOVA followed by Tukey post-test was applied. For the comparison of 2 different data sets Student’s t-test was applied. *p < 0.05, **p < 001, ***p < 0.001
ACKNOWLEDGEMENT

This work was supported by Science Foundation Ireland. JNJ1935 was a kind gift of Dr. Mike Rabinowitz (Janssen Pharmaceutical Research and Development, LLC, San Diego). The plasmids coding for PHD1-V5, PHD2-V5, PHD3-V5 and FIH-V5 were kindly supplied by Dr. Eric Metzen (University of Duisburg-Essen). The plasmid coding for FLAG-EGLN2 was a generous gift from Dr. William G. Kaelin, Jr (Dana-Farber Cancer Institute, Harvard Medical School, Boston). Prof. Luke O’Neill (Trinity College Dublin) generously provided the plasmid coding for Myc-TRAF6. We thank Dr. Dong-Er Zhang (The Scripps Research Institute, La Jolla) for making the pFlagCMV2-UbcH13 (pFLAG-UBC13) plasmid available through Addgene (plasmid 12460) and Dr. Wade Harper (Harvard Medical School, Boston) for making the pFLAG-HA-OTUB1 plasmid available (Addgene, plasmid 22551). We would like to thank Amaya Garcia and Kieran Wynne for their technical assistance in the sample preparation for mass spectrometric analysis.
REFERENCES


FIGURE LEGENDS

Figure 1. Hydroxylase inhibition attenuates IL-1β-induced NF-κB activity in vivo and in vitro. (A) IL-1β was administered to NF-κB-Luciferase reporter mice for 4 h with or without DMOG pre-treatment (8 mg/mouse, 24 h) and luciferase activity was visualized by in vivo imaging. (B) In vivo NF-κB activity was quantified by measurement of photon release from NF-κB-luciferase reporter mice. (C) Measurement of IL-1β-induced NF-κB-dependent transcriptional activity by NF-κB Gaussia luciferase reporter assay in cells treated with DMOG or JNJ1935. (D) Matrix inhibition assay was used to measure the effect of DMOG on increasing concentrations of IL-1β-induced NF-κB-dependent transcriptional activity using a NF-κB Gaussia luciferase reporter assay. Samples were pre-treated for 1 h with DMOG before IL-1β treatment. Samples were collected 10 h after the addition of IL-1β. (E) Measurement of IL-1β-induced NF-κB activity by NF-κB Gaussia luciferase reporter assay 8 h following stimulation in cells grown in graded hypoxic environments.

Data are represented as mean + SEM. (N=3-6 throughout; *p<0.05, **p<0.01, ***p<0.001 by One-Way ANOVA followed by Tukey post-test)

Figure 2. IL-1β-induced NF-κB activity and endogenous gene expression is regulated in a combinatorial manner by PHD1 and FIH. NF-κB Gaussia luciferase reporter assay was used to determine the impact of (A) combinatorial knockdown and (B) combinatorial overexpression of PHD1 and FIH on IL-1β-induced NF-κB activation. (C) Western blot analysis of whole cell IL-1β-induced IκBα protein expression in cells where PHD1 and FIH have been knocked down alone and in combination. (D)
Densitometric analysis of IκBα protein expression 24 h after IL-1β treatment with and without combinatorial knockdown of PHD1 and FIH.

Data are represented as mean + SEM. (AU = arbitrary unit; NS = not significant; N=4 throughout; *p<0.05, **p<0.01, ***p<0.001 for (A), (B) by One-Way Anova)

**Figure 3.** Hydroxylase inhibition leads to a reduction of TRAF6-induced NF-κB activity. (A) NF-κB Gaussia luciferase reporter assay in HeLa cells demonstrates that NF-κB activity induced by overexpressing TRAF6 is inhibited by addition of DMOG 6 h after transfection with TRAF6 plasmid. (B) Expression levels of components of the TRAF6 complex in HeLa cells treated with DMOG and IL-1β.

Data are represented as mean + SEM. (AU = arbitrary unit; N=4 throughout; *p<0.05, **p<0.01, ***p<0.001 by One-Way ANOVA followed by Tukey post-test)

**Figure 4.** Hydroxylation of UEV1A, UBC13 and OTUB1. Tandem mass spectrometric analysis shows hydroxylation (A) of UEV1A on two proline residues (data was obtained from searching a large-scale qualitative dataset downloaded from the Trance repository), (B) of one proline residue in UBC13 in a FLAG-UBC13 immunoprecipitation and (C) of one asparaginyl hydroxylation in OTUB1 in a FLAG-HA-OTUB1 immunoprecipitation. (D) The hydroxylated proline residues of UEV1A identified in the large-scale qualitative dataset from the Trance repository are conserved over 5 isoforms (here shown as alignment using ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/). (E) Position of hydroxylated residues within the different proteins and their isoforms. AA residue (OH) = hydroxylated amino acid residue
A) 
Control | IL-1β | IL-1β + DMOG

B) 
<table>
<thead>
<tr>
<th>NRE Activity (Counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

C) 
NRE Activity (AU)

D) 
NRE Activity (Fold change to 0 ng/mL IL-1β)

E) 
NRE activity (AU)

O₂ (%)
Fold over control (AU)

Fold over control (AU)

siNT

siPHD1 + siFIH

siNT + IL-1β (1 ng/ml)

siPHD1 + siFIH + IL-1β

NRE activity (AU)

Time (h)

***

siNT

siPHD1 + siFIH

siNT + IL-1β (1 ng/ml)

siPHD1 + siFIH + IL-1β

NRE activity (AU)

Time (h)

***

siNT

siPHD1 + siFIH

siNT + IL-1β (1 ng/ml)

siPHD1 + siFIH + IL-1β

NRE activity (AU)

Time (h)

***

pcDNA3

FLAG-PHD1 + FIH-V5

pcDNA3 + IL-1β (1 ng/ml)

FLAG-PHD1 + FIH-V5 + IL-1β

NRE activity (AU)

Time (h)

***

siNT

siPHD1 + siFIH

siNT + IL-1β (1 ng/ml)

siPHD1 + siFIH + IL-1β

NRE activity (AU)

Time (h)

***

pcDNA3

FLAG-PHD1 + FIH-V5

pcDNA3 + IL-1β (1 ng/ml)

FLAG-PHD1 + FIH-V5 + IL-1β

NRE activity (AU)

Time (h)

***
Figure A: Time course of NRE activity following treatment with IL-1β in cells treated with vehicle or DMOG. The activity is measured in Arbitrary Units (AU) over time (h).

Figure B: Western blot analysis of HIF-1α, TRAF6, UBC13, UEV1A, OTUB1, and β-actin in cells treated with Vehicle or DMOG with or without IL-1β, at different time points (1, 6, 15 h).

Key:
- Control + DMSO
- Control + DMOG
- Myc-TRAF6 + DMSO
- Myc-TRAF6 + DMOG
A) UEV1A:

![ UEV1A Mass Spectrogram ]

B) UBC13:

![ UBC13 Mass Spectrogram ]

C) OTUB1:

![ OTUB1 Mass Spectrogram ]

D) UEV1A:

- Isoform 1: RTGM1IGPRTIENIIYSLKIECGPKYEAAPYRVTINNGCVSSGNHSGGVDPR6AIS 180
- Isoform 3: RTGM1IGPRTIENIIYSLKIECGPKYEAAPYRVTINNGCVSSGNHSGGVDPR6AIS 106
- Isoform 2: RTGM1IGPRTIENIIYSLKIECGPKYEAAPYRVTINNGCVSSGNHSGGVDPR6AIS 129
- Isoform 4: RTGM1IGPRTIENIIYSLKIECGPKYEAAPYRVTINNGCVSSGNHSGGVDPR6AIS 62

E) Protein Isoform AA residue (OH)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Isoform</th>
<th>AA residue (OH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UEV1A</td>
<td>1</td>
<td>P153, P154</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>P75, P80</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>P102, P103</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>P102, P103</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>P36, P36</td>
</tr>
<tr>
<td>UBC13</td>
<td>-</td>
<td>P59</td>
</tr>
<tr>
<td>OTUB1</td>
<td>1</td>
<td>N22</td>
</tr>
</tbody>
</table>
Table 1. Co-immunoprecipitation of components of the IL-1β signaling pathway with PHD1, 2, 3, FIH or UBC13.

<table>
<thead>
<tr>
<th>Known Interactors</th>
<th>Interacting protein</th>
<th>PHD1</th>
<th>PHD2</th>
<th>PHD3</th>
<th>Interacting protein</th>
<th>UBC13</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHD1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>PHD1</td>
<td>-</td>
</tr>
<tr>
<td>PHD2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>PHD2</td>
<td>-</td>
</tr>
<tr>
<td>PHD3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>PHD3</td>
<td>-</td>
</tr>
<tr>
<td>FIH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>FIH</td>
<td>-</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>HIF-1α</td>
<td>-</td>
</tr>
<tr>
<td>ARNT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ARNT</td>
<td>-</td>
</tr>
<tr>
<td>HSP90*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ARNT</td>
<td>-</td>
</tr>
<tr>
<td>FKBP38</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Tankyrase-1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tankyrase-2</td>
<td>+</td>
</tr>
<tr>
<td>Notch2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Notch2</td>
<td>+</td>
</tr>
<tr>
<td>RIPK4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>RIPK4</td>
<td>-</td>
</tr>
<tr>
<td>UBC13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>UBC13</td>
<td>+</td>
</tr>
<tr>
<td>UEV1A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>UEV1A</td>
<td>+</td>
</tr>
<tr>
<td>OTUB1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>OTUB1</td>
<td>+</td>
</tr>
<tr>
<td>lctlβ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>lctlβ</td>
<td>+</td>
</tr>
</tbody>
</table>

PHD1, 2, 3, FIH or UBC13 were immunoprecipitated and the co-precipitants were analyzed using Mass Spectrometry. Previously described hydroxylase targets/interactors served as controls demonstrating effective co-immunoprecipitation. HSP90*: several different isoforms of HSP90 were found which were combined in this table as “HSP90”. For further information see dataset S1.
Supporting Information

Materials and Methods

Plasmids, siRNAs, antibodies, and antibody-coupled agarose beads

The non-targeting siRNA (siNT) and the siRNAs targeting PHD1 (siPHD1), PHD2 (siPHD2) or PHD3 (siPHD3), respectively, were purchased from Dharmacon (ON-TARGETplus SMARTpool). The siRNA targeting FIH (siFIH) was produced by Eurogentec according to a previously reported sequence (1) (sequence F1).

The PHD1-V5, PHD2-V5, PHD3-V5 and FIH-V5 coding plasmids were a kind gift of Dr. Eric Metzen (University of Duisburg-Essen) while the plasmid encoding FLAG-tagged PHD1 (pFLAG-EGLN2) was generously provided by Dr. William G. Kaelin, Jr. (Dana-Farber Cancer Institute, Harvard Medical School, Boston). The NF-κB Firefly-luciferase reporter constructs were purchased from Promega while the Gaussia-luciferase reporter constructs were produced by Dr. Alex Cheong and recently described (2). The pFlagCMV2-UbcH13 (pFLAG-UBC13) plasmid was made available through Addgene by Dr. Dong-Er Zhang (The Scripps Research Institute, La Jolla) and the pFLAG-HA-OTUB1 plasmid by Dr. Wade Harper (Harvard Medical School, Boston). The plasmid coding for Myc-Traf6 was a kind gift from Prof. Luke O’Neill (Trinity College Dublin). The pUEV1A-tGFP plasmid was purchased from OriGene.

All antibodies used were purchased from commercial sources: Anti-HIF-1α (BD Biosciences), anti-HIF-2α (Novus Biologicals), anti-α-Tubulin (Santa Cruz Biotechnology), anti-TBP (TATA-box binding protein) (Abcam), anti-PHD1 (Novus Biologicals), anti-PHD2 (Novus Biologicals), anti-PHD3 (Novus Biologicals), anti-
FIH (Abcam), anti-β-Actin (Sigma-Aldrich), anti-V5 (Sigma-Aldrich), anti-FLAG (Sigma-Aldrich), anti-IκBα (Cell Signaling), anti-JNK1/2/3 (Cell Signaling), anti-P-JNK1/2/3 (Cell Signaling), anti-IKKα (Cell Signaling), anti-P-IKKα/β (Cell Signaling), anti-ERK1/2 (Cell Signaling), anti-P-ERK1/2 (Sigma-Aldrich), anti-p50 (Cell Signaling), anti-TRAF6 (Cell Signaling).

All antibody-coupled agarose beads for immunoprecipitation were purchased: Anti-FLAG M2 Affinity Gel (Sigma), Anti-V5 Agarose Affinity Gel (Clone V5-10) (Sigma).

Transfection

For both the transfection of plasmids and siRNA Lipofectamin® 2000 reagent (Invitrogen) was used according to the manufacturer’s instruction. The following concentrations of siRNAs were used (in 6-well tissue culture plates): 20 nM siPHD1, 5 nM siPHD2, 5 nM siPHD3, 25 nM siFIH. For transfections in different plate formats the amount was adjusted accordingly.

For transfection of plasmids in 6-well tissue culture plates 200 ng each of PHD1-V5, FLAG-PHD1, FIH-V5, NF-κB- and HRE-Gaussia-luciferase reporter construct were used. For transfections in different plate formats the amount was adjusted accordingly.

In vivo imaging

Female NF-κB transgenic mice expressing a transgene encoding Firefly luciferase under the control of a concatamer of NF-κB response elements (Caliper LS) were used. For IL-1β treatment mice were intravenously injected with 10 μg/kg recombinant murine IL-1β (R&D Systems) in sterile PBS 4 h before imaging. For the
inhibition of hydroxylases mice were pre-treated intraperitoneally with 8 mg/mouse DMOG 24 h before the injection of IL-1β. Subsequently, the mice were injected with 150 mg/kg luciferin intraperitoneally. *In vivo* luciferase activity was measured 10 min after the luciferin injection under anesthesia using the In Vivo Imaging System (IVIS) 200 (Caliper LS). Afterwards, organs were harvested and used for *ex vivo* imaging. All images recorded were analyzed using Living Image Software (version 3.0.2; Caliper LS). Institutional animal research ethics committee approval was obtained.

*Luciferase reporter assays*

HeLa cells were transiently transfected with a plasmid coding for either a Firefly-derived or a *Gaussia*-derived luciferase under the control of NF-κB or HIF (depending on the experiment). *Gaussia*-derived luciferase is in contrast to Firefly-derived luciferase secreted out of the cells. Therefore, to measure the activity of *Gaussia*-derived luciferase the supernatant cell media was collected at different time points while cells transfected with a construct coding for Firefly-derived luciferase were lysed in order to measure the luciferase activity (2). Using this characteristic of the *Gaussia*-derived luciferase it was possible to measure multiple time points from the same sample. Experiments using the *Gaussia*-derived luciferase construct were normalized to protein concentration and to the sum of all values of the time course of the positive control if not indicated otherwise (for detailed information see Bruning et al. 2011) (2). The experiment in Fig. 2F was normalized to the expression of a *Cypridina*-derived luciferase under the control of a CMV promoter, which was transfected in parallel with the *Gaussia*-luciferase construct. This luciferase is secreted similarly to the *Gaussia*-derived luciferase but uses a different substrate allowing it to be measured in samples containing *Gaussia*-derived luciferase (3-5).
Experiments using Firefly-derived luciferase were normalized to the activity of co-transfected β-Galactosidase under the control of a SV40 promoter.

**Immunoprecipitation**

To screen for proteins that interact with PHD1, 2, 3, FIH or UBC13 samples were treated as followed: HEK293 cells were transfected with either empty vector control (pcDNA3.1; Invitrogen), PHD1-V5, PHD2-V5, PHD3-V5 or FIH-V5 overexpression plasmids for 24 h followed by exposure to 2 mM DMOG (to trap interactants) for 2 h before V5-specific immunoprecipitation (IP). HeLa cells were transfected with either empty vector control or FLAG-UBC13 overexpression plasmid (Addgene plasmid 12460) (6) and treated with 50 µM Ascorbate followed by FLAG-specific immunoprecipitation (Table 1 and Fig. 4B). For the detection of OTUB1 asparaginyl hydroxylation FLAG-HA-OTUB1 (Addgene plasmid 22551) (7) was overexpressed in HEK293 cells for 24 h and treated with 50 µM Ascorbate for 8 h prior to immunoprecipitation using the FLAG tag (Fig. 4C).

To investigate a potential DMOG-sensitive interaction between UBC13 and UEV1A HeLa cells were transfected with FLAG-UBC13 and UEV1A-tGFP (OriGene) for 24 h followed by FLAG-specific immunoprecipitation and Western Blot analysis (Fig. S5).

To investigate DMOG-sensitive interactions of proteins with FIH V5-FIH was overexpressed for 24 h in HeLa cells with or without treatment with 2 mM DMOG 2 h prior to lysis. V5-FIH was precipitated using V5-specific agarose beads followed by Mass Spectrometric analysis (Fig. S6).

In order to immunoprecipitate the protein of interest 10 µl of agarose beads coupled with the antibody of interest were added to the cleared lysates which were incubated
for 1 h at 4°C on a vertical rotator. The samples were centrifuged for 30 s at 500 rpm to spin down the agarose. The supernatant was taken off using a vacuum pump and a gel-loading tip. The samples were washed twice with 300 µl lysis buffer (1 % Triton X100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl₂) followed by three washing steps with 300 µl washing buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl₂). This was either followed by sample preparation for Mass Spectrometric analysis (see below) or the samples being boiled in 30 µl 1x NuPage LDS Sample Buffer (diluted from 4x in NLB) (Invitrogen) without reducing reagents for 5-8 min. The agarose beads were centrifuged as above and the supernatant was transferred to new tubes. 10 mM DTT were added followed by another boiling step for 5-8 min. Subsequently the samples were used for Western Blot.

Mass Spectrometric analysis of co-immunoprecipitated and/or hydroxylated proteins

Following immunoprecipitation samples were treated as followed for mass spectrometric analysis (MS). After washing twice with 300 µL ice cold PBS, beads with bound proteins were eluted in two steps. First, by using 60 µl of eluting buffer I (50 mM Tris-HCl (pH 7.5), 2 M Urea and 50 µg/ml Trypsin (modified sequencing grade trypsin, Promega) and incubated while shaking at 27°C for 30 minutes, and secondly by adding twice 25 µl of elution buffer II (50 mM Tris-HCl (pH 7.5), 2 M Urea and 1 mM DTT). Both supernatants were combined and incubated overnight at room temperature. Samples were alkylated (20 µl iodoacetamide, 5 mg/ml, 30 min in dark). Then, the reaction was stopped with 1 µl 100 % Trifluoracetic acid (TFA) and 100 µl of the sample was immediately loaded into equilibrated handmade C18 StageTips containing Octadecyl C18 disks (Supelco). Samples were desalted by using two times 50 µl of 0.1 % TFA and eluted with two times 25 µl of 50 % AcN and 0.1
% TFA solution. Final eluates were combined and concentrated until volume was
reduced to 5 μl using a CentriVap Concentrator (Labconco). Samples were diluted to
obtain a final volume of 12 μl by adding 0.1 % TFA. The samples were run on a Q-
Exactive mass spectrometer (Thermo Scientific) connected to a Dionex Ultimate 3000
(RSLCnano) chromatography system (Thermo Scientific). Each sample was loaded
onto Biobasic Picotip Emitter (120 mm length, 75 μm ID) packed with Reprocil Pur
C18 (1.9 μm) reverse phase media column and was separated by an increasing
acetonitrile gradient, using a 53 min reverse phase gradient at a flow rate of 250
nl/min. The mass spectrometer was operated in positive ion mode with a capillary
temperature of 220°C, a capillary voltage of 1900 V applied to the capillary. All data
was acquired with the mass spectrometer operating in automatic data dependent
switching mode. A high-resolution MS scan (350-2000 Da) was performed using the
Orbitrap to select the 12 most intense ions prior to MS/MS analysis using the Ion trap.
Raw files were analysed using the MaxQuant software suite (8). MS/MS-spectra were
searched against the human uniprot database. Variable modifications included M, P,
W, Y, D, N, H-oxidations.
Supporting references


Supporting Figure Legends

**Figure S1. Regulation of cytokine-induced NF-κB activity by hydroxylase inhibition.**

NF-κB *Gaussia* luciferase reporter assay was used to investigate the impact of hypoxia (1 % O₂, 12 h) on (A) TNFα- (5 ng/ml) and (B) IL-1β-stimulated (1 ng/ml) NF-κB transcriptional activity in HEK293 cells. The control was collected from the normoxic sample 3 h after cytokine addition. (C) *Ex-vivo* imaging of heart, lung, liver, spleen and kidney tissues taken from NF-κB luciferase reporter mice exposed to IL-1β with and without DMOG pre-treatment. (D) Ex-vivo hepatic NF-κB activity was quantified by measurement of photon release from liver tissues taken from NF-κB-luciferase reporter mice.

Data shown as mean + SEM. (N=3-4 throughout, *p < 0.05, **p < 0.01, ***p < 0.001 by (A), (B) Student’s t-test or (C), (D) One-Way ANOVA followed by Tukey post-test)

**Figure S2. Comparison of the effects of DMOG and JNJ1935 on HIF.** (A-C) Western blot analysis demonstrates that pharmacologic inhibition of hydroxylase activity by DMOG or JNJ1935 for 6 h leads to increased expression of HIF-1α and HIF-2α in (A) nuclear, (B) cytosolic and (C) whole cell extracts. (D) Measurement of HIF-dependent transcriptional activity by HIF *Gaussia* luciferase reporter assay in cells exposed to DMOG or JNJ1935 for 24 h.

Data are represented as mean + SEM. (NS = Non-specific; N=3, throughout; *p<0.05, *p<0.01, ***p<0.001 by One-Way ANOVA followed by Tukey post-test)
**Figure S3.** Functional knockdown of oxygen-sensing hydroxylases and the impact of hydroxylase knockdown on IL-1β-induced NF-κB activity. (A-D) Western Blot analysis demonstrates efficiency of siRNA-mediated knockdown of (A) PHD1, (B) PHD2, (C) PHD3 or (D) FIH, respectively and demonstrates the effect of isoform-specific knockdown on HIF-1α protein expression in HeLa cells. (E) The isoform specific knockdown of individual hydroxylases by siRNA-mediated RNA interference was measured by Western Blot analysis. (F) Western blot analysis demonstrates selective combinatorial knockdown of PHD1 and FIH isoforms in HeLa cells. (G-J) NF-κB Firefly luciferase reporter assay was used in a 96-well high-throughput screening assay to investigate the impact of the knockdown of (G) PHD1, (H) PHD2, (I) PHD3 and (J) FIH on IL-1β-induced NF-κB transcriptional activity. (K), (L) NF-κB Gaussia luciferase reporter assay was used to determine the impact of individual knockdown of PHD1 (K) and FIH (L) on IL-1β-induced NF-κB activation over time. (M) Western Blot analysis of cytosolic and nuclear fractions demonstrated localization of PHD1 to both the cytosolic and nuclear compartment in cells with and without siRNA-mediated combinatorial knockdown of PHD1 and FIH. α-Tubulin was used to demonstrate successful fractionation of cytosol and nuclei. The samples were all treated with 1 ng/ml IL-1β for 10 min.

Data are represented as mean + SEM. ((G-J, M) N=3, (K, L) N = 4, *p<0.05, **p<0.01, ***p<0.001 by One-Way Anova followed by Tukey test)
Figure S4. Functional overexpression of oxygen-sensing hydroxylase isoforms and the effect of single isoform overexpression on IL-1β-induced NF-κB activity. (A) Western Blot analysis of individual and combinatorial overexpression of V5-tagged PHD1 (PHD1-V5), FLAG-tagged PHD1 (FLAG-PHD1) and V5-tagged FIH (FIH-V5) at two different time points. (B) Analysis of the functional individual and combinatorial overexpression of FLAG-PHD1 and FIH-V5 using a HIF Gaussia luciferase reporter assay. (C), (D) NF-κB Gaussia luciferase reporter assay was used to determine the impact of individual hydroxylase overexpression of PHD1 (C) and FIH (D) on IL-1β-induced NF-κB activation.

Data are represented as mean + SEM. ((A), (B) N=3, (C), (D) N=4, *p<0.05, **p<0.01, ***p<0.001 by One-Way Anova followed by Tukey test)

Figure S5. DMOG does not effect the interaction of UEV1A and UBC13. (A) Schematic representation of the key signaling nodes in the IL-1β-induced signaling pathway. (B) Co-immunoprecipitation of overexpressed UEV1A and UBC13 in HeLa cells pre-treated with DMOG for 1 h before addition of IL-1β for 10 min; analyzed by Western Blot.

Figure S6. DMOG-sensitive interaction of proteins of the IL-1β signaling pathway with FIH. HEK293 cells were transfected with either mock (control) or FIH-V5 overexpression plasmids for 24 h with and without treatment with 2 mM DMOG for 2
h before V5-specific immunoprecipitation. The precipitants were analyzed for associated proteins by Mass Spectrometry. The values were normalized to the relative amount of precipitated FIH. (A) Relative amount of precipitated FIH present in each sample. (B) – (D) DMOG-sensitive interaction of (B) IκBα, (C) IκBε, (D) p105, (E) p100, (F) IκBβ and (G) OTUB1 with FIH.

Data are represented as mean + SD. (*p<0.05, **p<0.01, ***p<0.001 by Student’s t-test)

**Figure S7. Single-site hydroxylation of UEV1A.** (A, B) Tandem mass spectrometric analysis shows single-site hydroxylation of two different proline amino acids in the UEV1A protein in a large-scale qualitative dataset downloaded from the Trance repository. A hydroxylation of both prolines at the same time was also observed (see Figure 4).
**A** TNFα–induced NF-κB activity

![Graph showing % of maximum NF-κB activity in normoxia](image)

**B** IL-1β–induced NF-κB activity

![Graph showing % of maximum NF-κB activity in normoxia](image)

**C** IL-1β–induced NF-κB activity

![Images of tissue sections with color bar](image)

**D** NRE activity (Counts)

![Graph showing NRE activity (Counts)](image)
**Table S1. Hydroxylation of proteins of the IL-1β-signaling pathway.**

<table>
<thead>
<tr>
<th>Hydroxylated protein</th>
<th>Hydroxylated amino acid(s)</th>
<th>Sequence containing hydroxylated amino acid</th>
<th>Localization probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTUB1</td>
<td>P (2x)</td>
<td><em>EFCQQVEF_PoxMCK</em></td>
<td>0.83461; 0.931064</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>VYLLYRPoxGHOxyYDILYK</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td><em>QPELGSDNIEPN(ox)CLAYDEAIMAQQDR</em></td>
<td>0.659119</td>
</tr>
<tr>
<td></td>
<td>D (2x)</td>
<td><em>EYAE_DoxDoxNIYQQK</em></td>
<td>0.924875; 0.924875</td>
</tr>
<tr>
<td></td>
<td>H (2x)</td>
<td><em>FPEH_Dox_DoxGPRYVK</em></td>
<td>1; 0.931064</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>VYLLYRPoxGHOxyYDILYK</em></td>
<td></td>
</tr>
<tr>
<td>UBC13</td>
<td>P (2x)</td>
<td><em>ETQRLAE_PoxVPoxGIK</em></td>
<td>0.86164; 0.957552</td>
</tr>
<tr>
<td>UEV1A</td>
<td>P (2x)</td>
<td><em>YPEAPoxPoxFVRFVTK</em></td>
<td>0.64497; 0.670384</td>
</tr>
<tr>
<td>IkBβ</td>
<td>D</td>
<td><em>EEEEEESEED(ox)WK</em></td>
<td>1</td>
</tr>
<tr>
<td>IkKa</td>
<td>D</td>
<td><em>EVYSLMN(ox)EDE</em></td>
<td>1</td>
</tr>
<tr>
<td>TNIP1 (ABIN2)</td>
<td>H</td>
<td><em>TVYESGAEPDAGRQLTH(ox)LGRLALE</em></td>
<td>0.868868</td>
</tr>
<tr>
<td>MKK3</td>
<td>D</td>
<td><em>MCDoxFGISGYLVDSVAK</em></td>
<td>0.999978</td>
</tr>
<tr>
<td>MKK6</td>
<td>D</td>
<td><em>MCDoxFGISGYLVDSVAK</em></td>
<td>0.999978</td>
</tr>
<tr>
<td>MK2 (MAPKAPK2)</td>
<td>P (2x)</td>
<td><em>TTSHNSFTI_PoxCYT_PoxYYVAPE</em></td>
<td>0.960567; 0.965604</td>
</tr>
<tr>
<td>HSP27</td>
<td>P (2x)</td>
<td><em>LATQSNTIP(ox)VTFESRAQLG(ox)EAAK</em></td>
<td>0.996229; 0.860748</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td><em>GPSWD(ox)PFREDWYPHSR</em></td>
<td>0.845665</td>
</tr>
<tr>
<td>ERK2 (MAPK1)</td>
<td>P</td>
<td><em>VADP(ox)DoxHoxDoxHoxTGFLTEYVATR</em></td>
<td>0.987356</td>
</tr>
<tr>
<td></td>
<td>D (2x)</td>
<td><em>VADP(ox)DoxHoxDoxHoxTGFLTEYVATR</em></td>
<td>0.987356</td>
</tr>
<tr>
<td></td>
<td>H (2x)</td>
<td><em>VADP(ox)DoxHoxDoxHoxTGFLTEYVATR</em></td>
<td>0.987356</td>
</tr>
<tr>
<td>ATF2</td>
<td>H</td>
<td><em>SKIEEPSVETTHQD(ox)SPLPH(ox)PESTDSDEK</em></td>
<td>0.688776</td>
</tr>
<tr>
<td>ELK1</td>
<td>D</td>
<td><em>LV(ox)AEVVAR</em></td>
<td>1</td>
</tr>
</tbody>
</table>

A previously published dataset of the proteome of HeLa cells grown in normoxic conditions (28) was searched for M, P, W, Y, D, N, H-oxidations as possible modifications. Proteins of the IL-1β signaling pathway are shown along with the amino acids found to be hydroxylated, the sequence detected and the localization score for the modified amino acid.