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The FIH hydroxylase is a cellular peroxide sensor that modulates HIF transcriptional activity

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Hypoxic and oxidant stresses can coexist in biological systems, and oxidant stress has been proposed to activate hypoxia pathways through the inactivation of the 'oxygen-sensing' hypoxia-inducible factor (HIF) prolyl and asparaginyl hydroxylases. Here, we show that despite reduced sensitivity to cellular hypoxia, the HIF asparaginyl hydroxylase—known as FIH, factor inhibiting HIF—is strikingly more sensitive to peroxide than the HIF prolyl hydroxylases. These contrasting sensitivities indicate that oxidant stress is unlikely to signal hypoxia directly to the HIF system, but that hypoxia and oxidant stress can interact functionally as distinct regulators of HIF transcriptional output.

Keywords: FIH; HIF; hydroxylation; peroxide

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INTRODUCTION

In metazoan cells, response to hypoxia is primarily mediated by hypoxia-inducible factor (HIF)-dependent alteration of gene transcription. In the presence of O_2 (normoxia), HIF activity is suppressed by enzymatic hydroxylations (OHs) on HIF-α subunits that alter either protein stability or transactivation: in HIF-1α, OH at P402 and P564 by the prolyl hydroxylase domain (PHD) enzymes signals proteolytic degradation by the von-Hippel-Lindau (pVHL) E3 ligase, whereas OH at N803 by factor inhibiting HIF (FIH) regulates activity of the C-terminal transactivation domain, CAD [1]. Exactly how PHD and FIH activity is inhibited by hypoxia is unclear. These Fe^{2+}- and 2-oxoglutarate (2OG)-dependent dioxygenases have apparent K_mO_2 values qualifying them to function in an O_2-dependent manner across physiological O_2 tensions [2], but this property is shared by other dioxygenases [3].

Reactive oxygen species (ROS) might function as regulators of HIF OH. In hypoxia, it has been proposed that increased mitochondrial ROS produced from the ubiquinone (coenzyme Q) oxidation–reduction cycle at complex III [4] contributes to, or even acts as, the primary mechanism for inactivation of the HIF hydroxylases [5]. However, some studies have reported decreased rather than increased production of ROS in hypoxia [6]. Furthermore, although dysregulation of HIF has been reported in response to inhibitors of mitochondrial electron transport, there is disagreement as to whether this stems from reduced ROS production, or increased cellular O_2 availability generated as a result of reduced O_2 consumption.

A key argument supporting a role for ROS is that, in normoxia, HIF can be activated by external agents that generate ROS or oxidant stress, but have no predicted effect on cellular O_2 levels [7]. Exogenous application of H_2O_2 can induce HIF in normoxia, [8] and studies of genetic or chemical interventions have provided evidence that ROS can inhibit HIF hydroxylase activity [9–11].

To better understand the role of H_2O_2 in regulation of the HIF hydroxylases, we compared the effects of H_2O_2 on both asparaginyl and prolyl OH. Surprisingly, HIF asparaginyl OH was substantially more sensitive to low concentrations of H_2O_2 than prolyl OH, whereas in moderate hypoxia asparaginyl OH was less sensitive to inhibition than prolyl OH [12]. These contrasting responses argue against a direct role for H_2O_2 in transducing the response to hypoxia, but indicate that functional interaction between the two stimuli might tune HIF transcriptional output.

RESULTS

Differential sensitivity of HIF-1α OH sites to peroxide

To compare the effect of peroxide on HIF-1α prolyl and asparaginyl OH, U2OS cells expressing doxycycline (dox)-inducible HIF-1α (344–826 V5) were exposed to bolus additions of *tert*-butyl hydroperoxide (T-hydro 10 μM) every 25 min for 3 h. To minimize degradation of prolyl OH HIF-1α, cells were also treated with the proteasomal inhibitor MG132. Immunoblotting (IB) of extracts confirmed no alteration in HIF-1α synthesis by peroxide (Fig 1A). Extracts were then probed with hydroxy-residue-specific antibodies [12]. Although this showed modest inhibitory effects of peroxide on P402-OH/P564-OH, strikingly greater inhibition of N803-OH was revealed (Fig 1A supplementary Fig S1A online). Consistent with the modest effects on P402/P564, T-hydro at this concentration only weakly induced HIF-1α

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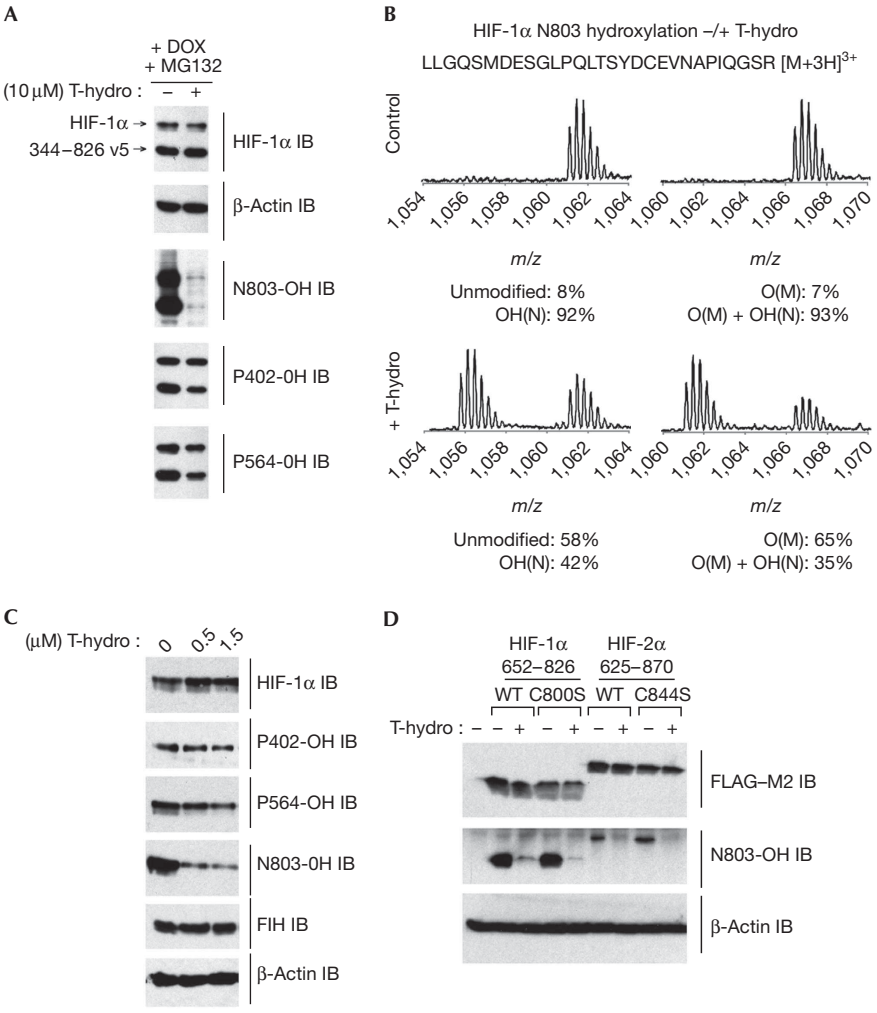

Fig 1 | Treatment of cells with peroxide reveals preferential inhibition of N803-OH compared with P402/P564. (**A**) Immunoblotting (IB) of extracts from U2OS 344–826 V5 cells treated with MG132 and −/+ T-hydro at 10 μM for 3 h. (**B**) Mass spectrometry (MS) of purified 344–826 V5 confirms inhibition of N803-OH by T-hydro. The N803 tryptic peptide is shown with summed spectra for both its hydroxylated, OH(N) and unmodified [M + 3H]3 + precursor ions. Methionine-oxidized forms of the peptide 'O(M)' are separated by chromatographic retention time and their spectra are also displayed. Percentage hydroxylation was calculated using the area under extracted-ion chromatograms for these ions. (**C**) N803-OH is more sensitive to inhibition by peroxide in RCC4 cells. (**D**) C800 is not required for peroxide-dependent inhibition of N803-OH. Weak crossreactivity of the anti-N803-OH antibody enabled assessment of the effects of C844 in HIF-2α. Dox, doxycycline; FIH, factor inhibiting hypoxia-inducible factor; HIF, hypoxia-inducible factor; OH, hydroxylation; T-hydro, *tert*-butyl hydroperoxide; WT, wild type.

in normoxic U2OS cells (supplementary Fig S1B online), although, as reported by others, [7,8] higher doses did induce HIF-1α (supplementary Figs S1C and S5D online).

To confirm directly that loss of N803-OH immunoreactivity did indeed represent a loss of OH, direct assessment was made by mass spectrometry (MS). 344–826 V5 protein purified from cells treated as above was digested with trypsin and peptides analysed. The MS analysis verified direct effects of T-hydro on N803-OH status. HIF-1α protein from control cells was almost completely OH at N803, whereas protein from T-hydro-treated cells was largely unmodified (Fig 1B).

To ensure that differential effects were not linked to MG132 or HIF-1α overexpression, OH status was analysed in VHL-defective RCC4 cells. This enabled direct comparison of prolyl with asparaginyl OH without proteasomal blockade. As the HIF-1α $t_{1/2}$ is 1–2 h in RCC4, T-hydro was applied for 4 h to ensure that OH of newly synthesized HIF-1α was assessed. A 10-μM concentration reduced total HIF-1α, probably reflecting effects on protein synthesis, and thus lower concentrations were used. HIF OH was more sensitive to peroxide in these cells, but differential sensitivity of prolyl versus asparaginyl OH remained striking (Fig 1C).

Cysteine 800 in HIF-1α does not regulate N803-OH

Redox-sensitive regulation of HIF-1α transactivation has been described and proposed to be mediated by reversible oxidation of C800 in HIF-1α [13]. Given the proximity, we considered that peroxide might be inhibiting OH of N803 via modification of

C800 (a reversible oxidative modification of C800 could have been overlooked as a result of the standard reduction step used in MS sample preparation, Fig 1B). To address this, FLAG-tagged HIF-1α and HIF-2α proteins, either wild type or with a C800S mutation (HIF-1α) or C844S mutation (the equivalent residue in HIF-2α), were transiently expressed in U2OS cells. OH status at N803 was then examined by IB of extracts from control and peroxide-treated cells (Fig 1D). Cysteine mutation was found to have no effect on OH of N803 (HIF-1α) or N847 (HIF-2α) either in untreated cells or cells treated with peroxide.

Peroxide inhibits OH of multiple FIH substrates

FIH-dependent OH of ankyrin repeat domain-containing proteins is well characterized [14]. To enable a parallel assessment of the effects of peroxide on different FIH substrates, HEK 293 cells expressing a Rabankyrin 5/HIF-1α CAD fusion protein were used [15]. Stable isotope labelling by amino acids in cell culture permitted specific analysis of fusion protein synthesized during the 3 h peroxide exposure. Following purification, tryptic digest and MS, the percentage OH values were quantified at three sites in Rabankyrin 5 (N316, N485 and N649) and at N803 in HIF-1α from the area under extracted-ion chromatograms for each set of peptide ions (supplementary Fig S2A online). OH at all sites was greatly reduced by peroxide with no apparent reduction in FIH level (supplementary Fig S2B online). Quantitative data from replicate experiments showed an approximately threefold reduction of Rabankyrin 5 N485-OH, the peptide in which both hydroxylated and unhydroxylated forms were most readily quantified (Fig 2). Overall, ankyrin OH was found to be at least as sensitive to peroxide as HIF-1α N803-OH.

Peroxide rapidly inhibits FIH in a range of cell types

Striking suppression of FIH-dependent OH across different substrates suggested an effect on the catalytic activity of FIH and indicated that the total cellular HIF prolyl and asparaginyl activities might be differentially sensitive to H_2O_2. To test this we developed a site-specific assay of hydroxylase activity in cell extracts, using GAL-tagged HIF-1α substrates prepared by *in vitro* transcription and translation. For comparative studies of prolyl and asparaginyl OH, *in vitro* transcription and translation substrates were prepared in wheat germ extract (WGE, containing neither activity), whereas for analysis of asparaginyl OH alone rabbit reticulocyte lysate (RRL) was used. Extracts from control or peroxide-treated cells were reacted with GAL–HIF-1α substrates and OH visualized by IB. FIH and PHD activity (assessed by production of N803-OH and P564-OH, respectively) was detected in control U2OS cells (Fig 3A). As reported, activity was dependent on exogenous Fe^{2+} [16], but not on 2OG, most likely because of carryover of this factor in crude extracts. Effects of peroxide were then examined. A single exposure of cells to T-hydro 10 μM caused a profound inhibition of FIH, but not PHD, activity, which was not restored by exogenous Fe^{2+} (Fig 3A, lanes 6–8). As FIH protein levels were unaltered, this provides clear evidence of differential sensitivity of PHD and FIH catalytic activity to peroxide. Pretreatment of cells with the reducing agents, ascorbate and *N*-acetyl cysteine, did not seem to protect FIH from inhibition by H_2O_2 at least under the conditions of these experiments (supplementary Fig S3 online).

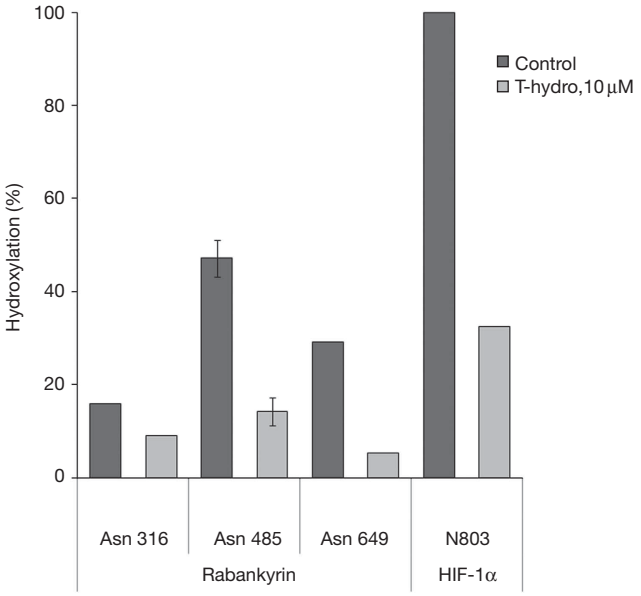

Fig 2 | Peroxide inhibits OH of Rabankyrin 5. Summary of the percentage OH quantified at N316, N485 and N649 in Rabankyrin and at N803 in HIF-1α by MS from control and peroxide-treated cells. Error bars for N485 represent standard error ($n = 4$ replicates). Quantitative data were also obtained for N316, N485 and N803 ($n = 2$ replicates) and is included to demonstrate that whenever OH could be accurately quantified it was found to be reduced by peroxide. Specific effects on OH were confirmed by a parallel assessment confirming equal abundance of control peptides −/+ peroxide (data not shown). HIF, hypoxia-inducible factor; OH, hydroxylation; T-hydro, *tert*-butyl hydroperoxide.

The maximum concentrations of peroxide derived from exposure to 10 μM T-hydro are within reported ranges for endogenous H_2O_2, although these are cell-type specific [17]. In normal physiology, immune cells experience H_2O_2 exposure, and thus we next studied cells of immune origin. Analysis of Jurkat cells revealed, first, that they were at least as sensitive as other cell lines (supplementary Fig S4 online), with differential inhibition of FIH activity versus PHD activity being observed at 3 μM T-hydro (Fig 3B). Second, it was also revealed that inhibition was rapid, with almost complete inhibition evident after 5 min (Fig 3C). In keeping with very short exposure to H_2O_2 inhibiting FIH, the effects of H_2O_2 and T-hydro were similar (Fig 3D, supplementary Fig S4 online), thus excluding a role of the *tert*-butyl moiety.

Peroxide modifies the FIH enzyme complex

To further analyse the inhibition of FIH, a refined *in vitro* assay was developed using purified enzyme, enabled by the use of U2OS cells expressing dox-inducible FLAG-tagged FIH. Extracts from cells exposed to T-hydro were assayed and inhibition of FLAG–FIH confirmed (Fig 4A). FLAG–FIH was then immunopurified and assayed for activity. Although FLAG–FIH purified from control cells was active, FLAG–FIH from peroxide-treated cells remained inactive even after readdition of cofactors (Fig 4A). As peroxide was unlikely to have persisted during purification, this indicates that inhibition results from a direct modification of FIH or complexes thereof.

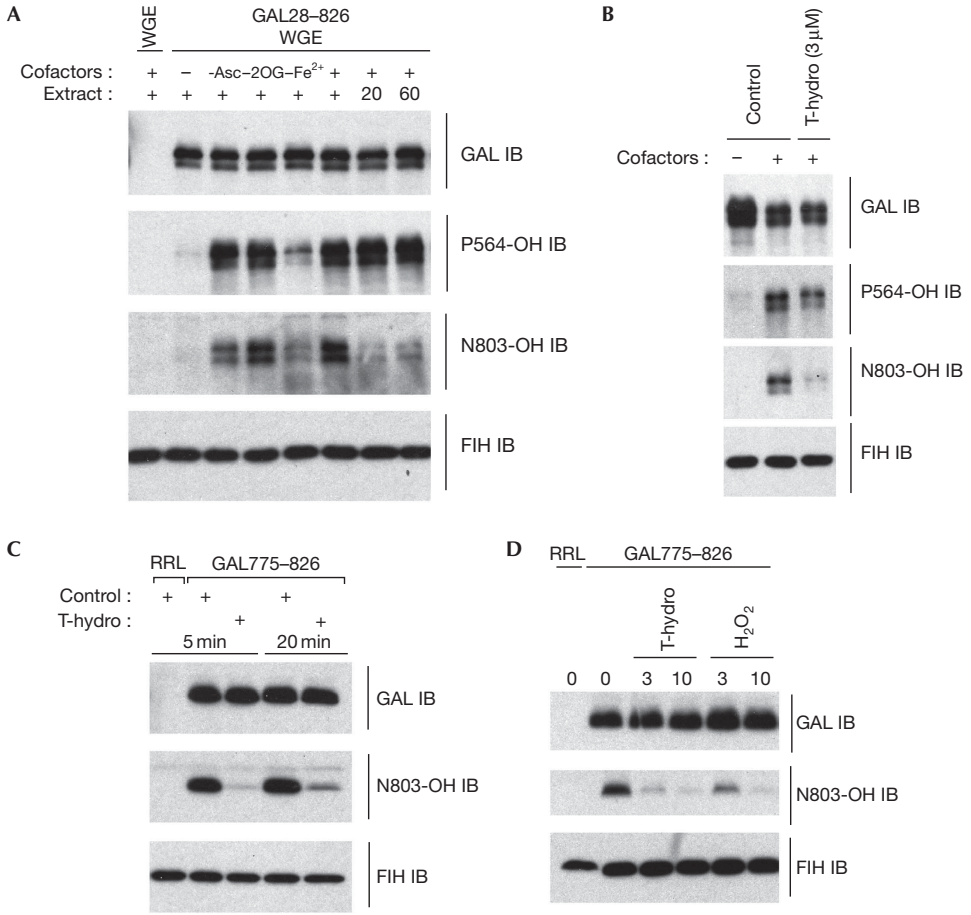

Fig 3 | Assay of cellular PHD and FIH activity reveals differential and rapid inhibition of FIH by peroxide. (**A**) WGE or GAL28–826 WGE was incubated with cofactors (ascorbate, 2OG and Fe^{2+}, +), or omissions as indicated, and extract from either control U2OS (+) or cells exposed to a single dose of 10 μM T-hydro and collected after 20 or 60 min. OH reactions were screened by IB. (**B**) OH reactions using Jurkat extracts from control, or cells exposed to a single dose of T-hydro 3 μM for 20 min. (**C**) OH reactions using Jurkat extracts from cells exposed to 10 μM T-hydro for 5 or 20 min. FIH activity was assessed by reaction with RRL GAL775–826. (**D**) OH reactions using Jurkat extracts from cells incubated with either T-hydro or H_2O_2 (μM, as indicated) for 20 min. FIH, factor inhibiting hypoxia-inducible factor; HIF, hypoxia-inducible factor; IB, immunoblotting; OH, hydroxylation; RRL, rabbit reticulocyte lysate; T-hydro, *tert*-butyl hydroperoxide; WGE, wheat germ extract; 2OG, 2-oxoglutarate.

As H_2O_2 can promote oxidant stress by iron-dependent Fenton chemistry, we tested whether depletion of chelatable iron from cells at the time of peroxide exposure affected inactivation of FIH. Cells were treated with desferrioxamine or 2,2′-dipyridyl (both at 100 μM) during induction of FLAG–FIH and before T-hydro exposure, after which FLAG–FIH was purified and assayed with full cofactor supplementation as described above. Both chelators completely blocked the inactivation of FIH by peroxide. (supplementary Fig S5 online, Fig 4B). In comparison, exposure of cells to severe hypoxia (0.1% O_2) before T-hydro exposure had no protective effect (Fig 4B).

Peroxide also increases HIF target gene expression

As inhibition of FIH by peroxide persisted in hypoxia, this suggested that interaction between these stimuli might regulate the innate hypoxic gene response. To test directly whether peroxide can modulate N803-OH status under hypoxia, RCC4 cells at 1% O_2 were treated with 0.5–4.5 μM T-hydro and HIF-1α OH status was analysed (Fig 5A). Under these conditions, in the absence of peroxide, hydroxylation at P402 and P564 was 80–90% complete, whereas N803-OH was complete, as has been described previously [12]. As in normoxia, P402-OH/P564-OH was barely affected by peroxide, in contrast with profound inhibition of N803-OH (Figs 1C and 5A). This suggested that peroxide might modulate N803-OH that ordinarily persists when HIF-1α is induced by hypoxia in VHL-competent cells. In U2OS cells, consistent with poor inhibition of PHD activity at 10 μM T-hydro, no induction of HIF-1α was observed in normoxia (Fig 5B). However, during hypoxic upregulation of HIF-1α, combination with T-hydro ablated N803-OH (Fig 5B). After 4 h hypoxic exposure, the induction of PHD3, adrenomedullin and CA9 observed in control cells was significantly potentiated by peroxide treatment (CON short interfering RNA (siRNA), Fig 5C). Analysis of a hypoxia response element-linked luciferase reporter gene confirmed the hypoxia response element dependence of the peroxide effects (supplementary Fig S6 online). To test whether

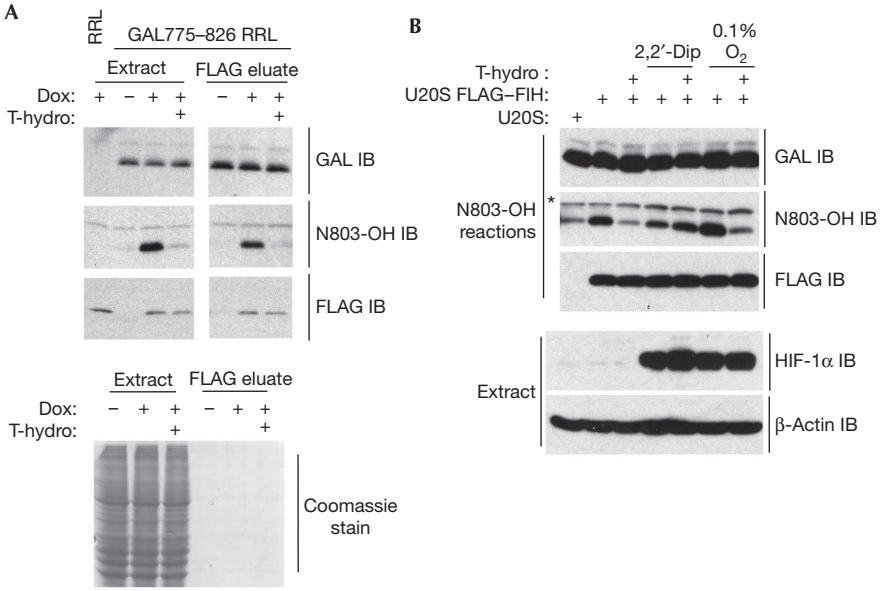

Fig 4 | The FIH enzyme complex is the site of inhibition by peroxide. (**A**) Hypotonic extracts were prepared from U2OS FLAG–FIH cells treated with dox for 24 h –/+ 10 μM T-hydro (applied in two bolus additions 40 min apart at the end of the time course). Extracts were titrated into OH reactions with RRL GAL775–826 to enable specific assay of overexpressed FLAG–FIH. Purified FLAG–FIH (FLAG eluate) was tested in parallel. Coomassie stain of extracts and FLAG eluates (lower panel). (**B**) U2OS control or FLAG–FIH cells were treated with dox, either alone or in combination with 2,2′-dipyridyl (2,2′-Dip) or 0.1% O_2 and then exposed to T-hydro as in **A**. HIF-1α IB of extracts confirmed efficacy of the 2,2′-dipyridyl and 0.1% O_2 treatments. FLAG–FIH was then purified and FLAG eluate tested for activity. * Indicates a nonspecific band. Dox, doxycycline; FIH, factor inhibiting hypoxia-inducible factor; HIF, hypoxia-inducible factor; IB, immunoblotting; OH, hydroxylation; RRL, rabbit reticulocyte lysate; T-hydro, *tert*-butyl hydroperoxide.

potentiation was FIH dependent, siRNA was used. In FIH siRNA-treated cells, hypoxic induction of target genes was already maximal and no incremental effects of peroxide were seen (Fig 5C).

DISCUSSION

The interplay between hypoxic and oxidant stress is of fundamental importance to oxygen homeostasis in biology. Our analyses have revealed that the 'O_2-sensing' PHD and FIH enzymes manifest markedly different sensitivities to inhibition by H_2O_2. Concentrations of peroxide as low as 0.5 μM reduced activity of FIH substantially; inhibition was rapid, but not rapidly reversible.

Although replenishment of cofactors did not restore FIH activity in either extract or purified enzyme, pretreatment of cells with iron chelators protected against inactivation of FIH. This interaction between iron and H_2O_2 indicates the involvement of Fenton chemistry in the mode of inhibition, as has been suggested in oxidant inactivation of other dioxygenases, including the PHDs [9]. Oxidations could occur on the catalytic iron centre itself, on susceptible amino acids or both. Although evidence of iron oxidation in PHD2 was reported by electron paramagnetic resonance spectroscopy [9], we have so far been unable to relate electron paramagnetic resonance signals to either PHDs or FIH in cells or extracts (data not shown). As FIH activity is not immediately restored by re-provision of Fe^{2+} and other factors, the mechanism of inhibition might be more complex, perhaps involving more sites of attack. Although FIH has a predicted low pKa cysteine (C236), and we have found that cysteine oxidation on FIH does occur in H_2O_2-exposed cells, neither mutation of C236 nor of C216 or C226 ablated the activity of FIH or sensitivity to H_2O_2 (supplementary Fig S7 online).

Irrespective of the precise mechanism of FIH inactivation, the work has important implications for the biology of HIF signalling. Although our data do not directly address the nature of changes in ROS production in hypoxia, the findings argue against increased production of H_2O_2, or oxygen radicals derived from H_2O_2, being the primary mechanism by which hypoxia regulates the HIF hydroxylases. First, the greater sensitivity of HIF asparaginyl OH than HIF prolyl OH to H_2O_2 contrasts with their responses to hypoxia. Second, HIF OH was similarly sensitive to H_2O_2 in both normoxia and hypoxia. Third, whereas reintroduction of O_2 to hypoxic cells leads to rapid (within 1 min) OH of HIF-1α [12], inhibition of FIH by H_2O_2 was not rapidly reversed either by removal of H_2O_2 or re-provision of cofactors. Finally, to compare and contrast these responses to those of a well-characterized redox-responsive transcription factor, we analysed induction of both HIF-1α and NF-E2-related factor 2 (NRF2) during peroxide exposure and after graded hypoxia. Whereas the low doses of T-hydro that inhibit FIH also induced NRF2, graded hypoxia had little or no action on NRF2 despite robust upregulation of HIF-1α (Fig 5D). Although our data do not exclude a role for oxidant stress in modulating PHD activity, taken together our findings indicate that the main mechanisms of inhibition of HIF hydroxylases by hypoxia and H_2O_2 are likely to be distinct.

The finding that hypoxia and H_2O_2 have qualitatively different effects on HIF OH also has important implications for HIF

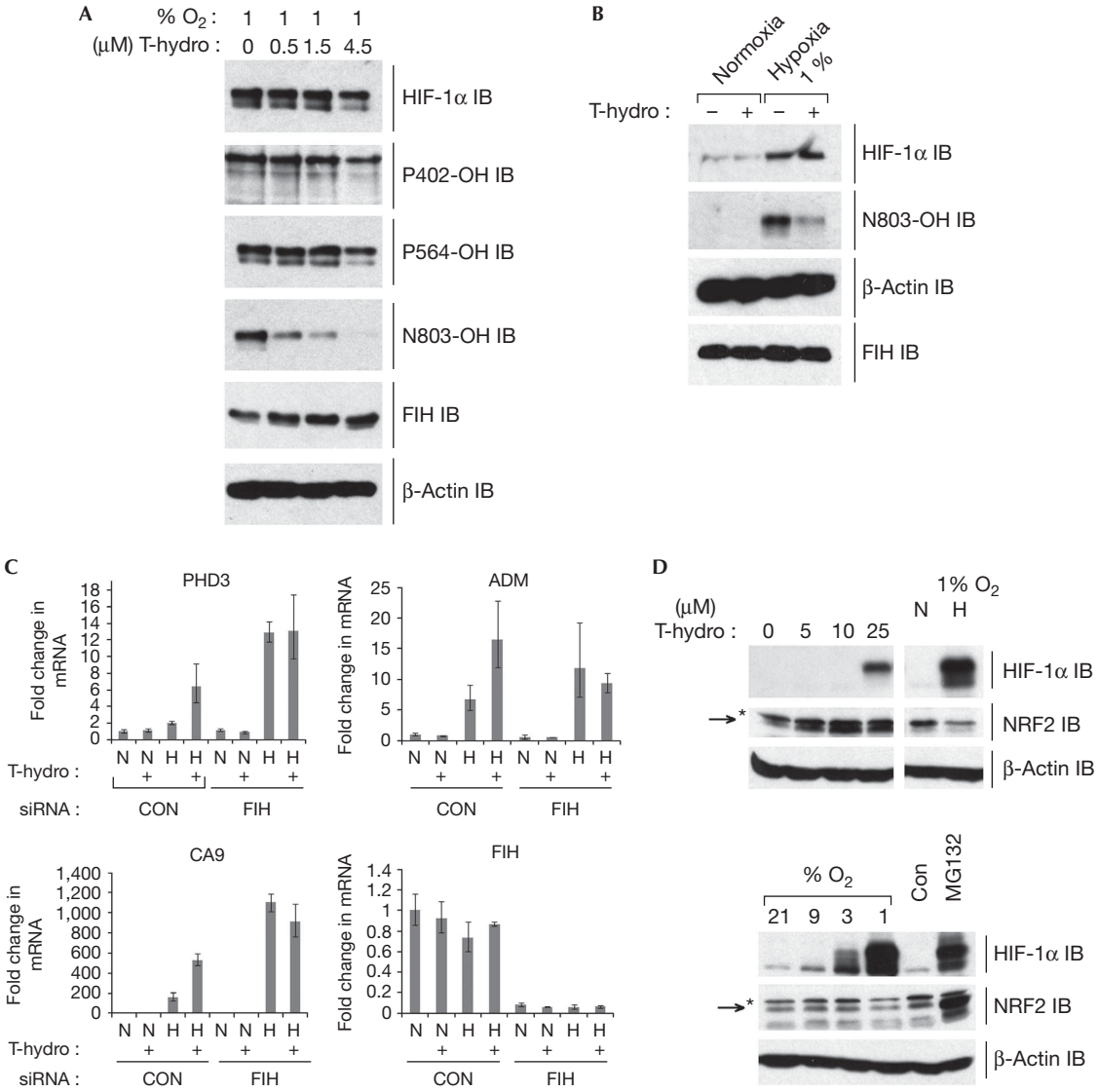

Fig 5 | Peroxide increases HIF target gene expression in an FIH-dependent manner. (**A**) RCC4 cells were incubated at 1% O_2 for 3 h before bolus T-hydro treatment (at doses indicated) for 4 h. Extracts were analysed by IB. (**B**) IB of extracts from U2OS cells incubated for 4 h either in normoxia or 1% O_2 and treated with −/+ T-hydro (10 μM, added every hour). (**C**) Reverse transcription–quantitative polymerase chain reaction showing PHD3, adrenomedullin (ADM), CA9 and FIH mRNA levels after treatment of U2OS cells with control or FIH siRNA and incubations as in **B**. mRNA levels normalized to cyclophilin are expressed as fold change relative to normoxic control siRNA levels ($n = 3$ biological replicates). Data are mean ± s.d. (**D**) IB of extracts from U2OS cells incubated for 4 h either in normoxia, graded hypoxia, T-hydro (at concentrations indicated) or with MG132. The position of NRF2 is marked with an arrow, and a nonspecific band is marked as *. At 25 μM, T-hydro cells showed morphological change. CON, control; Dox, doxycycline; FIH, factor inhibiting hypoxia-inducible factor; HIF, hypoxia-inducible factor; IB, immunoblotting; NRF2, NF-E2-related factor 2; OH, hydroxylation; siRNA, short interfering RNA; T-hydro, *tert*-butyl hydroperoxide.

signalling in situations where these stresses coexist. Oxidant stresses including elevated H_2O_2 levels well within the range required to inhibit FIH arise in a number of hypoxic pathophysiological settings [17]. Although lower sensitivity of N803-OH to hypoxia was proposed to contribute to tuning of the hypoxic transcriptional response [1], such a role is puzzling given that N803-OH persists under all but the most severe levels of hypoxia. Although we have not fully explored mechanisms by which reduced N803-OH promotes transcription of HIF target genes, our finding that sensitivity of FIH to H_2O_2 also persists in hypoxia defines an interface that might regulate HIF transcription in response to H_2O_2 or related stresses.

As FIH-catalysed ankyrin OH was also extremely sensitive to H_2O_2, it is possible that other biological processes might be affected. The existence of non-HIF signalling functions of FIH is supported by studies of targeted FIH inactivation in mice, which

reveal a metabolic phenotype that seems unrelated to the action of FIH on HIF [18]. Finally, as FIH (and not the PHDs) is a member of the large JmjC subfamily of dioxygenases, it will be of interest to determine whether comparable sensitivity to H_2O_2 is shared by these enzymes and affects other biological functions, such as epigenetic regulation.

METHODS

Cell manipulations. Standard culture was used for RCC4, U2OS and Jurkat cells. U2OS HIF-1α (344–826 V5) also with N416R, M561A and M568A mutations was derived and cultured using described protocols [12]. T-hydro and H_2O_2 (Sigma) were applied to cells at 80% confluence on 6 cm plates (4 ml medium) or 25 cm plates (25 ml medium). 2,2′-Dipyridyl and dox (used at 1 μg/ml) were also from Sigma. MG132 (Enzo Life Sciences) was used at a concentration of 25 μM. p3XFlag–CMV10 HIF-1α 652–826 or C800S mutant and HIF-2α 625–870 and C844S mutant were transfected for 3 h using FuGENE 6 (Roche) and then treated with −/+ T-hydro for a further 3 h before collection. Transfection of 40 nM siRNA duplex against *Drosophila* SIMA, or FIH [19] and hypoxic incubations was performed as described in [12].

Immunoblotting/purification and MS. RCC4 cells were lysed in urea/SDS buffer [12] and U2OS in NP-40 buffer [16]. Anti-V5-Agarose or Anti-FlagM2 affinity gel was used for purifications, and 3 × Flag peptide was used for elution (all from Sigma). Purified 344–826 V5 digested in solution with trypsin was analysed as described [12]. MS analysis of Rabankyrin 5/HIF-1α CAD FLAG protein was performed as described [15]. Anti-HIF-1α, β-actin horseradish peroxidase, anti-P402-OH, anti-P564-OH, anti-N803-OH and anti-Gal4 DBD antibodies are described in [12]. Anti-FIH [19], anti-FlagM2-horseradish peroxidase (Sigma) and anti-NRF2 (Abcam) were also used.

***In vitro* OH.** GAL28–826 and GAL775–826 substrates were prepared in WGE and RRL, respectively (TNT, Promega). OH reactions were incubated at 37 °C for 10 min in a total volume of 50 μl, containing 4 μl of RRL or WGE, 40 μl hypotonic extract [16] or Flag eluate, 4 mM L-ascorbate, 160 μM 2OG and 40 μM $FeSO_4$. Reactions were stopped with Laemmli buffer.

Reverse transcription–quantitative polymerase chain reaction. Cells were lysed in TRIzol (Sigma), and mRNA was extracted by phase separation. Equal amounts of mRNA template were used for complementary DNA synthesis using the High Capacity cDNA Kit (Applied Biosystems). Expression analyses were performed using TaqMan Assay on a StepOne thermocycler (both Applied Biosystems) using the ΔC_t method.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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Author contributions: N.M., C.J.S. and P.J.R. designed research. N.M., R.S.S., R.S., L.J.A., M.X.M., Y.-M.T. and B.M.K. performed experiments. D.C.T. and N.M. analysed data. N.M. and P.J.R. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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