Inhibition of the Oxygen-sensing Asparaginyl Hydroxylase Factor Inhibiting Hypoxia-inducible Factor (FIH): A Potential Hypoxia Response Modulating Strategy

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ABSTRACT

Factor Inhibiting Hypoxia-inducible factor (FIH) is a JmjC domain 2-oxogluarate and Fe(II) dependent oxygenase that catalyzes hydroxylation of specific asparagines in the C-terminal transcriptional activation domain of Hypoxia-Inducible Factor alpha (HIF-α) isoforms. This modification suppresses the transcriptional activity of HIF by reducing its interaction with the

transcriptional coactivators p300/CBP. By contrast with inhibition of the HIF prolyl hydroxylases (PHDs), inhibitors of FIH, which accepts multiple non-HIF substrates, are less studied; they are of interest due to their potential ability to alter metabolism (either in a HIF dependent and/or independent manner) and, providing HIF is upregulated, to modulate the course of the HIF mediated hypoxic response. Here we review studies on the mechanism and inhibition of FIH. We discuss proposed biological roles of FIH including its regulation of HIF activity and potential roles of FIH catalyzed oxidation of non-HIF substrates. We highlight potential therapeutic applications of FIH inhibitors.

KEYWORDS

Factor inhibiting hypoxia-inducible factor (FIH); hypoxia/oxygen sensing; 2-oxoglutarate (2OG) / α-ketoglutarate oxygenase inhibitors; JmjC histone demethylase, post-translational protein hydroxylation; prolyl hydroxylase domain enzymes (PHDs); epigenetics.

1. INTRODUCTION

All aerobic life forms, including metazoans, are dependent on a continuous supply of O_2 for survival.[1] How eukaryotes adapt to variations in the ambient dioxygen (O_2) concentration has been of longstanding interest in physiology.[2] In 2019, the Nobel Prize in Physiology or Medicine was awarded for the discovery of a mechanism by which animal cells sense and adapt to chronically limited O_2 supply.[3] Decreased O_2 availability results in cellular hypoxia, the condition when O_2 demand exceeds supply. Hypoxia can occur continuously or intermittently and can be acute or chronic in nature.[4] In animals the maintenance of O_2 homeostasis across the entire body is important for health; in higher animals the cardiovascular system plays a key role in delivering O_2

to tissues. O_2 availability is also important in development. Chronic hypoxia, that is when cellular O_2 demands exceed the supply for a prolonged period, can occur in healthy physiology (e.g. on travelling to high altitude), but also occurs in multiple disease contexts, including cardiovascular diseases, diabetes (ischaemic tissue), and cancer (tumors).[4, 5] The evolution of efficient O_2 sensing mechanisms that counter the effects of hypoxia were likely important events during the evolution of aerobic life.

In animals, key cellular regulators of chronic O_2 homeostasis are transcription factors named hypoxia-inducible factors (HIFs), the levels of which are (normally) highly sensitive to shifts in O_2 tension.[2, 4, 5] HIF is an α,β-heterodimeric transcription factor which mediates responses to hypoxia both at cellular and systemic levels. Levels of HIF-α, but not HIF-β, are upregulated in a wide variety of cells and tissues under hypoxic conditions. [2, 4, 5] In addition to its role in adaptation in healthy physiology, upregulated HIF-α with consequently increased HIF-α,β transcriptional activity is associated with a variety of pathologies, including cancer, cardiovascular disease and metabolic diseases, and multiple other ischemia related pathologies.[5]

Both the stability of HIF-α and transcriptional activities of HIF-α,β are negatively regulated by O_2 and 2-oxoglutarate (2OG) dependent hydroxylases[6-12], which were first identified as important oxygen sensors for the HIF pathway in the early 2000s[10, 11]. The HIF prolyl hydroxylase domain enzymes (PHDs or EGLN enzymes) and factor inhibiting HIF (FIH)[8, 11], both play significant, but different, roles in cellular adaption to hypoxia.[11] When cells suffer from hypoxia, the HIF-α related hydroxylation reactions mediated by the PHDs and FIH are suppressed due to lowered availability of O_2. The decrease in PHD activity correlates with increased HIF-α stability and consequent HIF-α,β transcriptional activity, leading to the activation of HIF target genes, including in higher

animals those involved in erythropoiesis, angiogenesis, and metabolism.[6] FIH catalysis also modulates HIF transcription but by a different mechanism to that of the PHDs.

2. ROLE OF FIH IN HIF MEDIATED OXYGEN SENSING

Both the PHDs and FIH are members of the 2OG-dependent oxygenase structural superfamily, which is one of the largest known sets of non-heme, Fe(II) dependent enzymes.[9] In human cells there are three PHD isoforms (or EGLNs)[11, 12], PHD1-3, but only one FIH isoform[13]. There are three human HIF-α isoforms (HIF-1-3α), of which HIF-1α and HIF-2α are best studied – both being responsible for upregulation of specific sets of genes, though in a context dependent and potentially overlapping manner.[2, 5] HIF-1α and HIF-2α have two transcriptional activation domains – an N-terminal activation domain (NTAD) and a C-terminal activation domain (CTAD).[2, 4-6] The PHDs appear to be more important in NTAD regulated gene expressions, while FIH is more important in CTAD mediated regulation of gene expression.[2] PHD2 (which has a characteristic MYND finger domain, not present in PHD1 and 3), is highly conserved throughout animal evolution[12, 14, 15], but FIH is only sporadically present in early animals.[16]

Despite their related, but distinct roles in HIF mediated hypoxia sensing and response, the PHDs and FIH belong to different 2OG oxygenase structural subfamilies, providing an apparent example of convergence to related, but different, functions. The PHDs belongs to the prolyl hydroxylase (PH) structural family VIII of 2OG oxygenases, which are typically monomeric and which includes other prolyl hydroxylases along with small molecule oxidant oxygenases.[9, 17] By contrast, FIH belongs to the Jumonji C (JmjC) structural family VI of 2OG oxygenases[18-21], which are often dimeric, and which includes other protein hydroxylases and N-methyl lysine demethylases acting on histone substrates (demethylation proceeds via initial hydroxylation).[17]

The PHDs catalyze C-4 *trans* hydroxylation of two prolyl residues in the oxygen dependent degradation domain of HIF-α (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in HIF-2α) in reactions that regulate levels of HIF-α. PHD catalyzed prolyl-hydroxylation of HIF-α strongly (probably >100 fold for HIF-1α) promotes its binding to the von Hippel-Lindau (VHL) ubiquitin ligase complex, so targeting HIF for ubiquitination and consequent proteasomal degradation, a process which is highly efficient in most cells under normoxic conditions.[10, 11]

FIH catalyzed asparagine-hydroxylation is a second type of O_2 sensitive HIF-α modification that regulates the transcriptional capacity of HIF-α, but by inhibiting its binding to p300/CREB-binding protein (p300/CBP), which are histone acetyl transferases that promote transcription.[22, 23] In normoxia FIH catalyzes C-3 hydroxylation of an asparagine residue in the CTAD of HIF-α (Asn803 in HIF-1α and Asn851 in HIF-2α).[13, 23, 24] Just as with PHD catalysis, FIH catalyzed CTAD asparagine-hydroxylation slows in hypoxia, though to a lesser extent than PHD catalysis, so decreasing the O_2 dependent inhibition of the interaction between HIF-α and p300/CBP.[23] In hypoxia, HIF-α is less efficiently hydroxylated by the oxygen-dependent FIH; consequently, HIF-α binds (more tightly) to p300/CBP, so promoting expression of HIF target genes.[6, 23] (Figure 1) Thus, at least in the current model, for FIH to have a 'direct' role in HIF-α,β mediated transcription, HIF-α must first be upregulated at a sufficiently high level for it to be involved in transcription. This requirement is consistent with both cellular and biochemical studies implying FIH is less sensitive to limiting O_2 availability than are the PHDs.[22, 25-27] Along with other mechanisms of transcriptional regulation, some potentially involving other hypoxia sensitive 2OG oxygenases, in particular the JmjC KDMs[17, 28, 29], FIH likely contributes to the context dependent upregulation of specific sets of HIF target genes. [22, 23] Other possible 'indirect' roles for FIH in the hypoxic response, including via the regulation of small-molecule metabolism, are discussed below.

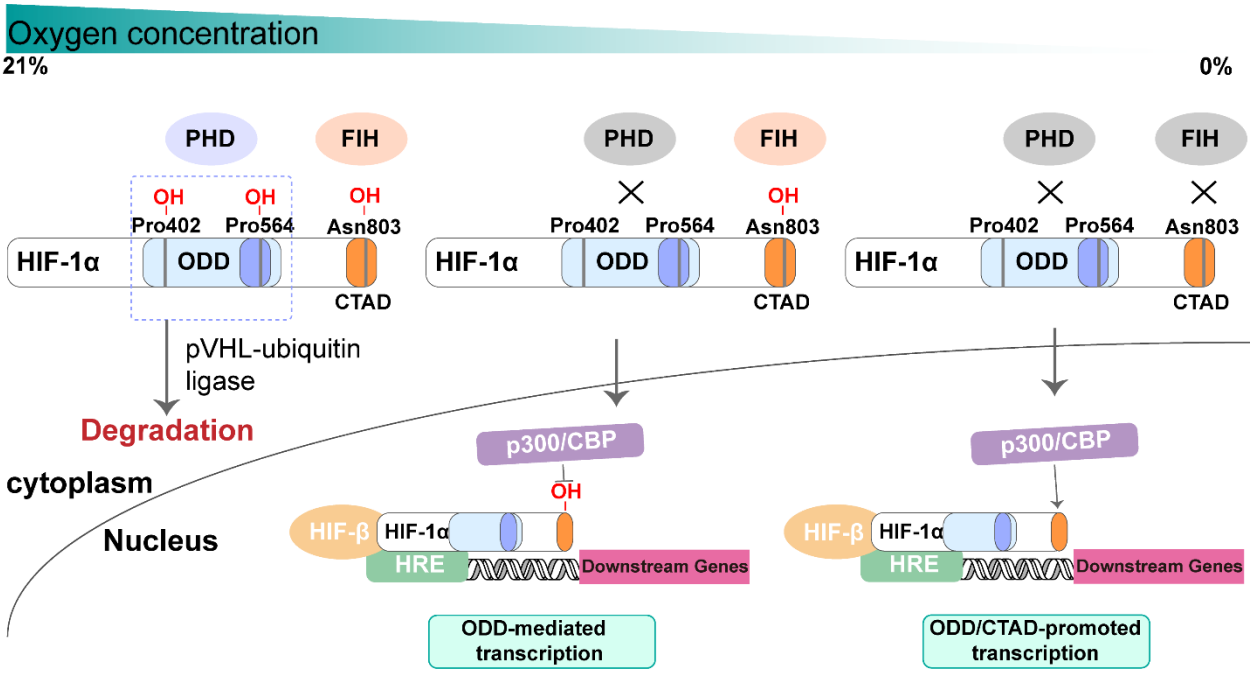

Figure 1. Overview of the roles of the 2-oxoglutarate and Fe(II) dependent oxygenases, factor inhibiting HIF (FIH) and the HIF prolyl hydroxylases (PHDs), in the dioxygen dependent regulation of HIF mediated transcription. The scheme is exemplified with HIF-1α; analogous processes occur in the case of human HIF-2α. CTAD: C-terminal activation domain of HIF-α; HRE: hypoxic response element sequence in HIF target gene; pVHL-ubiquitin ligase: von Hippel Lindau protein-ubiquitin ligase complex; p300/CBP: histone acetyl transferases that promote transcription.

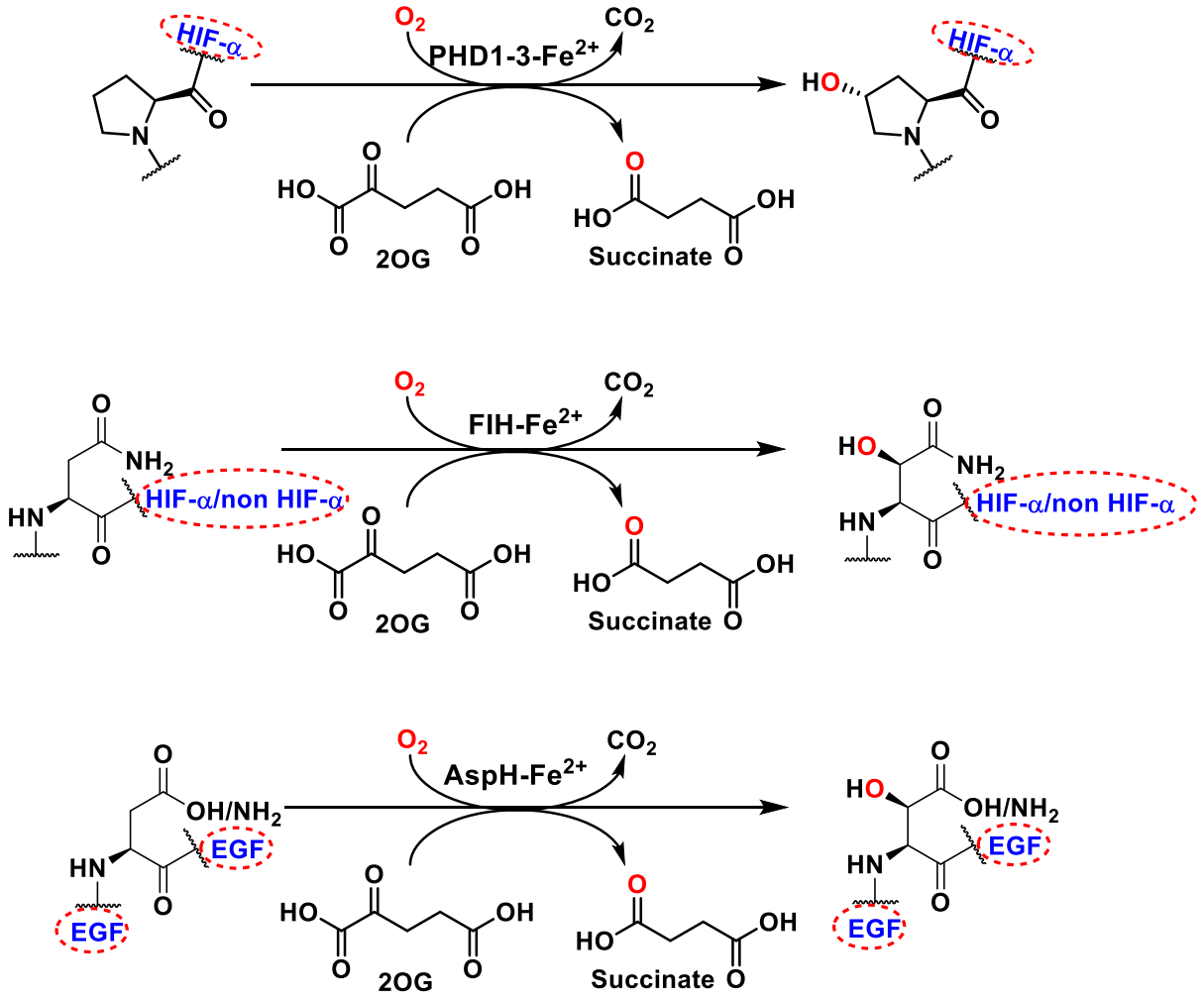

Figure 2. Stoichiometry of PHD1-3 catalyzed HIF-α prolyl-hydroxylation, FIH catalyzed HIF-α asparaginyl-hydroxylation, and AspH-catalyzed post-translational hydroxylation of asparaginyl- and aspartyl residues in epidermal growth factor (EGF)-like domains. Note FIH also accepts residues other than asparagine in its non-HIF substrates.[30]

Because they are 2OG-dependent hydroxylases, both FIH and the PHDs[11-13, 22] share the same Fe(II) cofactor, employ 2OG and dioxygen as cosubstrates and produce carbon dioxide and succinate as coproducts (Figure 2). [11-13, 22] They have related active sites (though these differ substantially in detail – see below) both employing a conserved two-histidine, one carboxylate

(from an aspartate) motif to coordinate the single active site Fe(II); these residues which form a "facial triad" of residues that occupy three of the six possible coordination sites in an octahedral coordination geometry.[20, 21, 31, 32] It should be noted that although 2OG oxygenases employ similar, but not identical, Fe(II) binding ligands, the kinetics and mechanistic details of their catalysis can vary substantially.[17, 26, 27] It is reported that the PHDs, especially PHD2, have a high affinity for Fe(II) and 2OG while FIH binds them less tightly.[33] FIH and, in particular PHD2, are the best studied of the human HIF hydroxylases. Both catalyze (at least) highly stereoselective reactions and incorporate an oxygen atom from atmospheric O_2 into their alcohol products.[24, 34, 35] The apparent *Km* values of PHDs for O_2 are slightly above the concentration of dissolved O_2 in the air, while that for FIH for O_2 is about 40% of its atmospheric concentration (note these and other kinetic studies were performed with HIF-α fragments)[26, 34, 36, 37]. These differences along with more detailed kinetic studies are consistent with cellular observation of the roles of, at least, PHD2 and FIH in HIF-α hydroxylation,[8, 22] i.e. FIH is more active in hypoxia than the PHDs (or at least PHD2). The available evidence implies PHD2 is especially well suited to a role as a hypoxia sensor as it binds Fe(II) and 2OG efficiently and does not oxidize 2OG efficiently in the absence of substrate; at least under isolated protein conditions the reaction of O_2 with the PHD2-Fe(II)-2OG-HIF-α complex appears to be rate limiting, consistent with its proposed hypoxia sensing role.[8, 11, 26, 38, 39] Modelling studies imply the rate of O_2 transport to the PHD2 active site followed by its binding to Fe(II), which likely involves displacement of a metal ion ligated water, are central to the slow / dioxygen regulated reaction of PHD2.[39, 40]

Consistent with the cellular studies, the *Km*(app)(O_2) values are lower for FIH than for PHD2 with all HIF-1α and 2α OOD fragment derived substrates.[8, 25, 26] Further, under pre-steady-state conditions, the O_2-initiated FIH reaction is substantially faster than that of PHD2.[8, 38] The more

rapid reaction of FIH with O_2 compared to the PHDs, is consistent with crystallographic studies in which a vacant coordination site ready for O_2 binding, adjacent to the oxidized substrate C-H bond is observed with FIH (at least for some substrates), but not for PHD2.[20, 41] It should also be noted, however, that FIH has other substrates that likely complicate its kinetics in cells (see below) as may the dimeric nature of FIH compared to the monomeric PHDs.[20, 21] The reported activities of both the PHDs and FIH depend on the length of the HIF-α fragments used; it is reported that FIH requires relatively longer peptide for efficient catalysis. The activity of the HIF-2α CTAD is reported to be reduced compared to the HIF-1α CTAD for isolated FIH, suggesting that FIH may act less effectively on HIF-2α than on HIF-1α.[25]

FIH and the PHDs manifest differential sensitivity of their HIF-α hydroxylation sites to peroxide, with FIH catalyzed HIF-α asparagine hydroxylation being strikingly more sensitive to peroxide than PHD catalyzed prolyl hydroxylation.[42] Peroxide rapidly suppresses FIH activity leading to decreased HIF-α asparagine hydroxylation and enhanced transcriptional activity, suggesting that FIH may have roles in sensing both peroxide and hypoxia. However, further studies on the sensitivity of other 2OG oxygenases are required as the available data may, in part, reflect the relative insensitivity of PHD catalysis to peroxide.

Genetic knockout and knockdown studies indicate that FIH and the PHDs play different roles within the regulation of oxidative metabolism. Loss of a PHD leads to the accumulation of HIF-α, which increases glycolysis and reduces O_2 consumption.[43] By contrast, the loss of FIH is reported to cause an overall increase in basal cellular respiration leading to increased O_2 consumption[44, 45], apparently resulting from changes in uncoupled mitochondrial respiration. The loss of FIH is also reported to result in increased mitochondrial activity and an increased glycolytic reserve.[46] The

effects of FIH on metabolism may be associated, in a context dependent manner, with its non-HIF substrates[47] and / or a potential role for its catalysis in directly regulating 2OG / succinate levels.

Table 1. Comparison of selected properties of FIH and PHD2 (the best characterized of the human PHDs).

Comparison		FIH	PHDs (PHD2)	Ref.
Similarities		Both are Fe(II) and 2OG dependent dioxygenases producing succinate and CO_2 as coproducts.		19
		Both are hypoxia sensors regulating HIF transcription.		12, 13
		Both have the conserved 2OG oxygenase distorted core DSBH fold and similar Fe(II) coordination chemistry, but there are key differences in their active sites, including in their 2OG and substrate binding modes.		23, 33, 34
		The PHDs (PHD2) have a high affinity for Fe(II) and 2OG and FIH less so.		24
Differences	Hydroxylation sites	Asn803 in HIF-1α Asn851 in HIF-2α (CTAD)	Pro402 and 564 in HIF-1α Pro405 and 531 in HIF-2α (ODD)	10, 11, 25, 26
	HIF activity regulation	HIF-α transactivational capacity	stability of HIF-α	6, 10, 11, 24, 25
	Preferred oligomeric form	dimeric	monomeric	9, 19

	Efficiency on HIF-1α and HIF-2α	Evidence for reduced relative effectiveness on HIF-2α	Effective on both HIF-1α and HIF-2α (but PHD3 selectively hydroxylates CODD)	27
	FIH is active at lower O_2 levels	Active *Km* (O_2): 90 ± 20 μM	Inactive *Km* (O_2): 229 ± 60 μM, i.e. slightly above the atmosphere O_2 concentration (~ 200 μM)/slow reaction with O_2	8, 26, 27, 28, 38, 39
	Sensibility to peroxide	Sensitive	Less sensitive	44
	Oxidative metabolism regulation	FIH loss accelerates rate of O_2 consumption	PHDs loss gives rise to the classical cellular response to hypoxia, i.e., increased glycolysis	45, 46, 47, 48
	Regulation of HIF target genes	Particularly important in CTAD mediated expression, (e.g., glut1, hk2, and ldha) / regulates CBP/p300 mediated expression	Also important in NTAD mediated expression, e.g., transferrin	6. 24. 25

3. MECHANISM OF FIH CATALYZED PROTEIN HYDROXYLATION

As with most other 2OG-dependent hydroxylases including the PHDs, FIH catalyzed asparagine hydroxylation requires Fe(II), O_2 and 2OG. FIH catalysis involves initial complexation

of Fe(II) followed ordered sequential active site binding of first 2OG, then HIF-α, then O_2 to the active site.[11] In the resting state the Fe(II) is complexed by the a 2-His-1-Asp triad (His199, His279 and Asp201) with the remaining three coordination sites occupied by two-three labile water molecules. [20, 21] 2OG binds in a bidentate manner via its C1 carboxylate and C2 ketone oxygen forming an octahedral coordination structure.[20, 21, 48] Subsequently, the HIF-α CTAD binds in a manner positioning Asn803 adjacent to the Fe(II); the CTAD Asn803 is held by hydrogen bonds including with Asp201 and Gln239 of FIH.[20] It is proposed that O_2 binds to an open coordination site with on the Fe(II) (potentially induced by substrate binding as precedented with other 2OG oxygenases[17, 49], but notably not PHD2[41]) yielding an Fe(III) linked superoxide. The distal superoxide oxygen then attacks the C-2 carbonyl of 2OG, resulting in oxidative decarboxylation with formation of succinate, carbon dioxide (which likely efficiently leaves the active site) and a Fe(IV)=O intermediate, the latter of which is the active oxidant.[35, 41, 50] The ferryl species then stereo-specifically abstracts a hydrogen-atom from C-3 of HIF-α Asn803 to form a substrate radical and ferric hydroxide; a radical rebound process then gives the hydroxylated product and restores the Fe(II) state.[8, 51] The hydroxylated product and succinate are then released[8, 52] and the FIH-Fe(II) complex can transition into a new catalytic cycle (Figure 3).

It is important to note that although FIH appears to follow the general path of the consensus 2OG oxygenase mechanism it may well have unusual features, including some relating to its capacity to hydroxylate multiple different (non-HIF) substrate-residues (see below). The dimeric nature of FIH may also introduce complexity in its kinetics.[20, 21] Further, as well as hydroxylation reactions, at least in isolated form, FIH can catalyze other reactions (see below), including desaturation of some residues to give α,β-unsaturated side chains.[30] FIH also has the potential to catalyze as yet undefined reactions, e.g. although - it hydroxylates the deubiquitinase ovarian

tumor domain-containing ubiquitin aldehyde binding protein 1, it also reacts to form a covalently linked protein complex with this particular substrate.[53] Thus, both the scope of FIH substrates and reactions may be remarkably wide. The precise active site features that enable this breadth of activity are yet to be defined.

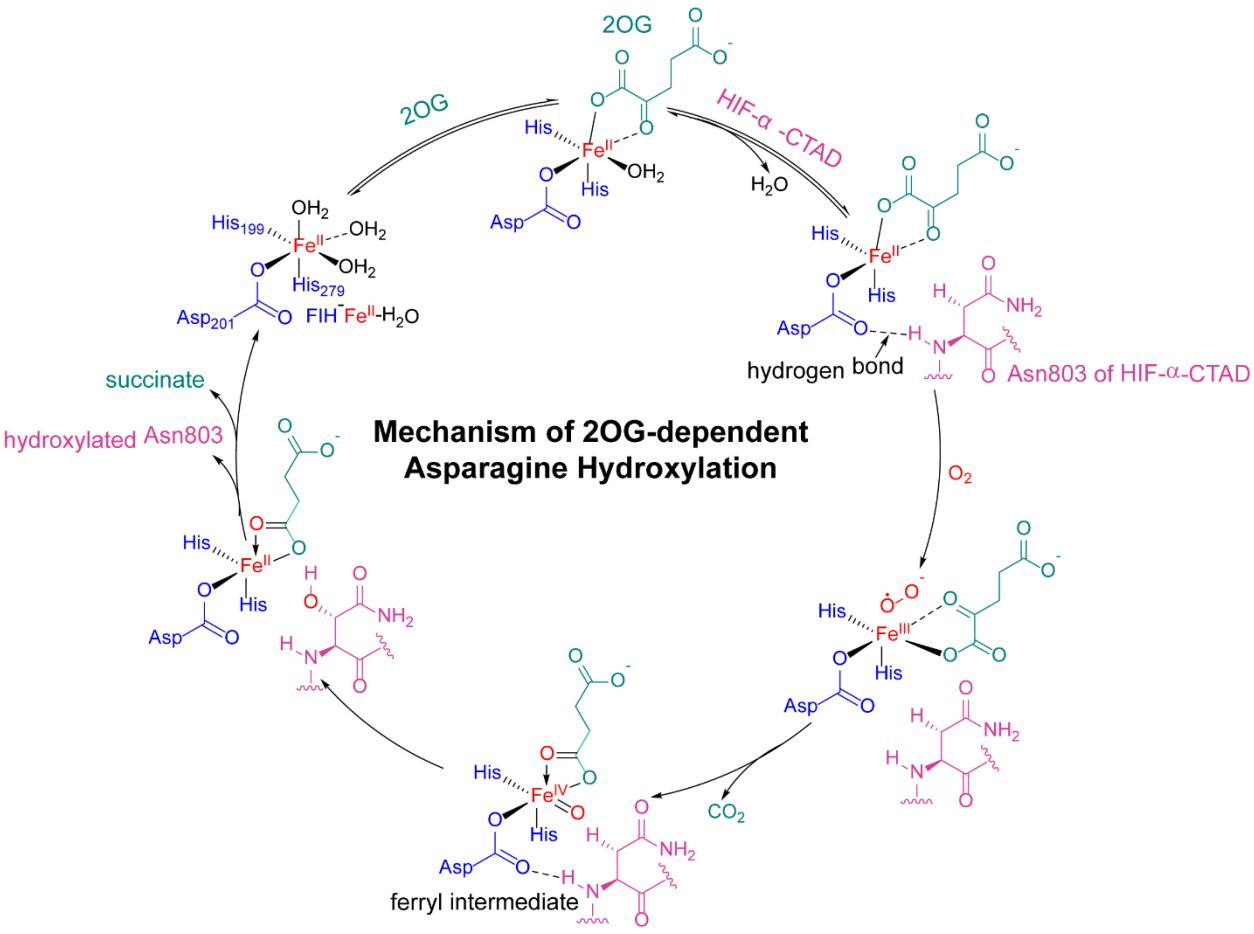

Figure 3. Outline mechanism for FIH catalyzed asparagine-residue hydroxylation.

4. STRUCTURAL FEATURES OF FIH

Crystal structures of human FIH (349 residues, monomeric mass ~40 kDa) were the first of a JmjC subfamily 2OG oxygenase to be reported.[13, 20, 54] The core conserved double stranded β-helix fold is comprised of eight β-strands and supports Fe(II) and 2OG binding; it is located in the C-

terminal half of FIH, and forms two β-sheets. Six additional strands in the N-terminal region extend the two β-sheets of the core DSBH, which is surrounded by helical elements. α-Helices located to the C-terminus of the DSBH core of each monomer interlock via significant hydrophobic helix-helix interactions to form the FIH dimer interface.[20, 21] (Figure 4)

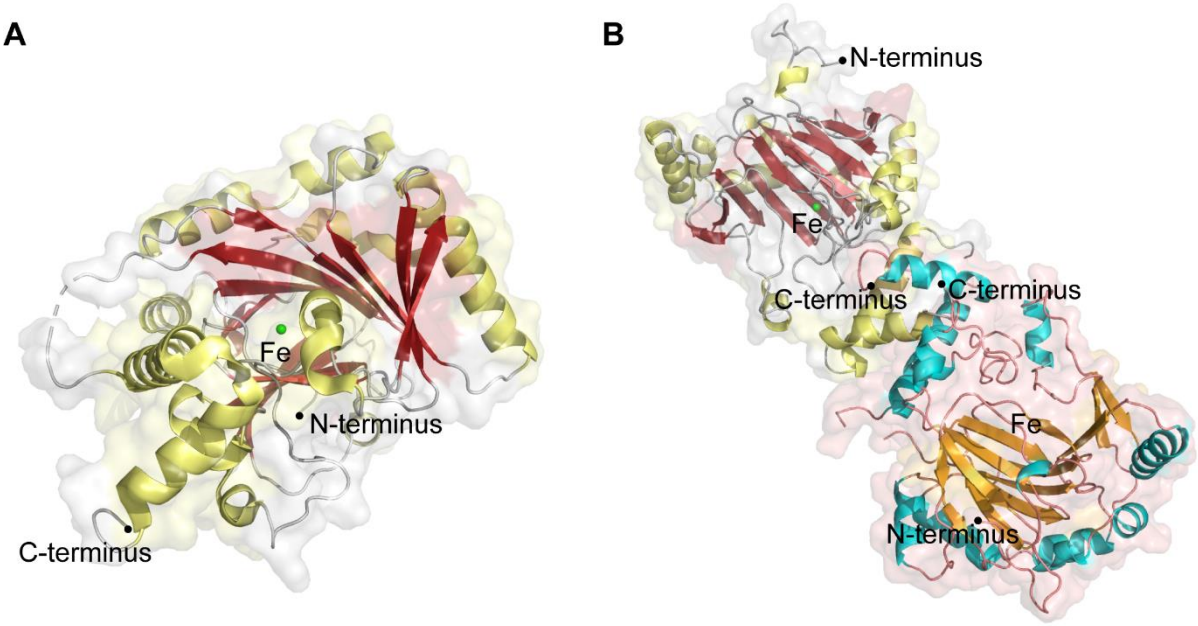

Figure 4. Crystallographically derived views of the FIH structure. A) View of an FIH monomer complexed with Fe(II); the other monomer in the dimer is not shown. The core double-stranded β-helix or jellyroll motif β-strands are in red, and the active site metal ion is a green sphere; B) View of the FIH dimer, the major solution form, with one monomer colored as in A, and the other in orange and blue. (PDB ID: 1H2N[20])

At least in the crystalline state, FIH has a relatively open active site pocket which is connected to the C-terminal dimerization domain by a wide and long groove, wherein the substrate binds (see below).[20, 54] Comparison of crystal structures of FIH (PDB ID: 1H2K[20]) and PHD2 (PDB ID: 3OUR[55]) in complex with *N*-oxalylglycine (NOG, a near isosteric analogue of 2OG) reveals clear

differences in their NOG and O_2 cosubstrate binding modes (Figure 5). Instead of the NOG C5 carboxylate forming an electrostatic interaction with the guanidino group of an arginine and an alcohol as occurs in PHD2 (Arg383, Tyr329)[20, 41] and related 2OG oxygenases, with FIH the NOG C5 carboxylate is positioned to form three hydrogen bonds / polar interactions with the side-chains of Tyr145, Thr196, and Lys214.[20, 21] Although there are variations, this type of NOG/2OG C5 carboxylate binding mode is typical of JmjC family NOG oxygenases, which often have more than one substrate type[18]. As predicted by sequence analyses[21], the Fe(II) of is coordinated by three residues (His199, His279, and Asp201). With both FIH and PHD2, the oxalyl group of NOG binds in a bidentate manner, though the relative coordination position of the C1 carboxylate is different in the two enzymes, a property proposed to related to their relative rates of their reaction with dioxygen (Figure 5).[37-40]

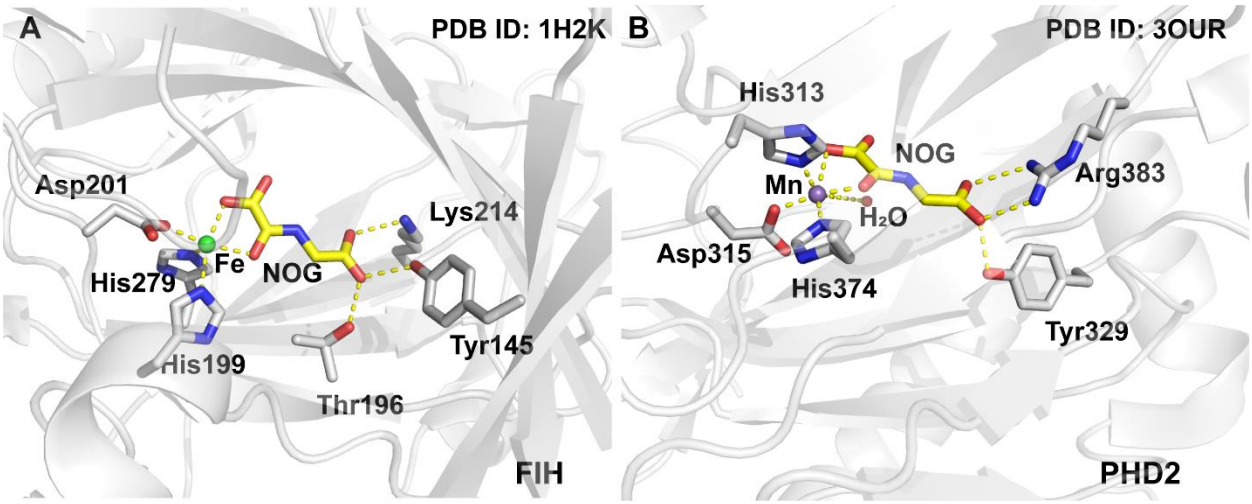

Figure 5. Comparison of the *N*-oxalylglycine (NOG) binding modes for FIH and PHD2. A) View from a crystal structure of FIH complexed with the 2OG cosubstrate analogue NOG (PDB ID: 1H2K[20]); B) View from a crystal structure of PHD2 complexed with the cosubstrate 2OG analogue NOG (PDB ID: 3OUR[55]).

In 2002, Elkins et al. reported a crystal structure of FIH complexed with the CTAD of HIF-1α (PDB ID: 1H2K[20]) (Figure 6A) in the presence of an iron ion and NOG.[20] Two distinct FIH-CTAD interaction sites were revealed in this complex structure. One extends to the dimerization interface and involves the hydroxylation site (CTAD795-806, Site 1), and the second is located to the C-terminal side of this site (CTAD813-822, Site 2) (Figure 6A). Kinetic analysis demonstrated that the length of the CTAD fragment influences the efficiency of FIH catalysis; CTAD fragments with more than 20 residues are required for efficient hydroxylation by FIH.[20] The CTAD fragments containing Site 1 are hydroxylated by FIH; however, the efficiency of their hydroxylation is less than fragments containing both Sites 1 and 2. Analysis of FIH-CTAD786-826 structure reveals that the electron density for Site 2 is at a lower level and quality than Site 1, likely reflecting weaker binding at Site 2. Electron density for the residues linking sites 1 and 2, i.e. CTAD807-811, was not clearly observed, indicating that direct interactions with FIH of these residues is likely weak.[20] At Site1, the CTAD residues adopt a largely extended conformation involving backbone hydrogen bond interactions with FIH.[20] Asn803 is buried at the active site and is located close to the Fe(II). Interestingly, the HIF-α CTAD is directly linked to the Fe(II) binding site as a result of the hydrogen bond between the backbone nitrogen of Asn803 and the carboxylate oxygen of the Fe(II) ligating Asp201 (Figure 6B). Productive binding of Asn803 is helped by hydrophobic interactions with FIH residue Tyr102 and the formation of additional hydrogen bonds with the side-chains of Arg238 and Gln239 of FIH (Figure 6B).[20, 56] In contrast to Site 1, Site 2 is located in hydrophobic pocket which binds the side-chains of three highly conserved residues in CTAD (Leu818, Leu819, and Leu822). It is important to note that although the crystallographic analyses reveal apparently precise FIH-CTAD interactions, especially close to the Fe(II) / active site, these do not preclude acceptance of multiple other substrates by FIH.

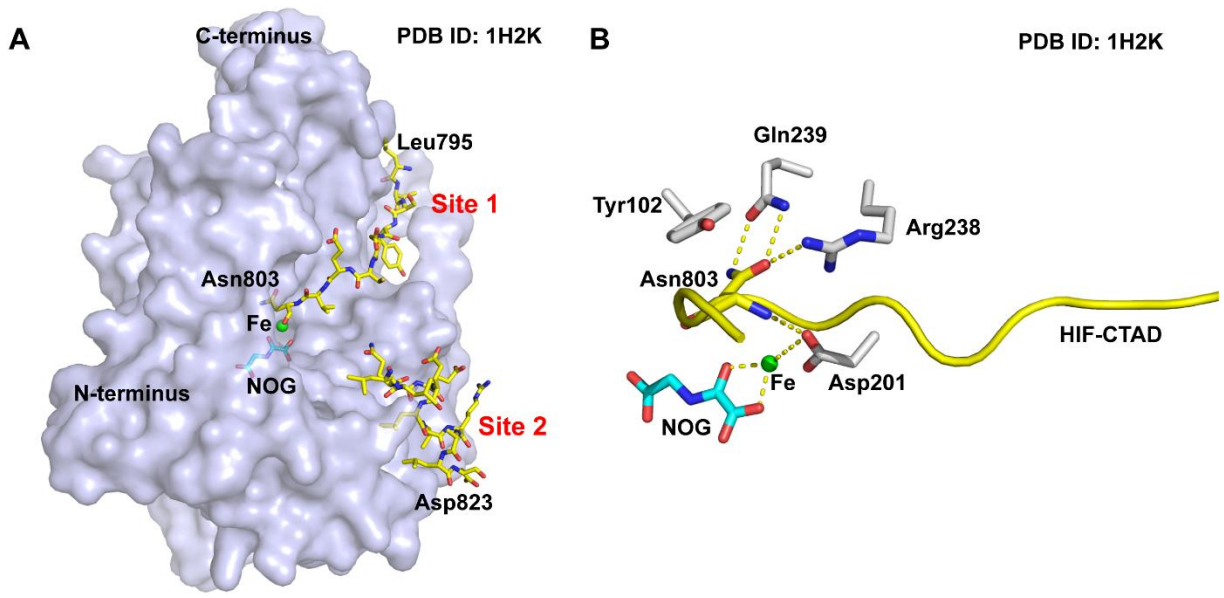

Figure 6. Crystallographic analysis of an FIH-HIF-1α C-terminal transcriptional activation domain (CTAD) complex. A) The FIH-HIF-1α CTAD complex (PDB ID: 1H2K[20]) structure with the CTAD fragment as stick model in yellow above a van der Waals surface of FIH. Asn803 is buried at the active site, being located adjacent to the Fe(II) cofactor; B) Interactions between HIF-1α Asn803 and FIH. Asn803 is located between the FIH residue Tyr102 and Fe(II); it is positioned to form hydrogen bonds with Asp201, Arg238, and Gln239.

A pVHL-binding site on FIH, located on the N-terminal side relative to the HIF-binding site, has been reported; although further validation studies are required this report suggests that FIH may act as a molecular bridge in interactions between pVHL and HIF-CTAD.[57] Moreover, in addition to binding prolyl-hydroxylated HIF-1α oxygen-dependent degradation domains (ODDs)[6], pVHL is also reported to bind to histone deacetylases (HDAC)[58], which play important roles in regulating expression.[54] It is also of interest that JMJD6, which like FIH is a JmjC oxygenase, but

which is involved in the regulation of mRNA splicing (and maybe other processes) is reported to oxidize pVHL.[59, 60]

5. IN-VITRO SCREENING FOR FIH INHIBITORS

Valid and efficient in-vitro screening assays are important for developing FIH inhibitors and in assigning FIH substrates. Multiple assays have been developed for 2OG oxygenases as reviewed in Rose et al.[61] These often employ monitoring of cosubstrate (O_2, 2OG) depletion and/or coproduct (succinate, CO_2) formation, either directly (e.g. by MS) or indirectly (e.g. by enzyme-coupled assays). Such assays can be useful, but do not always accurately reflect substrate oxidation, as 2OG turnover can occur non-enzymatically[62] or be decoupled from that of substrate oxidation. Hence, there is a preference for assays that directly monitor substrate depletion / product formation, ideally in a simultaneous manner, such as can be achieved by MS (or in some cases antibody) based assays.[63] Examples of assays that have been applied to the discovery and development of FIH inhibitors are given in Figure 7. In one assay, inhibitors of FIH can be evaluated by a 2OG depletion-based method, in which post-reaction derivatization of 2OG by *O*-phenylenediamine (OPD) (Figure 7B) gives a fluorescent product, 3-(2-carboxyethyl)-2(1*H*)-quinoxalinone, formation of which can be monitored by the excitation at 340 nm and emission measured at 420 nm.[64] The depletion of 2OG by FIH also can be detected by NMR[65] or by monitoring conversion of 1-[^{14}C]-2OG to [^{14}C]-CO_2 (Figure 7D).[66] FIH activity can also be monitored by measuring O_2 consumption.[25]

To more directly, assess the efficacy of FIH inhibitors to suppress Asn803 hydroxylation, MS and NMR based methods have been employed[24, 67, 68], For example matrix-assisted laser desorption/ionization-time of flight-MS (MALDI-TOF-MS) and solid phase extraction coupled to

MS methods have been used to monitor hydroxylation (as done with other 2OG oxygenases, including the PHDs[68]) inhibition assays have been developed (Figure 7A).[67]

Experience with other 2OG oxygenases would suggest there is scope for optimization of the presently reported FIH turnover assays, including for further development into efficient high throughput formats. There is also scope for developing assays that monitor FIH catalyzed HIF-α CTAD and ankyrin (and other non-HIF substrates), ideally simultaneously, to identify inhibitors selective for the two groups of substrates (see below).

There is also more scope for work on assays for FIH activity in cells. As yet there are no commercially available antibodies for the hydroxylated products of FIH in cells, though the literature shows these can be made.[22] Monitoring HIF target genes strongly upregulated by FIH is one possibility[22] and an in cell FIH catalytic assay based on the unusual cross linking reaction of FIH with OTUB-1 has been reported.[45]

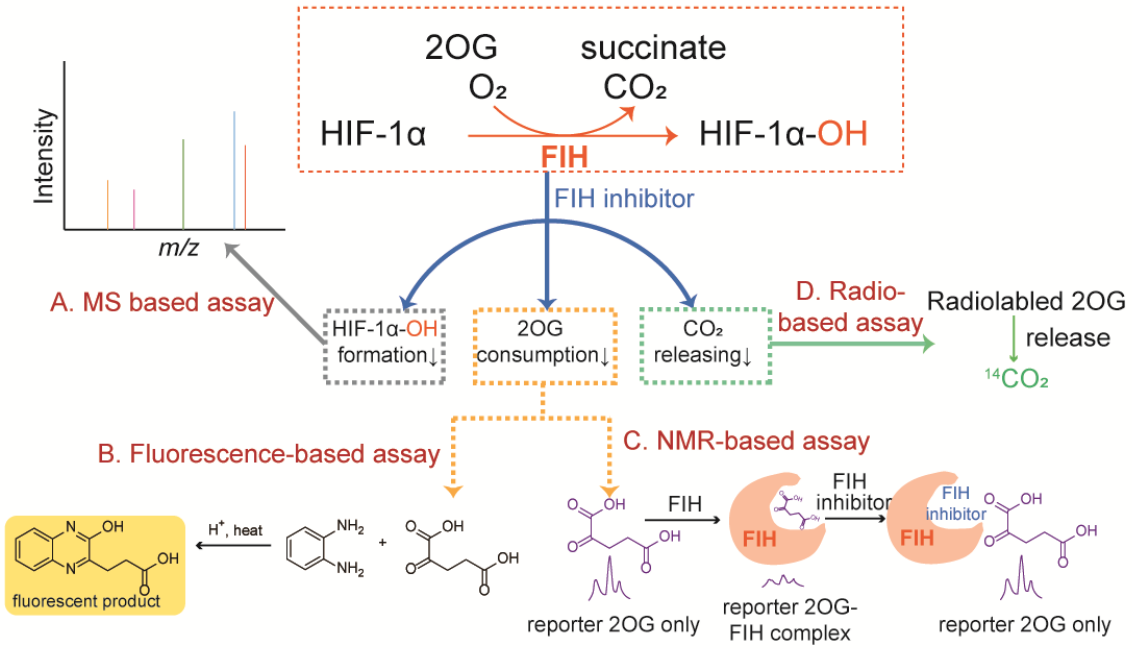

Figure 7. Reported in vitro screening methods for FIH inhibitors. A) MS based assays measuring substrate depletion / product formation; B) Monitoring of 2OG depletion by derivatization with *O*-phenylenediamine; C) NMR-based assay measuring 2OG consumption; D) Assays measuring decarboxylation of radiolabeled 2OG by detecting released CO_2.

6. NON-HIF SUBSTRATES OXIDIZED BY FIH

Many proteins with the highly conserved Asn-residues within an ankyrin repeat (AR) domain (ARD), a common structural motifs in eukaryotes, have been identified as substrates for FIH-mediated hydroxylation (and potentially other oxidation reactions)[69-71], suggesting that the function of these proteins may be regulated by FIH catalyzed post-translational modification.[71] ARDs are present in >200 human proteins and are comprised of 33 residue repeats that form a hairpin-α-helix-loop-α-helix motif which most likely unfolds to enable FIH catalyzed hydroxylation.[72, 73] Evidence has been reported that a larger set of mostly, but not exclusively, ARD proteins interact with FIH, with many, but not all, undergoing hydroxylation[74]. This finding is supported by multiple focused studies with cells and isolated proteins raises interesting and substantially unanswered questions, including as to how sufficient FIH is made available for efficient HIF-α hydroxylation; does competition between the HIF-α CTAD and ARD protein for the FIH active site modulate the hypoxic response; how can the active site of FIH can accommodate such a variety of substrates; and what are the roles of ARD non-HIF-α FIH catalyzed hydroxylations?

FIH has been shown to accept consensus ARD substrates and, at least in some cases, asparagine-hydroxylation can stabilize the ARD fold, in a manner somewhat analogues to that of proline-4-hydroxylation in thermodynamically stabilizing the collagen triple helix fold.[69, 75, 76] However, given that the extent of ARD hydroxylation in cells varies and is rarely complete, a

general role for ARD-hydroxylation analogues to that of collagen proline-4-hydroxylation, seems unlikely. Below we describe selected results with several of the non-HIF FIH substrates[77] in more detail, aiming to give an idea of the scope of FIH catalyzed hydroxylation, though it probably correct to state that in none of the non-HIF FIH substrates studied have a clear physically validated roles for (O_2 dependent) hydroxylation in signaling been shown, in contrast with the established roles for the PHDs and FIH in the HIF system.[11, 75]

6.1 IκB proteins as FIH substrates

NF-κB is a transcription factor that plays a crucial role in regulating expression in response to inflammation, differentiation, and proliferation; there is evidence that the NF-κB system is regulated by O_2 availability.[78] NF-κB contains a conserved 300-amino acid Rel homology domain (RHD) that binds with IκB proteins, resulting in the inactive state of NF-κB. In a pioneering study on FIH catalyzed ARD hydroxylation, the ARDs of the IκB family proteins, IκBα and the NF-κB precursor protein p105 (which inhibits NF-κB via interactions involving its ARD), were shown to undergo FIH catalyzed hydroxylation at specific asparagine-residues.[71]

FIH catalyzed hydroxylation of asparagine-678 of p105 is supported by studies with an FIH inhibitor, dimethyloxalylglycine (DMOG) and mutagenesis work.[71] It was observed that 2OG decarboxylation activity with IκBα was at least 2-fold greater than p105, indicating that IκBα might contain more than one hydroxylation site. Mutation of IκBα asparagine-201 to alanine-201, the putative IκBα hydroxylation site best aligning with that of p105, reduced but did not ablate the decarboxylation of 2OG, consistent with the hypothesis that more than one hydroxylation site in IκBα. Subsequently, MS/MS analysis supported the presence of two IκBα hydroxylation sites, asparagine-244 and asparagine-210, with a higher hydroxylation level being observed at asparagine-244 (> 95%) than for asparagine-210 (approximately 60%).[71]

Overall, the studies on IκBα/p105 revealed that FIH catalyzed asparagine-hydroxylation extends beyond HIF systems, that FIH can catalyze more than one hydroxylation on the same protein, that the efficiency of FIH catalyzed hydroxylation varies from site to site, and (on the basis of bioinformatics and studies with peptides) that the scope of the FIH catalyzed may be broad.[71] In terms of at least some of these properties, FIH appears to resemble another previously characterized 2OG dependent human C-3 asparagine/aspartate-residue hydroxylase (AspH) which catalyzes the hydroxylation of epidermal growth factor like domain proteins, but which comes from a different structural subfamily than does FIH.[79] No clear signaling roles for either FIH catalyzed IκBα or AspH catalyzed epidermal growth factor like domain have yet been established.

6.2 Notch proteins as FIH substrates

The Notch protein family are transmembrane receptors associated with cell fate decisions, differentiation, and proliferation.[80] Four Notch proteins (N1-N4) are present in mammals, which stimulate the cytoplasmic release of the Notch intracellular domain (ICD), which is recruited to target genes by the transcription factor CSL. The Notch ICD coordinates the assembly of a nuclear transcriptional activation complex involving Mastermind-like (MAML) protein.[80] It is reported that Notch signaling is sensitive to changes in O_2 levels, and both direct and indirect interactions between the Notch and HIF signaling pathways are reported.[72, 81]

Notch-1, Notch-2, and Notch-3 are all reported to bind to FIH, in manner enhanced by cell treatment with DMOG.[72] Work with Notch-1, the best-characterized family member, shows it is hydroxylated by FIH at asparagine-1945 and asparagine-2012, in manner dependent on cellular oxygen levels.[72] Notch-1 ICD substituted at residues Asn1945 and Asn2012 shows significantly reduced transcriptional activity and impairs Notch mediated control of neurogenesis and

myogenesis. The available evidence indicates FIH plays a negative role in regulating the Notch pathway, though further evidence for a direct role of hydroxylation in this regard is required.[81]

To investigate the structural consequences of the FIH-mediated Notch ARD hydroxylation, a crystal structure of FIH in complex with a Notch-1 ARD fragment was determined, along with a structure of the hydroxylation product (Figure 8).[72] The product structure showed that the hydroxylation site is located in the β-hairpin type loop connecting the ARs in the overall ARD (Figure 8A). The hydroxylation of the asparagine residues did not significantly change the crystallographically observed conformation of the ARD fold, though it was noted that the hydroxylated aparagine-1945 could form a hydrogen bond to the side chain of aspartate-1943, likely resulting in stabilization of the β-hairpin type loop conformation and consistent with other studies showing asparagine-hydroxylation can stabilize the ARD fold.[72]

In the FIH: Notch N1 fragment complex structure, similarly to the FIH: HIF-1α CTAD complex, the target asparagine residue is at the apex of a tight turn and is buried at the active site with its sidechain methylene directly adjacent to the Fe(II) center (Figure 8B-D). There are changes in the conformations of both FIH (though these are local to the active site) and in the target Notch N-1 AR on formation of the enzyme-substrate complex, though as yet no structure of a complete ARD and FIH has been reported. The dissociation constants (K_Ds) for non-hydroxylated and hydroxylated Notch-1 ARD binding to FIH are 4.0 μM and 48.9 μM, respectively. [82] In comparison, the same study reported the K_d value of HIF-1α CTAD is 106 μM, indicating that Notch-1 ARD binds more tightly to FIH[82], especially in its non-hydroxylated form. The tighter binding suggests the possibility that hydroxylation of HIF-1α CTAD mediated by FIH may be modulated by FIH ARDs, including (but not limited to) ARDs in Notch proteins.

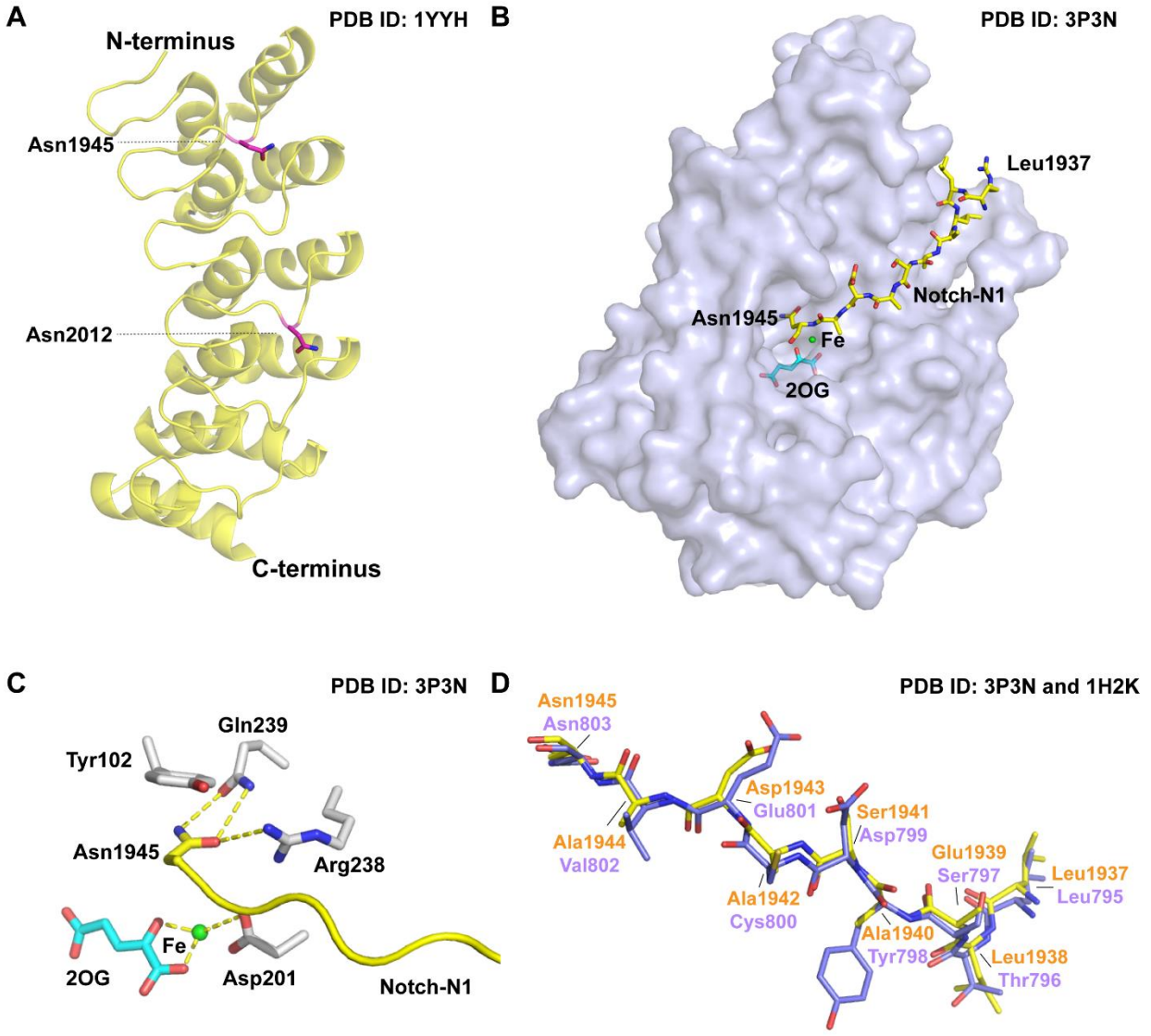

Figure 8. Crystallographic analysis of hydroxylated Notch-1 and a Notch-1 fragment in complex with FIH. A) Six ankyrin repeats (ARs) are observed in the structure of the hydroxylated Notch-N1 ankyrin repeat domain (ARD), the overall fold of which is the same as in its unhydroxylated form (PDB ID: 1YYH); B) FIH-Notch-N1 complex under anaerobic conditions (PDB ID: 3P3N). Notch-N1 fragments are shown as sticks in yellow above a van der Waals surface of FIH. Asn1945 is buried at the active site and is located close to the Fe(II); C) Intermolecular interactions between

Notch-N1 Asn1945 and FIH. Notch-N1 Asn1945 is adjacent to Tyr102 in FIH and the Fe(II). It forms hydrogen bonds with Asp201, Arg238, and Gln239; D) Stick representation of superimposed Notch-N1 (1937-1945, yellow) and HIF-α CTAD (795-803, purple) fragments in complex with FIH.

6.3 Apoptosis-stimulating p53-binding Protein 2 (ASPP2) as an FIH substrate

The apoptosis-stimulating p53-binding protein (ASPP) family members are conserved regulators of the tumor suppressor p53.[83] Potential ARD hydroxylation sites for FIH are found in all ASPP members, including ASPP1, ASPP2, and iASPP.[84] ASPP1 and ASPP2 show high sequence homology, whereas the sequence of iASPP in the vicinity of the potential hydroxylation site differs from ASPP1/ASPP2. Indeed, it is reported that ASPP2 can be hydroxylated by FIH, but iASPP is not.[84] Mutation analyses indicate that FIH hydroxylates ASPP2 at Asn986 and that hydroxylation is suppressed when cells are treated with DMOG, suggesting the ASPP2 hydroxylation is FIH-dependent. There is evidence that the interaction of the ASPP2 ARD with FIH leads to changes in ASPP2 interaction profiles and changes in its subcellular localization.[84]

The K_m value for ASPP2 hydroxylation is 75 μM, comparable to those for other identified FIH substrates (120 μM for that of HIF-1α CTAD[82]). It was reported that FIH binds more strongly to the ASPP2 ARD than to HIF-1α CTAD on the basis of GST-tagged "pulldown" assays.[84] In contrast to the extended conformation of HIF-1α CTAD in solution, ASPP2 has a defined fold, as do all other ARDs. However, as with Notch, the ASPP2 ARD fragment interacts with FIH in a largely extended conformation similar to those observed for HIF-1α and Notch, implying (partially) unfolding of the ARD is require to enable productive FIH catalysis (Figure S1A-C).

Hydroxylation of Asn986 in ASPP2 does not affect the stability and total cellular protein levels of ASPP2[84]. Hydroxylation mediated by FIH is reported to enhance the interaction of ASPP2 with

partitioning defective 3 homolog (Par-3, which has a role in cell polarity), while the interaction of ASPP2 with the tumor suppressor p53 was not affected by the hydroxylation mediated by FIH (Figure S1D).[83] The role of FIH in regulating these interactions under (patho)physiological conditions remains undefined.

6.4 TRPV3 as an FIH substrate

On the basis of bioinformatic studies, it was suggested that transient receptor potential (TRP) channel proteins might be FIH substrates with predicted hydroxylation sites within their cytoplasmic ARDs.[85] TRPV3, which is assigned as a temperature sensing channel, plays important roles in skin barrier formation, thermosensation, hair growth, and nociception. TRPV3 is also associated with inflammation, ischemia, and wound healing.[86] It was suggested that TRPV3-ARD polypeptides could interact with FIH similarly to other ARDs.[85] Evidence was accrued from isolated proteins and in cells that TRPV3 Asn242 is hydroxylated by FIH (though this was not demonstrated at endogenous TRPV3 levels). The interaction of TRPV3 with FIH was reported to inhibit channel activity, but the mechanism for this regulation has not been determined.[86]

6.5 OTUB1 as an FIH substrate

The ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 (OTUB1) is a ubiquitously and highly expressed deubiquitinase (DUB), which regulates cellular energy metabolism and is associated with cancer prognosis.[53] FIH interacts with OTUB1, which does not contain an ARD, with (DMOG inhibited) hydroxylation occurring at asparagine-22 of OTUB1, which is located at the junction of an α-helix and an unstructured region, i.e. in this regard OTUB1 is more similar to the CTAD of HIF-α than the ARD FIH substrates.[47] Evidence has been described that the DUB activity of OTUB1 is regulated by FIH. The hydroxylation of Asn22 does not alter

the stability or DUB deubiquitinase activity of OTUB1, but the interaction of OTUB1with other proteins, especially those associated with oxidative metabolism, is affected. Notably, FIH reacts with OTUB1 to likely form a covalently linked heterodimer[53], the nature of which is uncharacterized, but which has functional consequences for OTUB1 DUB activity. FIH:OTUB-1 heterodimer formation is highly sensitive to cellular O_2 levels, suggesting it has a role in O_2-dependent metabolism.[45] Cells overexpressing both FIH and Asn22 mutated OTUB1 manifest substantially increased the phosphorylation of adenosine 5‘-monophosphate (AMP)-activated protein kinase α (AMPKα), but that of FIH and wild type OTBU1 does not, suggesting that the FIH-dependent Asn22 hydroxylation of OTUB1 may contribute to the regulation of cellular metabolism.[47]

6.6 ASB4 as an FIH substrate

Ankyrin repeat and suppressor of cytokine signaling (SOCS) box proteins (ASBs) are a subclass of the SOCS superfamily, that is reported to mediate the ubiquitination of broad range of target proteins[87]; they are characterized by the presence of several N-terminal ARs as substrate-binding domains. ASB4 is a member of the ASB family that is produced in vascular development during a time window when the oxygen tension changes rapidly.[87] It is reported that asparagine-246 of ASB4 is an FIH substrate, but that its hydroxylation does not affect the stability or function of ASB4 in cells.[87]

6.7 HACE1 as an FIH substrate

ECT domain and ankyrin repeat-containing E3 ubiquitin protein ligase 1 (HACE1) is a member of the HECT E3 ubiquitin ligase family and plays a role in tumor suppression in some cancers.[88] The loss of HACE1 leads to the accumulation and long-lasting activation of the GTPase

Rac1, which is associated with the malignant progression of breast cancer.[88] Immunoprecipitation assays indicated that a hydroxylation-dependent interaction exists between FIH and HACE1, and that the interaction is strengthened in cells by addition DMOG. HACE1 asparagine-191, which is located in a favorable position for FIH hydroxylation, was indeed shown to be hydroxylated. Asparagine-191 hydroxylation does not affect the cellular levels of HACE1, but evidence has been described that Asn191 suppresses the ability of HACE1 to ubiquitinate the active form of Rac1, suggesting that FIH negatively regulates the interaction between HACE1 and Rac1. FIH-dependent hydroxylation of HACE1 is proposed to suppresses its function to reduce cell migration and subsequent invasion in breast cancers.[88]

6.8 Tankyrase-2 as an FIH substrate

Tankyrase is a poly-ADP-ribose polymerase family enzyme that uses nicotinamide adenine dinucleotide (NAD^+) as a cosubstrate to link ADP-ribose polymers to target proteins, leading to post-translational modification (PARsylation). There is evidence that the ARD of Tankyrase-2 is an FIH substrate, undergoing multiple hydroxylations (it has 13 'appropriately positioned' asparagines with respect to potential FIH catalyzed hydroxylation).[30, 74] Asn586, Asn706, and Asn739 of Tankyrase-2 are reported to be hydroxylated by FIH.[30, 74]

Interestingly, besides the reported multiple asparagine hydroxylation sites, two histidine residues (located at analogous positions to the asparagine-residues in the ARD) were also shown to undergo FIH catalyzed hydroxylation to different extents.[30] Studies with peptides suggest the oxidation scope of FIH may be wider than presently detected in cell-derived proteins, possibly to include di-hydroxylation, oxidation of D-residues and oxidation of hydrophobic residues.[89, 90]

6.9 Perspective on non-HIF substrates of FIH

The combined results from multiple groups, some of which are summarized above, provide compelling evidence that FIH has roles beyond that of regulation of the HIF system by HIF-α CTAD hydroxylation. At the catalytic level FIH appears to have a multitude of substrates and to catalyze hydroxylation and likely other oxidative reactions, not only of asparagine, but also of other residues. The results with OTUB1 indicate that FIH may catalyze a new type of oxidative cross linking.[53] Although there are reports of the PHDs having other substrates, and like FIH, the PHDs almost certainly interact with multiple proteins with which they may or may not modify, recent evidence implies that the functions of the human PHDs are largely focused on HIF-α modification.[6] The apparent relative complexity of FIH's apparent cellular roles is somewhat daunting - but given its established important role in the HIF mediated hypoxic response and that genetic studies in animal models imply FIH deletion is not lethal / highly toxic[44], the exploration of FIH as a drug target is of interest. Further, the apparent multitude of FIH's interactions in cells may complicate analyses involving genetic modification of its expression due to adaptation arising from FIH loss, making a case for the development of small-molecule FIH inhibitors of FIH to probe its function and therapeutic value.

7. FIH INHIBITORS

Compounds which function as inhibitors of FIH catalyzed HIF-α CTAD hydroxylation offer promise for increasing and/or altering the transcriptional activity of HIF via modulating HIF-α hydroxylation status. Selective inhibitors of FIH catalyzed hydroxylation of non-HIF substrates may promote FIH catalyzed HIF-α CTAD hydroxylation, so giving the opposing effect (by freeing up FIH for HIF-α CTAD hydroxylation), something that is of interest from the perspective of decreasing HIF activity for cancer therapy. Compounds (or combinations of compounds) that inhibit both the PHDs and FIH are of interest from the perspective of upregulating specific sets of

HIF target genes.[22] To date, however, FIH inhibition has been poorly investigated compared to that of the PHDs. This likely reflects: (i) the demonstrated ability of PHD inhibitors to upregulate HIF-α and HIF target genes, in particular that of EPO, in a therapeutically useful manner, (ii) the knowledge that FIH has multiple non-HIF substrates, some of physiological importance; thus FIH inhibition with a view to altering HIF activity may have unexpected toxic consequences, (iii) the perception that FIH inhibitors will not have a therapeutic effect in the absence of therapeutically upregulated HIF-α. However, since there is little real-time information on HIF-α levels in humans, the latter perception is open to challenge; further the roles of FIH outside of the HIF system and alteration of metabolism are uncertain and FIH deletion is not lethal in animals. There is thus a good case for the generation of potent and selective FIH inhibitors to test the therapeutic potential of FIH inhibition and to explore its roles in physiology and disease. Below, we classify reported small-molecule FIH inhibitors (to date all of which likely bind at active site) into three categories. The first comprises FIH inhibitors that are close 2OG analogues and which interact predominantly with 2OG binding residues; the second comprises a few compounds reported to be at least partially selective for FIH; the third comprises molecules known to bind to both the PHDs and FIH. Clearly there is scope for development of new types of FIH inhibitor, as precedented with other oxygenases.[61]

7.1 FIH inhibitors that are close 2OG analogues

FIH inhibitors (Figure 9) that are close mimics of 2OG are reported, mostly with weak or moderate potency and which are not selective for FIH. *N*-Oxalylglycine (NOG, **1**)[20] is a close 2OG analogue and broad spectrum 2OG oxygenases inhibitor which inhibits FIH with an IC_{50} value of 46 μM, as assessed by the MALDI-TOF-MS assay (FIH concentration: 1 μM).[91] X-ray analysis implies NOG binds to FIH similarly to 2OG, i.e. ligating the metal ion in a bidentate with its C5

carboxyl forming polar interactions with Lys214, Thr196, and Tyr145; however, care should be taken in assuming the crystallographically observed binding mode precisely reflects that in solution. Related FIH inhibitors (**2**-**14**) that mimic 2OG bind in similar, but not identical, modes as shown by crystallography (Figures 10-12). The IC_{50}s of **6**, **7**, and **8** are 189 μM, 1500 μM, and 8.6 μM using the MALDI-TOF-MS assay, respectively,[21, 52, 91, 92] with pyridine-2,4-dicarboxylate **8** being the most potent close 2OG analogue inhibitor of FIH reported.[92] Note, by contrast NOG is a more potent inhibitor of the PHDs than **8**. Most of these inhibitors manifest bidentate coordination in an analogous manner to 2OG[21, 52, 91, 92] (Figure 12A). However, **9**-**11**[93, 94] differ in their chelation mode (Figure 12B), likely due to their bicyclic nature. Daminozide (**12**), an agrochemical, which also inhibits some JmjC KDMs[95], and **13** are interesting as they cause conformational changes at the FIH active site – in the case of **12** its two *N*-methyl groups cause the side-chain of Asn205 to rotate (Figure 12A)[96]. In the case of the hydroxamate **13**, an X-ray structure reveals binding of two molecules of **13** within the FIH active site, causing the metal chelating Asp201 to move away from the iron (Figure 12B)[92]. Although the solution relevance of these structures remains to be validated, they reveal the potential for conformational changes and unanticipated binding modes of inhibitors to FIH.

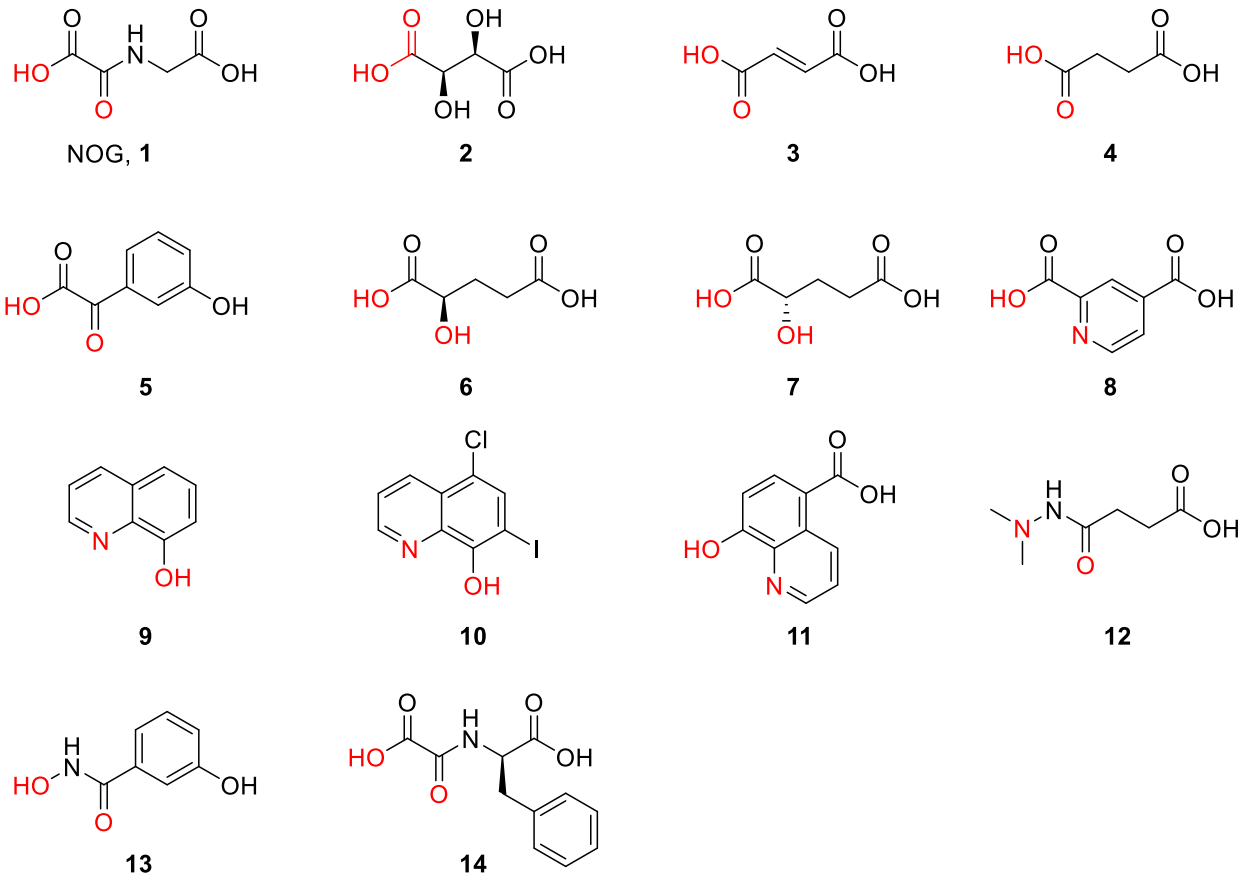

Figure 9. Structures of FIH inhibitors that are close 2OG analogues. Atoms proposed to be involved in chelating the active site Fe(II) are in red.

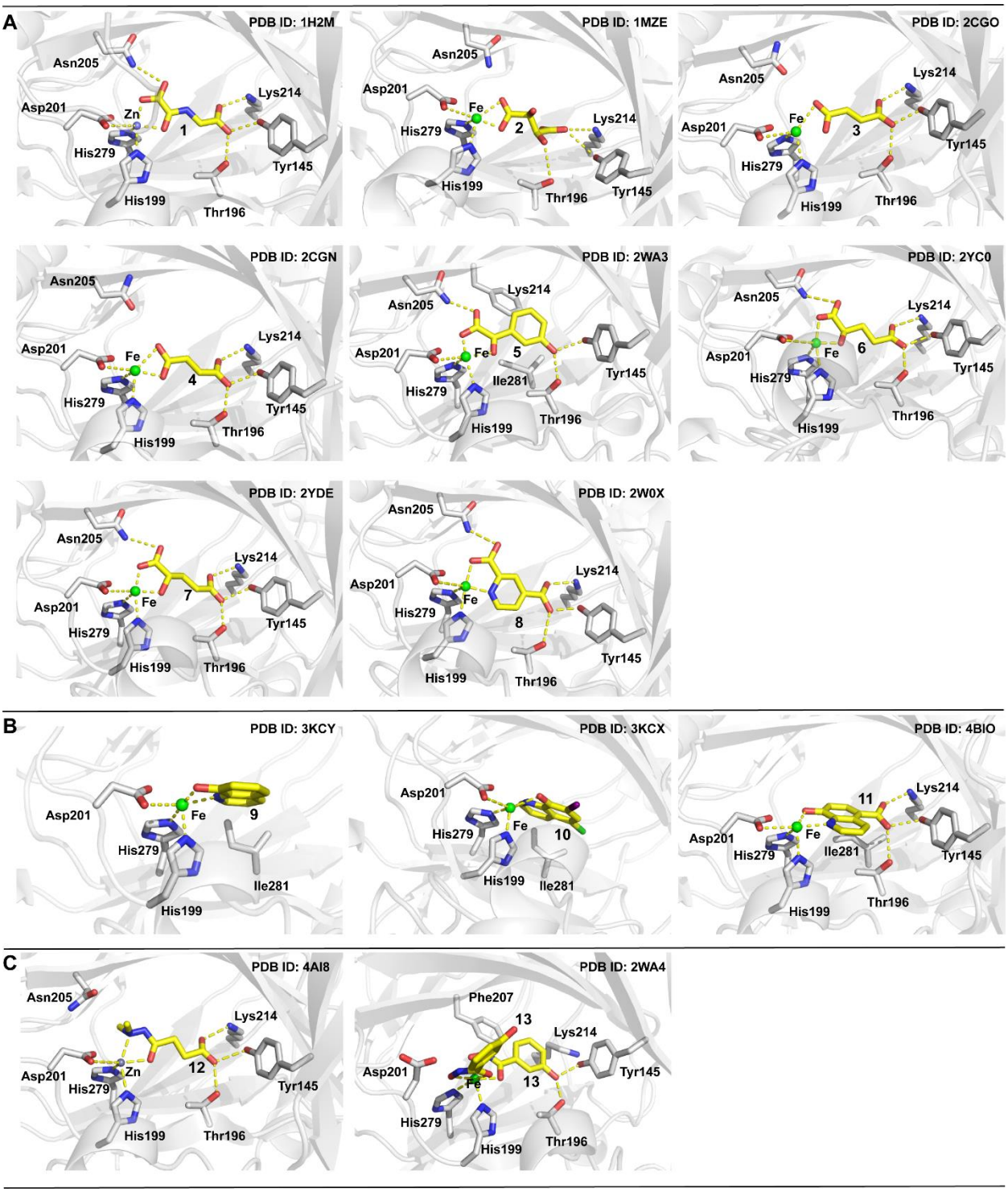

Figure 10. Crystallographically observed binding modes of 2OG analogues to FIH. A) **1-8** bind to FIH in a manner related to that of 2OG; B) **9-11** occupy the active site with different chelating orientations compared with 2OG, likely in some cases due to the presence of bulky chelating

groups; C) **12** and **13** binding to FIH is accompanied by conformational changes of specific residues (see Figure 11).

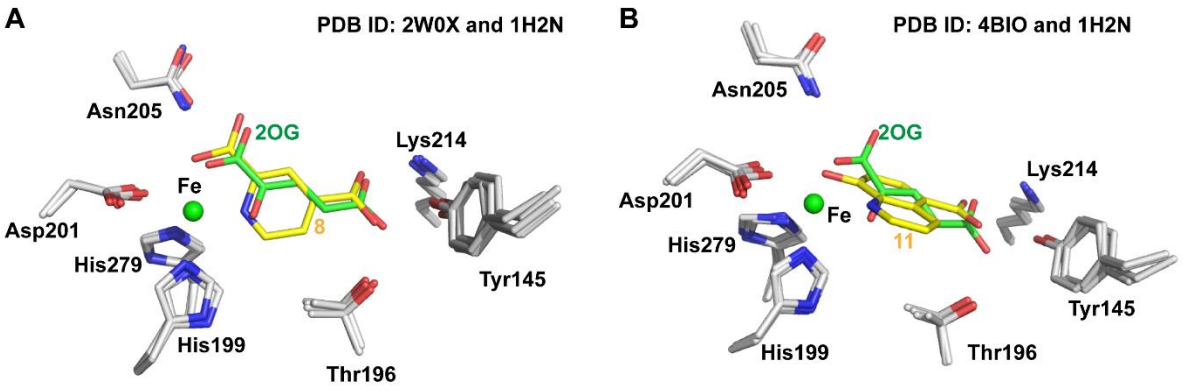

Figure 11. Crystallographic analysis of 2OG competing FIH inhibitors. A) 2OG (green, PDB ID: 1H2N[20]), and **8** (yellow, PDB ID: 2W0X[90]); B) Some of the 2OG competitors chelate the Fe(II) differently compared to 2OG. 2OG (PDB ID: 1H2N[20]) is in green sticks, **11** (PDB ID: 4BIO[93]) is in yellow sticks.

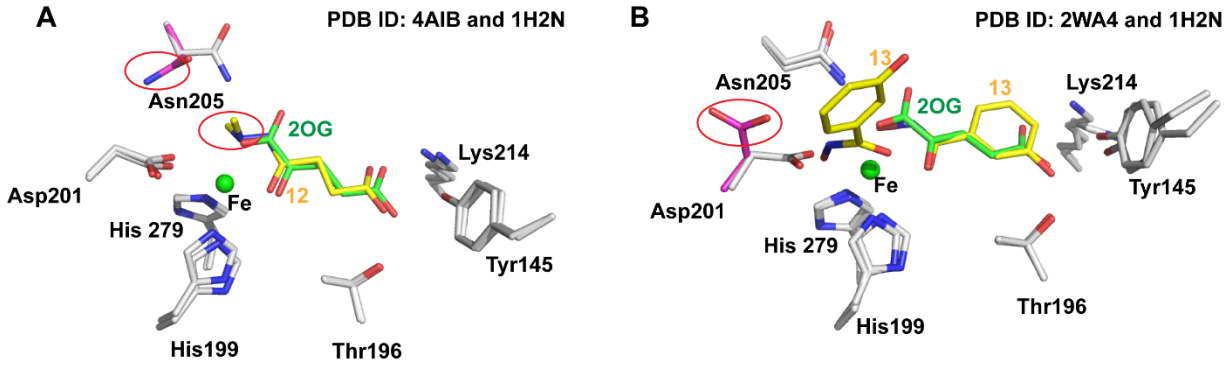

Figure 12. Conformational changes apparently induced by the binding of **12** and **13** to FIH. In both cases the structures for **12** (PDB ID: 4AIB[95]) and **13** (PDB ID: 2WA4[91]) are overlaid with that of 2OG (PDB ID: 1H2N[20]). 2OG is depicted in green sticks, and **12** and **13** in yellow sticks.

A) The presence of the two *N*-methyl groups in **12** causes a conformational change of Asn205 relative to the unligated structure; B) A conformational change of Asp201 occurs when **13** binds to FIH to accommodate a second molecule of **13** – note the solution relevance of binding of the second molecule of **13** is unclear.

7.2 Evidence that selective FIH inhibitors are feasible

Given the overall similarity of the active sites of many 2OG oxygenases, including for the PHDs and FIH, it may be perceived that it will be challenging to develop selective FIH inhibitors (Figure 13A). However, ongoing work, in particular on the PHDs, implies that the development of inhibitors that are highly selective for specific sets of 2OG oxygenases will be possible, including by active site targeting approaches which exploit subtle differences between 2OG oxygenases, including with respect to dynamic motions not always apparent from crystallographic analyses.[61, 97]

In 2005, McDonough et al. reported pioneering work that demonstrated selective inhibition of FIH over the PHDs, involving studies with a series of N-oxalyl amino acid derivatives.[66] The D-stereochemistry derivatives with hydrophobic side chains were more found to be effective - at inhibiting FIH than the PHDs, compared to the their L-enantiomers.[66] The *N*-oxalyl-D-phenylalanine derivative **14** is a selective FIH inhibitor; its cell-penetrating dimethyl ester prodrug form inhibits FIH catalysis in cells.[22, 98] An X-ray crystal structure of **14** complexed with FIH shows that the phenyl ring of **14** fits into a hydrophobic pocket formed by the sidechains of Leu186, Leu188, Trp296, and Tyr102 (as likely do the hydrophobic groups of related FIH inhibitors) Figure 13B), in a manner proposed to hinder dioxygen binding (though O_2 is unlikely to react in the absence of a keto-acid group).[66] The conformation of Tyr102 was observed to change relative to the unligated structure - subsequent structural studies have provided further evidence for

conformational dynamics of this residue.[90] Comparison of the structure of **14** with FIH with PHD2 structures (Figure 13D-E) reveals the basis of its selectivity for FIH, i.e. the presence of an unfavorable interaction between the phenyl ring of **14** and the Tyr310 residue in PHD2 will likely result in an unfavorable steric clash hindering binding of **14** to the PHD active site.

In 2008, Tsujita and coworkers reported a series of furan- and thiophene-2-carbonyl amino acid derivatives derived from computational studies with the potential to inhibit FIH.[99] Although biochemical studies were not reported (and need to be done to validate direct FIH inhibition), their results with engineered reporter cells suggest this series may be at least partially selective for FIH (Figure 13A). **15** is reported not to upregulate HIF-α under normoxic conditions, suggesting it does not inhibit the PHDs. However, when the SKN: HRE-MLuc cells are cultured under mild hypoxia conditions, addition of **15** promotes expression of certain HIF target genes, suggesting it may be an FIH inhibitor.

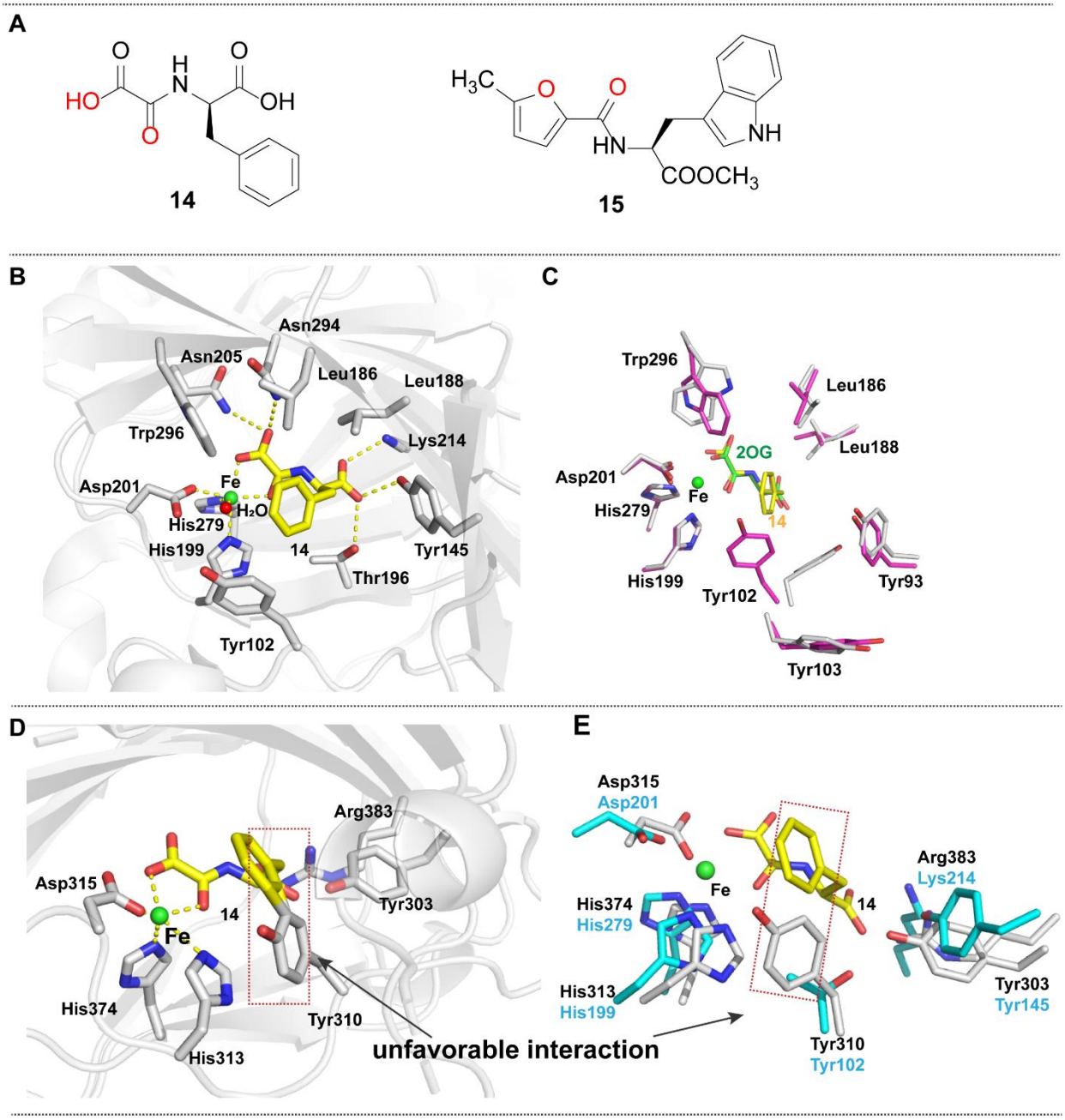

Figure 13. A) Structures of (partially) selective FIH inhibitors. The atoms (proposed in the case of **15**) to be involved in chelating Fe(II) are in red; note the methyl esters of **15**, which is likely a prodrug, may be hydrolyzed in cells to give the acid; B) Crystallographic analysis of *N*-oxalyl-D-phenylalanine **14** (yellow) complexed with FIH. Interactions between **14** and FIH (PDB ID: 1YCI[66]); C) The binding mode of **14** (yellow) superimposed with that of 2OG (green). D)

Superimposition analysis reveals why **14** does not bind favorably to PHD2. Modelled binding mode of **14** with PHD2 (PDB ID: 3OUJ[55]) based on its interaction with FIH. Unfavorable steric clashes between **14** and PHD2 are proposed due to the overlap between the phenyl ring in **14** and the PHD2 Tyr310 side chain; E) The modelled binding mode of **14** with PHD2 (gray) compared with its crystallographically observed binding mode with FIH (blue, PDB ID: 1YCI[66]).

7.3 Clinically relevant PHD inhibitors and FIH

Some clinically relevant PHD inhibitors (**16-18**, Figure 14) are reported to manifest weak inhibition of FIH.[68] Notably, Daprodustat (IC_{50} FIH = 21 μM; PHD2 = 0.067 μM) and Vadadustat (IC_{50} FIH = 29 μM; PHD2 = 0.029 μM)) are more potent inhibitors of FIH than Molidustat (IC_{50} FIH = 66 μM; PHD2 = 0.007 μM), as shown by solid-phase extraction coupled to MS based assays.[68] X-ray crystal structures of these three compounds complexed with FIH (Figure 15A) reveal similar bidentate metal coordination modes compared to that observed for Vadadustat and proposed for Molidustat and Daprodustat with PHD2; these compounds all bind to form electrostatic interactions in the 2OG C-5 carboxylate binding pocket; it should be noted that in the case of Molidustat this interaction involves its triazole ring rather than a carboxylate as in the other two PHD inhibitors for which there are FIH structures.[68] The cyclohexyl rings on Daprodustat and chlorophenyl ring of Vadadustat and morpholine ring of Molidustat do not make strong interactions with the FIH active site consistent with their low potency versus FIH compared to the PHDs. As with some of the close 2OG analogue FIH inhibitors, the conformation of Tyr102 was observed to change in the FIH complexes with the PHD inhibitors (e.g., Daprodustat) (Figure 15). It might be possible to exploit the conformational flexibility of Tyr102 and Tyr103 in the development of selective FIH inhibitors; interestingly conformational changes are also observed on inhibitor binding to the PHDs.[68]

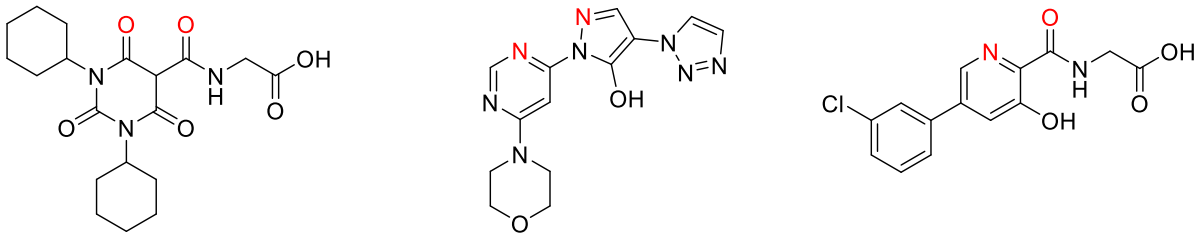

Figure 14. Structures of PHD inhibitors which have been used in clinical studies, which have also been shown to bind to and weakly inhibit FIH. The atoms involving in chelating Fe(II) are in red.

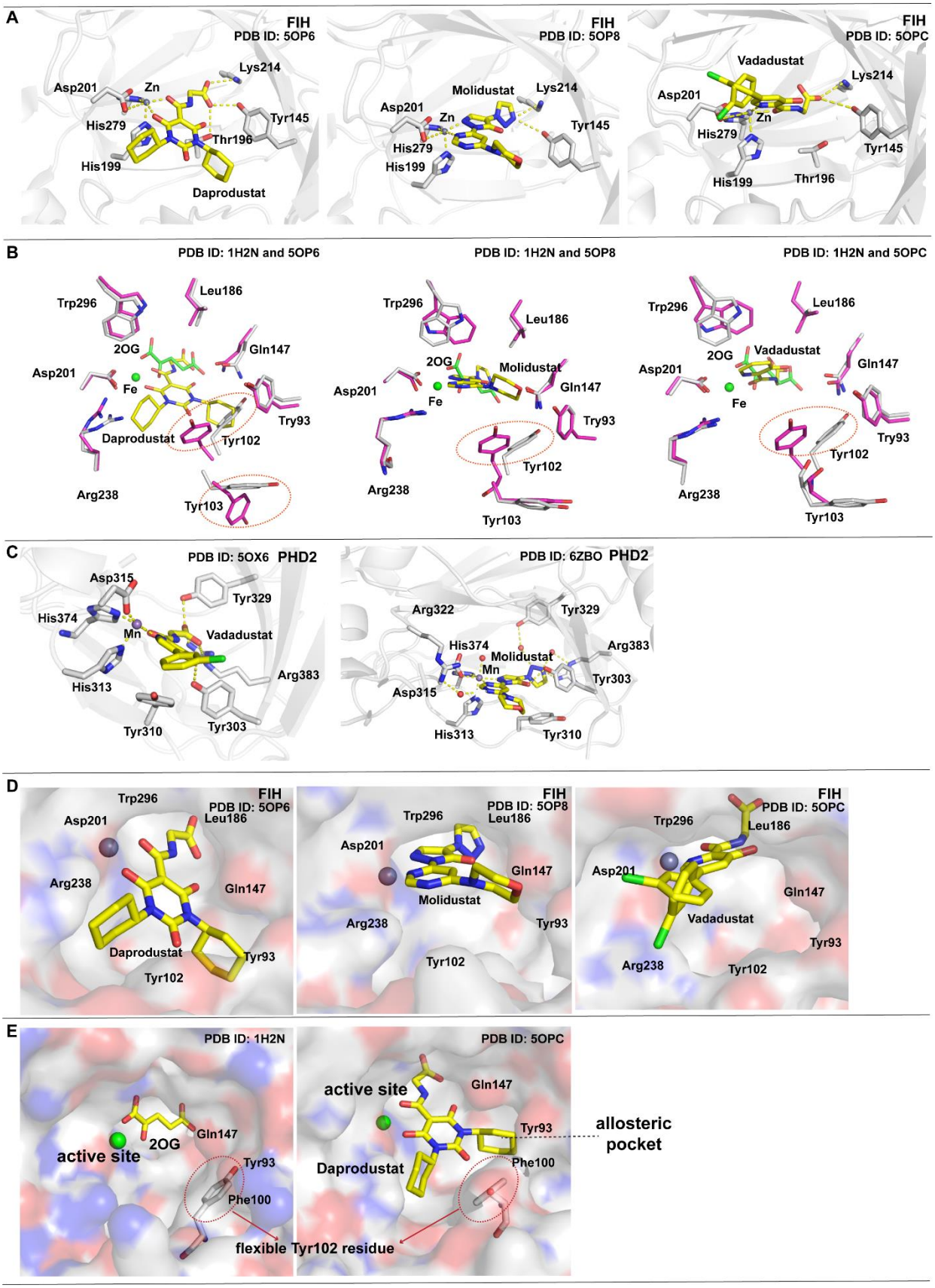
A
FIH
PDB ID: 5OP6
Lys214
Asp201
Zn
His279
Tyr145
Thr196
His199
Daprodustat
FIH
PDB ID: 5OP8
Molidustat
Lys214
Asp201
Zn
Tyr145
His279
His199
FIH
PDB ID: 5OPC
Vadadustat
Lys214
Asp201
Zn
His279
Tyr145
His199
Thr196
B
PDB ID: 1H2N and 5OP6
Trp296
Leu186
2OG
Gln147
Asp201
Fe
Try93
Daprodustat
Tyr102
Arg238
Tyr103
PDB ID: 1H2N and 5OP8
Trp296
Leu186
2OG
Molidustat
Asp201
Gln147
Fe
Try93
Tyr102
Arg238
Tyr103
PDB ID: 1H2N and 5OPC
Trp296
Leu186
2OG
Vadadustat
Asp201
Gln147
Fe
Try93
Tyr102
Arg238
Tyr103
C
PDB ID: 5OX6 PHD2
Asp315
Tyr329
His374
Mn
Vadadustat
Arg383
His313
Tyr310
Tyr303
PDB ID: 6ZBO PHD2
Arg322
Tyr329
His374
Arg383
Molidustat
Mn
Tyr303
Asp315
His313
Tyr310
D
FIH
PDB ID: 5OP6
Trp296
Asp201
Leu186
Arg238
Gln147
Daprodustat
Tyr93
Tyr102
FIH
PDB ID: 5OP8
Trp296
Leu186
Asp201
Gln147
Molidustat
Arg238
Tyr93
Tyr102
FIH
PDB ID: 5OPC
Trp296
Leu186
Asp201
Gln147
Vadadustat
Tyr93
Arg238
Tyr102
E
PDB ID: 1H2N
2OG
Gln147
active site
Tyr93
Phe100
flexible Tyr102 residue
PDB ID: 5OPC
active site
Gln147
Tyr93
allosteric
pocket
Daprodustat
Phe100

Figure 15. Crystallographic analysis of PHD inhibitors with FIH or PHD2. A) FIH complexed with Zn (II) and Daprodustat (PDB ID: 5OP6[68]), Molidustat (PDB ID:5OP8[68]), and Vadadustat (PDB ID: 5OPC[68]). Interactions of the inhibitors in the FIH active site are marked; B) The binding mode of the PHDs inhibitors (purple) superimposed with that of 2OG (yellow); C) PHD2 complexed with Mn (II) and Vadadustat (PDB ID: 5OX6[68]), and Molidustat (PDB ID: 6ZBO[100]); D) Views from crystal structure of the three inhibitors complexed with FIH; E) Active site and the allosteric pocket formed by Tyr102, Tyr93 and Phe100 of FIH. Note the conformational flexibility of the Tyr102.

8. THERAPEUTIC PERSPECTIVES FOR FIH INHIBITION

Manipulation of the natural responses to hypoxia has very substantial therapeutic potential as evidenced by the importance of EPO as a medicine and the more recent introduction of PHD inhibitors for the treatment of anemia in chronic kidney disease via HIF mediated EPO upregulation. The first-in-class PHD inhibitor Roxadustat was approved in 2018 for the treatment of anemia associated with chronic kidney diseases in China[101]; Subsequently, Vadadustat[102] and Daprodustat[103] were approved for the treatment of anemia in Japan. As described above these drugs are relatively weak inhibitors of isolated FIH, so it seems unlikely that their therapeutic effects are mediated via FIH, though this cannot be entirely excluded in long term PHD inhibitor treatments. It is of interest to test whether simultaneous PHD and FIH inhibition alters the therapeutic outcomes of the PHD inhibition with respect to anemia treatment.

Given the apparent complexity of the roles of FIH both within the HIF system and beyond, it is difficult to predict the outcomes of FIH inhibition in vivo, which may well be context dependent, e.g., they could be different under conditions of hypoxic stress when HIF-α is upregulated compared to when it is not. It is reported that the FIH gene is expressed in a wide range of tissues[44]

with significant variations in expression levels across tissues, with the highest levels of expression in skeletal muscle[46]; high levels of FIH expression also occur in brain, heart and lung.[44] Thus, the effects of FIH inhibition may be tissue / organ context dependent.

In the case of the HIF system, complexities with the role of FIH include with respect to its interactions with the histone acetyl transferases CBP/p300 which have widespread (and likely context dependent) roles in the regulation of transcription. As exemplified above outside of the HIF system, there is evidence FIH interacts with multiple important systems, including those involving Notch[71, 80] (which lays a key role in vascular development[104]), IκBα[70], and OTUB1[45, 47]. These complexities mean that the interpretation of the results of genetic interventions of FIH are challenging.

One observation from genetic studies is that FIH gene deletion / reduction causes clear differences in cellular metabolism, with a decrease in FIH correlating with increased oxidative metabolism and glycolysis.[46] The metabolic phenotypes caused by FIH deficiency suggests its inhibition might provide a possibility for metabolic manipulation.[44] Given the multiple apparent substrates of FIH, it is possible that its turnover directly affects metabolism by regulating 2OG/O_2/CO_2/succinate levels, perhaps in a localized manner; though given some other 2OG oxygenases have multiple substrates[61], if correct, this is a proposed function that would seem to be to unlikely to be restricted to FIH. In a potentially related observation, it is reported that loss of FIH causes a significant decrease in body mass and improves adiposity, suggesting that its inhibition might be a strategy for adiposity control.[105]

As with PHD inhibition, there is potential for FIH inhibition in terms of modulating biomedicinally important hypoxic response related processes including inflammation[106, 107], infection[108, 109], angiogenesis[110], and neuroprotection[111-114] (Figure 16). Further research on the

precise roles, if any, of FIH in these processes is important in terms of investigating the therapeutic potential of FIH inhibition. Exploring the potential of FIH inhibition, with or without concomitant PHD inhibition, for anemia treatment, would seem to be a promising initial investigation line.

Several lines of evidence indicate links between FIH activity and cancer.[115] FIH has been linked with tumor progression and may have a role as a tumor suppressor.[115, 116] Thus, although this review has focused on FIH inhibition, it should be noted that promotion of FIH catalyzed HIF-α CTAD hydroxylation is of interest, including with respect to suppressing HIF mediated transcription in tumors. However, it should also be noted that several studies indicate that FIH inhibition could protect the body from cancer, potentially via non-HIF FIH linked pathways.[117, 118] A recent report shows that delivering an FIH-insensitive form of HIF-2α with an anti-CD19 chimeric antigen receptor increases the cytolytic function of human $CD8^+$ T cells against lymphoma cells, including in a xenograft adoptive transfer mouse model. Thus there could be a role for HIF/FIH in enhancing the antitumor efficacy of therapeutic $CD8^+$ T cells against lymphoma cells via ectopic expression of the HIF transcription factor.[119]

We suggest that the therapeutic potential of FIH inhibition may perhaps best be tested via the development and application of potent and selective small molecule FIH inhibitors of different types suitable for in vivo use. As described above, although the development of FIH inhibitors suitable for safe clinical investigations is lagging behind that of the PHDs, the available evidence is that it should be viable.

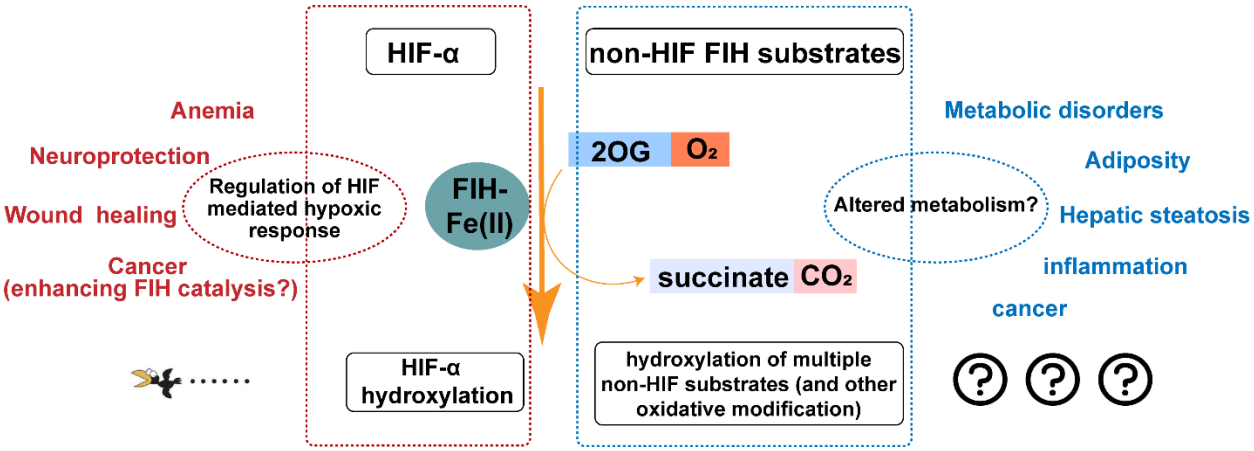

Figure 16. Summary of the possible therapeutic potential of FIH inhibitors. The role of FIH is best understood within the HIF system, where its therapeutic potential might first be best explored in vivo, though it is linked to multiple other biomedicinally important processes, suggesting modulation of its activity, perhaps best in substrate selective manner, may have broad therapeutic potential.

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.xxxxxxx.

Figures of crystallographic analysis of an ASPP2 fragment in complex with FIH.

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Notes

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ABBREVIATIONS

ARD, ankyrin repeat domain; ASBs, ankyrin repeat and suppressor of cytokine signaling box proteins; ASPP, apoptosis-stimulating p53-binding protein; CNS, central nervous system; CTAD,

carboxy-terminal transactivation domain; DMOG, dimethyloxalylglycine; DUB, deubiquitinase; EGF, epidermal growth factor; EPO, erythropoietin; FIH, factor inhibiting HIF; HACE1, ECT domain and ankyrin repeat-containing E3 ubiquitin protein ligase 1; HDAC, histone deacetylases; HFD, high-fat-diet; HIF, hypoxia-inducible factor; ICD, intracellular domain; KDMs, Histone lysine demethylases; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight-mass spectroscopy; MAML, mastermind-like; NAD^+, nicotinamide adenine dinucleotide; ODD, oxygen-dependent degradation domain; OPD, *O*-phenylenediamine; OTUB1, ovarian tumor domain containing ubiquitin aldehyde binding protein 1; Par-3, partitioning defective 3 homolog; PHDs, prolyl hydroxylase domains; RHD, rel homology domain; SOCS, suppressor of cytokine signaling; TRP, transient receptor potential; VHL, von Hippel-Lindau.

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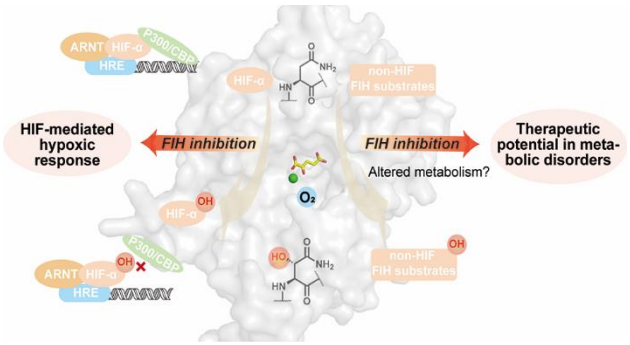

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