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Engineering Biological C−H Functionalization Leads to Allele-Specific Regulation of Histone Demethylases

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Supporting Information

ABSTRACT: Oxidative C−H hydroxylation of methyl groups, followed by their removal from DNA, RNA, or histones, is an epigenetic process critical to transcriptional reprogramming and cell fate determination. This reaction is catalyzed by Fe(II)-dependent dioxygenases using the essential metabolite 2-ketoglutarate (2KG) as a cofactor. Given that the human genome encodes for more than 60 2KG-dependent dioxygenases, assigning their individual functions remains a significant challenge. Here we describe a protein−ligand interface engineering approach to break the biochemical degeneracy of these enzymes. Using histone lysine demethylase 4 as a proof-of-concept, we show that the enzyme active site can be expanded to employ bulky 2KG analogues that do not sensitize wild-type demethylases. We establish the orthogonality, substrate specificity, and catalytic competency of the engineered demethylation apparatus in biochemical assays. We further demonstrate demethylation of cognate substrates in physiologically relevant settings. Our results provide a paradigm for rapid and conditional manipulation of histone demethylases to uncloak their isoform-specific functions.

The metabolite 2-ketoglutaric acid (2KG) **1** is an intermediate in the tricarboxylic acid cycle and an essential cofactor for more than 60 human Fe(II)-dependent oxygenases (KGOs).[1] These enzymes catalyze C−H hydroxylation on a range of substrates, including DNA, RNA, and proteins (Figures 1 and S1). KGO-mediated oxidative C−H activation is critical to gene regulation, hypoxic signaling, collagen biosynthesis, and fatty acid metabolism.[1] The oxidative removal of methyl groups from lysine residues in histones by *Jumonji* domain containing KGOs, also called lysine demethylases (KDMs), is important in nuclear reprogramming and differentiation, thus biochemically linking cellular metabolism to essential developmental processes (Figure 1).[2] Because of their fundamental importance in biology and misregulation in cancer and obesity, there has been an avalanche of efforts to develop methods, particularly small-molecule inhibitors, that target this class of proteins.[3] However, the biochemical degeneracy of KDMs (common cofactors and catalytic mechanism) has precluded the development of member- and isoform-specific chemical probes.

Biological functions of the >30 KDMs in humans are highly dynamic and context dependent (Figure S2). Mechanistic elucidation is further complicated by the presence of closely

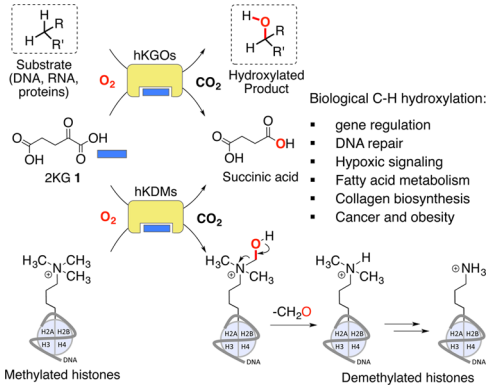

Figure 1. Biological C−H hydroxylations and their functions. Fe(II)- and 2KG-dependent enzymes (KGOs) oxidize C−H on a wide range of substrates to regulate a myriad of biological processes and diseases. One such example is C−H hydroxylation-mediated demethylation of nucleosomal histones by KDMs to regulate gene transcription.

related isoforms with nonoverlapping functions as well as the fact that KDMs act on both histone and nonhistone protein substrates.[4,5] For example, all five members of the KDM4 family (KDM4A-E) have highly homologous catalytic domains; however, KDM4A acts as a repressor of p53 transcriptional activity, while KDM4D stimulates p53-dependent gene expression. Full-length KDMs harbor multiple domains that regulate isoform-specific genome localization and substrate demethylation (Figure S2).[6,7] Furthermore, multiple KDMs can act both in synergistic and antagonistic manner for transcriptional reprogramming in response to fluctuating developmental cues.[8,9] Such isoform-specific functions and extensive crosstalk render it challenging for the existing genetic and pharmacological approaches that lack temporal control and specificity, respectively, to probe functions of KDMs in dynamic biological processes.

Allele-specific chemical genetics has emerged as a powerful tool that combines genetic and pharmacological perturbations to uncover isoform-specific protein functions.[10,11] Engineering protein−ligand interactions in a complementary manner (termed a "bump-hole" approach) can generate functional enzyme-cofactor/inhibitor pairs that are orthogonal to the wild-type system.[12,13] We reasoned the temporal control and specificity achieved through "bumped" ligand (2KG analogue)

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and "hole-modified" effector (engineered KDM) could overcome barriers to studying dynamic histone demethylation by KDMs (Figure 2a).

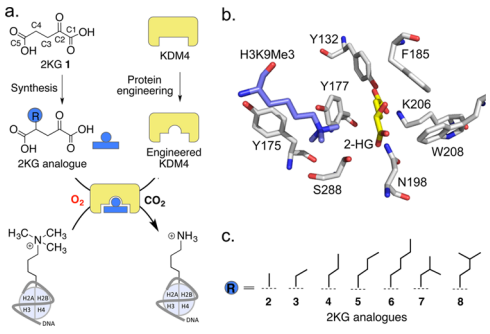

Figure 2. Engineering of demethylation apparatus. (a) Protein–ligand interface engineering to generate "hole-modified" KDM4 and "bumped" 2KG analogues for functional orthogonal demethylation system. (b) Active site structure of KDM4A bound with trimethyllysine peptide and 2-HG (PDB: 2YBP). (c) Representative 2KG analogues carrying "bumped" modifications at C-4.

KDMs rely on multiple substrates (methylated protein, 2KG and O_2) and are sensitive to Fe oxidation states. 2KG, being a small biochemical cofactor, resides close to the catalytic site. Engineering such a complex multicomponent catalytic system for allele-specific modulation has remained a significant challenge. Herein, we achieve an engineered demethylation apparatus by developing a suite of "bumped" 2KG analogues that specifically activate "hole-modified" KDM4 members. We also show the biochemical integrity of the engineered pairs, validate their orthogonality across human KGOs, and demonstrate their ability to regulate histone demethylation.

Structural analysis of KDM4s shows that their active sites contain conserved hydrophobic residues that are essential for 2KG recognition and catalysis but not substrate binding (Figure 2b).[14,15] We hypothesized that replacing one of these bulky amino acids with alanine or glycine would generate a "hole-modified" mutant enzyme that selectively utilizes a designed 2KG analogue as a cofactor (Figure 2c). From the crystal structure, it is also evident that the two internal methylenes (C3 and C4) in 2KG do not specifically interact with the enzyme and are therefore ideal sites for installing "bumped" modifications.

Based on the above analysis, we performed alanine scanning across eight positions (Y132, Y175, Y177, F185, N198, K206, W208, and S288) in KDM4A to generate a suitable "hole"-modified mutant (Figure 2b). We also synthesized 2KG analogues **2**–**8** bearing substituents with a range of steric bulk at C4 to serve as "bumped" cofactors (Figure 2c, Scheme S1). The reasoning behind selecting C4 for substitution instead of C3 is that the former is further away from the ketoacid moiety (C1–C2) that coordinates with the iron and is directly involved in C–H hydroxylation.

To identify functional enzyme–cofactor pairs, we screened 2KG (**1**) and analogues **2**–**8** against wild-type KDM4A and its alanine mutants in a combinatorial manner using $H3K9Me_3$ peptide as a substrate (Figure 3a). The reaction products were analyzed by MALDI mass spectrometry (Figures 3b,c and S3). Native KDM4A was completely inactive in the presence of the cofactor surrogates. Three of the mutants retained activity with 2KG, and one of those (F185A) was also active toward multiple 2KG analogues (Figures 3b and S3). The remaining mutants

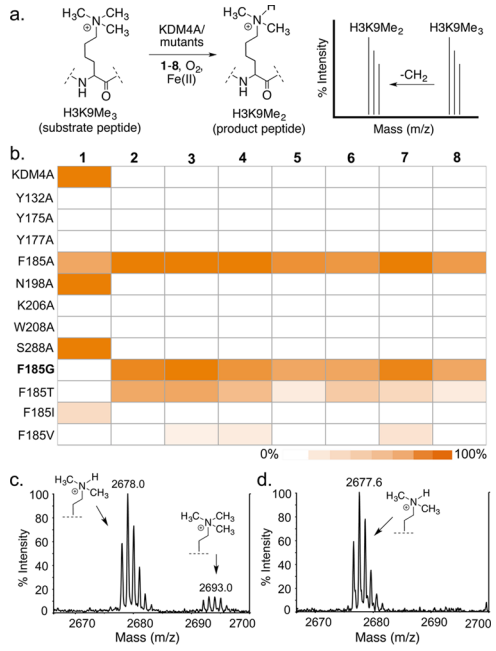

Figure 3. Demethylation activity of the engineered systems. (a) MALDI assay to determine the demethylation activity of KDM4A and its mutants toward **1**–**8**. (b) Heat-map diagram showing % activity of KDM4A and its mutants toward 2KG **1** and its analogues **2**–**8**. (c, d) Representative MALDI spectra for KDM4A-**1** and F185G-**3** pairs, respectively.

showed a complete loss of activity toward either 2KG or its analogues, likely due to substantial stereoelectronic perturbations. Thus, from an initial screening of eight mutants, we have identified F185A acting on multiple bulky 2KG analogues that do not sensitize the wild-type KDM4A.

Cellular concentration of 2KG is tightly regulated through the TCA cycle and conservatively rationed among >60 human KGOs. We reasoned that KDM4A variants that are functional only with 2KG analogues but inert toward endogenous 2KG would be ideal. Based on the promiscuity of F185A, we prepared four additional mutants at this site (F185T/V/I/G) and examined them against the same panel of cofactor analogues (Figures 3b and S3). Gratifyingly, F185G and F185T mutants showed no activity with **1**, but retained activity toward multiple analogues (Figure 3b,d). The robust activity of F185G with **3** or **7** led us to pursue further functional evaluation of these pairs. Collectively, our screening results demonstrate that structure-guided protein–ligand interface engineering is a powerful method to develop orthogonal enzyme-cofactor pairs for histone demethylases.

We next sought to establish the biochemical integrity of the engineered enzyme-cofactor pairs F185G-**3** and F185G-**7**. Catalytic efficiency is essential for demethylases to support dynamic chromatin-templated processes. We measured k_{cat} and K_M for $H3K9Me_3$ demethylation using a coupled fluorescence assay (Figures 4a and S4).[15] We observed a similar magnitude K_M for all the cofactors toward their respective enzymes and ~5–6 fold lower turnover (k_{cat}) values for the engineered pairs (Figures 4a and S5). Overall, the catalytic efficiency (k_{cat}/K_M) for F185G with **3** or **7** was only 6–10 fold lower than that of wild-type enzyme with **1**. Collectively, these results demonstrate robust demethylation by engineered pairs that are completely orthogonal to the endogenous system.

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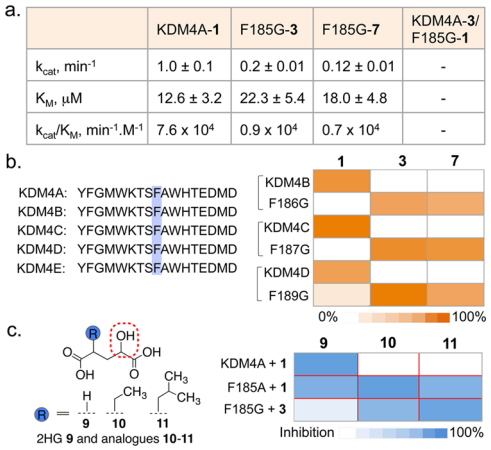

Figure 4. Biochemical characterization of the engineered system. (a) Kinetic constants of wild-type and engineered enzyme-cofactor pairs. (b) Generality of the approach: Sequencing showing F185 is highly conserved. Heat-map diagram showing % activity of KDM4B-D and their corresponding mutants toward 2KG **1** and its analogues **3** and **7**. (c) Allele-specific inhibition: 2HG **9** and its analogues **10** and **11**. Heat-map diagram showing inhibitory activity of **9**–**11** on enzyme-cofactor pairs.

To confirm that catalytic efficiency is tightly associated with substrate specificity, we synthesized a set of histone H3 peptides bearing methyl groups at positions R2, K4, K27, or K36. In a demethylation assay, wild-type KDM4A-**1**, F185G-**3**, and F185G-**7** showed similar activity profiles: each demethylated H3K9Me$_3$ and H3K36Me$_3$ peptides, but not the other substrates (Figure S6). Taken together, these results show that we have identified a functional KDM4A mutant that can efficiently utilize multiple 2KG analogues to demethylate specific substrates of the native enzyme.

Sequence and structural analysis revealed that F185 of KDM4A is well conserved in KDM4B-E (Figure 4b).[15] To demonstrate that similar orthogonal enzyme-cofactor pairs can be developed for the entire KDM4 family, we generated corresponding mutants of the remaining enzymatically well-characterized KDM4 members: KDM4B, C, and D (F186G, F187G, and F189G, respectively). First, we confirmed the demethylation activity of wild-type KDM4B-D toward H3K9Me$_3$ peptide using **1** (Figures 4b and S7). Under identical conditions, **3** and **7** failed to activate these wild-type KDM4s. In contrast, the F186G, F187G, and F189G mutants demonstrated robust demethylation activity using **3** and **7**, but were unable to utilize **1** (Figure 4b). The above findings demonstrate orthogonal engineered enzyme-cofactor pairs suitable for elucidating member-specific functions of KDM4s.

In the preceding sections, we showed that all four wild-type KDM4s examined are unable to utilize **3** and **7** as alternative cofactors. To ensure further that these "bumped" analogues do not activate other human demethylases, we tested three structurally and functionally distinct KGOs: TET2,[16] FTO,[17] and KDM6B,[18] which are DNA, RNA, and histone demethylases, respectively (Figure S8). These wild-type demethylases efficiently acted on their respective substrates in the presence of canonical cofactor **1** but not with the "bumped" analogues **3** or **7**. The fact that all the seven native demethylases (KDM4A–D, KDM6B, TET2, and FTO) examined thus far remained inactive toward **3** and **7** lends further support for the orthogonality of these analogues across human KGOs.

Allele-specific inhibitors, much like their cofactor counterparts, have found widespread application in elucidating enzyme functions.[19,20] This is particularly relevant for epigenetic enzymes such as demethylases because genetic knockout is often lethal.[21] Inspired by recent observations that 2-hydroxyglutarate (2HG) inhibits a wide range of demethylases in a 2KG-competitive manner,[22,23] we hypothesized that "bumped" 2HG analogues derived from **3** and **7** could selectively inhibit engineered KDM4A but not the wild-type protein. To test this hypothesis, we prepared putative inhibitors **9**–**11** by selectively reducing the 2-oxo moiety in **1**, **3**, and **7** (Figure 4c, Scheme S2). We then tested them against native KDM4A and its F185A and F185G mutants in a demethylation assay. For each enzyme, the selectivity profile among the inhibitors tracked well with that for the corresponding cofactors (Figures 4c and S9). The wild-type enzyme is inhibited only by **9**, the reduced analogue of the native substrate. F185A is inhibited by **9** but also bulky analogues **10** and **11**, mirroring the cofactor promiscuity of this mutant. Finally, F185G is inhibited by the bulky reduced cofactors **10** and **11** but not the smaller **9**. The fact that **10** and **11** selectively inhibit mutants with expanded active sites demonstrates that allele-specific cofactors and inhibitors can be identified for a range of histone demethylases.

To confirm that the engineered pairs are active on full-length protein substrates, we introduced a trimethylated thialysine analogue at position 9 in histone H3 (H3K$_c$9Me$_3$) by alkylation of the corresponding cysteine mutant with 2-bromoethyltrimethylammonium salt (Figure 5a).[24] LC-MS analysis confirmed

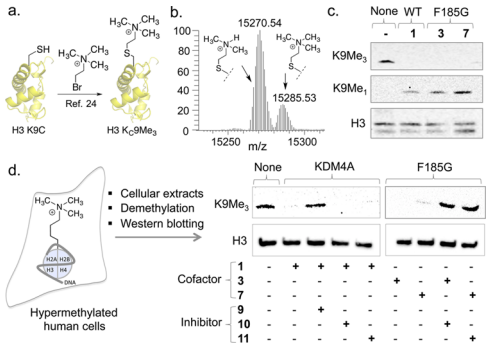

Figure 5. Activity of the engineered demethylation apparatus in biologically relevant systems. (a) Synthesis of site-specifically introduced trimethylthialysine in histone H3. (b) ESI LC-MS spectrum showing demethylation of H3K$_c$9Me$_3$ by the F185G-**3** pair. (c) Lysine demethylation in calf histones by KDM4A-**1** and F185G-**3**/**7** pairs. (d) Allele-specific activation and inhibition of KDM4A: nuclear histones in cell extracts can be conditionally demethylated using F185G and "bumped" cofactors **3**/**7** and inhibitors **10**/**11** as determined by Western blotting analysis. The activity of wild-type KDM4A-**1** was inhibited only by **9** but not by its bulky analogues **10** and **11**.

product formation (Figure S10). Such chemically methylated histone analogues have found widespread applications to probe functions of chromatin-modifying proteins. We observed robust demethylation of chemically methylated histone by F185G-**3** as confirmed by LC-MS (Figure 5b). We also examined the orthogonal pair against calf histone, a biologically more relevant preparation carrying other post-translational modifications. H3K9Me$_3$ present in calf histone was indeed demethylated by F185G when paired with **3** or **7** with efficiency comparable to the wild-type KDM4A and **1** as judged by Western blotting using

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site- and degree-specific antibodies with concomitant appearance of H3K9Me$_1$ signal (Figure 5c).

To examine whether the orthogonal enzyme-cofactor/inhibitor pairs are able to demethylate histones in a complex biological mixture, we generated hypermethylated human proteome in HEK293T cells using a cell-permeable demethylase inhibitor IOX1 (Figure 5d).[25] The lysate was incubated with either the wild-type or engineered pairs and analyzed for their demethylation activity on biologically relevant nucleosomal histones carrying other post-translational modifications using site- and degree-specific methylation antibodies. We observed comparable enzymatic activities for both the native and orthogonal systems as illustrated by significant loss of endogenous H3K9Me$_3$ (Figure 5d). The amount of trimethylated histone, however, remained unchanged for the negative controls with no enzymes or cofactors. Furthermore, the ability of F185G-**3** and F185G-**7** to demethylate endogenous histone was significantly reduced in the presence of the bulky 2HG analogues **10** and **11** (Figure 5d). In contrast, the activity of wild-type KDM4A-**1** was inhibited only by 2HG **9**. Taken together, the identified orthogonal pairs are capable of manipulating specific histone methylation status in biologically relevant settings without altering the wild-type demethylases.

KDMs belong to the oxygenase superfamily that has its phylogenetic origin in prokaryotes, highlighting their importance for life.[26] In humans, these enzymes catalyze diverse biochemical reactions, employing a conserved catalytic mechanism that has precluded development of specific chemical probes. To investigate member-specific functions of KDMs, we have employed protein−ligand interface engineering to develop enzyme-cofactor pairs orthogonal to wild-type KDMs and 2KG. As a proof-of-concept, we have successfully engineered catalytically active members of the KDM4 family and showed that they act on a set of "bumped" 2KG analogues to demethylate their cognate substrates. The engineered demethylase systems are fully orthogonal to the wild-type demethylases and 2KG without compromising the catalytic efficiency and substrate specificity, making them suitable for elucidating member-specific biological functions. Further, reduction of 2-oxo moiety in the bulky 2KG analogues led to the development of allele-specific inhibitors. The bulky 2HG analogues would be powerful probes to elucidate functions of demethylases that are essential to embryonic development. We anticipate that future experiments with full-length engineered KDM4s expressed in mammalian cells and cell-permeable 2KG and 2HG analogues will allow conditional and tunable manipulation for mapping isoform-specific functions in gene regulation and dynamic physiological processes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08653.

Experimental details and data (PDF)

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Notes

The authors declare no competing financial interest.

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