Advances and challenges in understanding histone demethylase biology

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ABSTRACT

Within the last decade we have witnessed significant progress in the field of chromatin methylation, ranging from the discovery that chromatin methylation is reversible, to the identification of two classes of oxidative chromatin demethylases. Multiple genetic and cellular studies emphasize the role of members of the amine oxidase and 2-oxoglutarate oxygenase enzyme families involved in methyl-lysine in physiology and disease. Advances in understanding of the underlying biochemistry have resulted in development of first series of clinical inhibitors and tool compounds which continue to resolve and help understand the complex relationships between chromatin modification, control of gene expression and metabolic states.

INTRODUCTION

Covalent modifications of chromatin components such as histones and DNA play central roles in transcriptional regulation, genome stability, X chromosome inactivation and imprinting, and are considered as part of a mechanism of epigenetic regulation [1, 2]. Among the multitude of combinatorial histone residue modifications, comprising acetylation, phosphorylation, ubiquitination and others, the methylation of arginine and lysine residues in particular, emerged as critical regulator of chromatin functions [3-8] leading to the hypothesis of a "histone code" [9, 10].

Histone lysine demethylases are members of two distinct enzyme families - The discovery of the first histone lysine demethylase (referred to as KDM 1 [11], lysine-specific demethylase 1 (LSD1, KDM1A), was described in 2004 by Shi *et al.* [12], shortly followed by Schüle *et al.* [13] and established two members of the enzyme family of flavine adenine dinucleotide (FAD) dependent amine oxidases as epigenetic regulators (Figure 1). Members of this enzyme family oxidize a range of biogenic amine containing substrates such as neurotransmitters, e.g. dopamine by MAOA or MAOB enzymes, or polyamines such as spermidine, cadaverine or putrescine by other members (Fig 1 A). Within this family, two members (KDM1A and KDM1B) have demonstrated activity towards Nε–methyl-lysine residues in histone 3 (H3), acting on mono- or dimethyl lysyl residues 4 or 9 (H3K4me1/2 or H3K9me1/2 by KDM1A, and H3K4me1/2 by KDM1B) [14, 15]. Shortly after, the discovery of the larger family of Jumonji-type of histone demethylases by Zhang et al [16-19] and others [20-28] highlighted the 2-oxoglutarate dependent oxygenases (2-OGs) as important mediators of chromatin and DNA modifications. This family of histone demethylases with more than 20 identified members in humans, contains a conserved catalytic JmjC domain composed of a double stranded beta helical fold (DSBH), and distinct members of demethylases (KDM2-7 highlighted in Fig 1B) show activity towards various methylated states of H3K4, H3K9, H3K27 and H3K36 (Figure 1).

These landmark discoveries provided a paradigm shift in histone lysine methylation: previously, this modification was considered to be a stable, post-translational modification of largely unknown significance: a modification which could only be removed by histone exchange or "dilution" during DNA replication [2, 29]. The Nε group of lysine can be methylated in 3 different states (mono-, di-, tri-) whereas

arginine residues can be mono-methylated or modified as symmetric/asymmetric di-methylated forms. The methylation state, residue position and the existence of specific recognition domains ("readers'), in addition to levels of specific methyltransferases ("writers") and demethylases ("erasers") dictate distinct functional and biological outcomes- for example, trimethylation of lysine 4 of histone 3 (H3K4me3) constitutes a "permissive" mark found around most transcriptionally active genes, whereas trimethylation of residues H3K27 and H3K9 is often found in "silenced" or transcriptionally inactive chromatin regions. From these and other groundbreaking genetic studies it is now clear that histone lysine methylation indeed is a reversible, dynamic and regulated process of importance for chromatin function [2], however the contribution of demethylase activity towards a physiological outcome is often not resolved or yet understood.

For further reading about the roles of KDMs in human biology and disease, we refer to several recent overviews [1, 2, 6, 7, 30, 31]. Instead, we will provide in the following review a brief account of the current chemistry efforts to develop inhibitors towards the different types of KDM enzymes. This attempt is fueled by the need to utilize selective chemical tools to understand the enzymatic role of demethylase activity. Moreover several lines of evidence suggest therapeutic utility of demethylase inhibitors in a variety of human pathologies, at present largely pursued in oncology and inflammation. We further discuss metabolic implications of demethylase activity under different physiological states, and then turn attention to the issue of substrate specificity and the role of demethylases within larger chromatin protein complexes.

The catalytic mechanism of KDMs - Amine oxidation catalyzed by flavin-containing amine oxidases is characterized by oxidation of the α-carbon bond of the substrate to form an imine intermediate, which, in turn, is hydrolyzed to form an unstable hemi-aminal that fragments into formaldehyde and the remaining lysyl-amine via a non-enzymatic process. In a complete catalytic cycle, the cofactor FAD is reduced to $FADH_2$ and then is re-oxidized to produce hydrogen peroxide as by-product [12, 32]. This mechanism requires a protonated nitrogen atom, explaining the selectivity of amine oxidase -type KDM1A and B towards mono- and dimethylated lysyl residues (Figure 1C, Figure 2).

The human 2-OG oxygenase family consists of over 60 different members, with a

significant proportion of bona fide histone demethylases (highlighted in Figure 1B), however the substrate spectrum of several members of this type of versatile oxygenases is not restricted to histone residues, and includes for example intermediary metabolites, nucleic acids, or amino acids residues in different proteins (such as prolyl and asparaginyl residues in matrix proteins (collagen) or transcription factors (HIF1α)) [30]. The discovery that distinct members of this family, namely the TET (ten-eleven translocation) oxygenases are critically involved in the demethylation process of the epigenetic mark 5-methyl-cytosine in DNA, opened new avenues in understanding the significance of this chromatin modification [33-35]. Moreover, the discovery of N^6-methyladenine in RNA and DNA, and oxidative reversal of this modification by 2-OG enzymes (such as ABH1, ABH5 or FTO, Figure 1) [36, 37] suggests that reversible nucleic acid modifications are more widespread than previously anticipated and also extends this principle into RNA biology.

Whereas the catalytic domain of the amine oxidases contains the typical α/β folding pattern typical of several types of oxidoreductases (and includes additional elements like the Tower domain found in KDM1A), the catalytic domain of JmjC- type oxygenases contains a highly conserved double- stranded β-helix (DSBH) fold with eight β-strands, also referred to as cupin, 'jelly-roll' or 'beta barrel' fold (Figure 2A-B). In humans, members of this family share few conserved sequence motifs necessary for iron and cofactor binding, and are mechanistically defined by their ability to hydroxylate their specific substrates using a reactive oxo-ferryl (IV) intermediate (Figure 1D). In brief, the catalytic sequence of methyl-lysine demethylation proceeds through distinct steps of 2-OG cofactor and molecular oxygen binding and activation, resulting in a reactive oxo-ferryl (IV) intermediate that reacts with the specific substrate atom, usually resulting in a hydroxylated substrate and concomitant CO_2 and succinate formation (Figure 1D, and Figure 2)[38, 39]. As in the case of amine oxidases, the hydroxylated substrate is an unstable hemi-aminal intermediate derived from the lysine methyl hydroxylation of the Nε- side-chain which then fragments into formaldehyde and a demethylated lysine side-chain. Importantly, this mechanism allows for demethylation of all possible Nε-methyl states (mono-, di- and tri-) found in methylated histone lysine residues and thus differs from the mechanism of monoamine oxidases.

Are histone demethylases druggable therapeutic targets? Ample genetic and experimental evidence has provided the rationale for several inhibitor development programs, both in academia and the private sector, with advancements into several clinical trials for KDM1 inhibitors [40]. Since KDM chemotype development has been the subject of several recent comprehensive reviews [38, 41] we will here only briefly touch upon these efforts. With regards to KDM1 inhibitors, most programs have focused on mechanism-based inhibitors using the cyclopropylamine template tranylcypromine or derivatives such as GSK-LSD1 (Figure 3) which form covalent FAD adducts within the KDM1 active site (Figure 2E), however reversible inhibitors such as GSK354 have also been reported. Clearly, high-quality chemical tools will aid in dissecting KDM biology, however development of required potent, selective, and cell-active molecules for 2-OG KDMs has been challenging thus far. With most chemical matter occupying part of the 2-OG binding site, chemotypes have tended to be polar expressions of an iron-chelating pharmacophore (Figure 2), which combines to present physicochemical properties incompatible with cell penetration and is often secured through the introduction of a pro-drug. Given the high levels of 2-OG and other TCA-cycle derived metabolites within the cell, any inhibitors designed to compete for cofactor binding, need to show high affinity to achieve sufficient on-target activity, as suggested through a recent chemoproteomics approach [42]. Substrate-binding competitive inhibitors appear to be more elusive and the possibilities for allosteric sites remain unproven. However, structure determinations of several of the major clinically relevant subfamilies of KDMs have enabled a better understanding of active site details (for example see KDM6B, Figure 2F) that allows to progress towards the challenge of highly selective inhibitors [43-50]. Although potent inhibitors have been discovered for most KDM sub-families, there are few compounds that meet criteria for chemical probes (Figure 3; see references [44, 47-49, 51-62]) due to a lack of selectivity and high potency in cells [63]. Taken together, there remains plenty of opportunity for the introduction of novel chemical probes for the demethylase family of enzymes.

Metabolic regulation of KDMs and epigenomic methylation signatures – the relationship between cellular metabolic states and chromatin modification is critical for understanding the long-term effects of transcriptional regulation, and as a consequence, the resulting cellular phenotypes. Chromatin modifying enzymes, i.e.

methyl- or acetyl- transferases as well as demethylases or deacetylases (i.e. class II HDACs, sirtuins) utilise as cofactors metabolic intermediates such as S-adenosylmethione (SAM), acetyl-CoA, 2-oxoglutarate or NAD+, respectively, whose concentrations are subject to nutritional status and impact on global protein acetylation and methylation patterns [64]. For example, the critical role of 2-oxoglutarate, and its impact on histone and DNA methylation with subsequent regulation of phenotypes has been recently demonstrated in pluripotency of stem cells [65] and in cancer [66]. Furthermore, several genetic studies highlight mutations in enzymes of the tricarboxylic acid (TCA) cycle such as fumarate hydratase, succinate dehydrogenase or isocitrate dehydrogenase (IDH) as drivers of malignant transformation and progression [66, 67]. Accordingly, the inhibitory effects of accumulated substrates (fumarate, succinate) or neomorphic products such as D-2-hydroxyglutarate as in the case of IDH [68, 69], on chromatin hydroxylating KDM or TET enzymes [34], could explain the aberrant chromatin methylation patterns as cause for oncogenic drives. This is analogous to the inhibitory control exerted by succinate, observed during myeloid activation. Metabolic reprogramming induced by toll-like receptor (TLR) agonists such as lipopolysaccharide (LPS) derived from gram-negative bacteria increases succinate levels resulting in inhibition of the KDM related, 2OG oxygenase enzyme prolyl hydroxylase (PHD2), which controls the oxygen sensing transcription factor HIF1α. Importantly, this leads to up-regulation of HIF1α target pathways under normoxic conditions [70, 71], with a concomitant expression of pro-inflammatory pathways during macrophage or dendritic cell activation. Oxygen levels themselves have a clear effect on global chromatin methylation states as observed during development and in several cancer types [72-76]. This can be related to the fact that several demethylases such as members of KDM subfamilies 3-6 themselves are HIF1α target genes [72, 76-82], however anaerobic metabolism can also increase concentrations of L-2-hydroxyglutarate via lactate or malate dehydrogenases, thereby inhibiting 2-OG enzymes such as KDMs. Changes in redox state could also affect susceptible amino acid residues, although the area of post-translational modifications of KDMs and resulting functional consequences requires further investigations. It is also conceivable that KDM enzyme activities (including LSD1/2, which are much less studied in this respect than 2-OG

type KDMs) are directly limited by oxygen levels, leading to important alterations in chromatin methylation (Figure 4).

Beyond histone lysine methylation: what are the functions of demethylases? So far we have highlighted several points relating to the catalytic function of KDMs, their interrelated dependencies on cofactors such as oxygen and 2-OG, as well as inhibition by various metabolic intermediates. We assume that continued research will deliver novel insights into the relationships between metabolic control and chromatin methylation states. Another question is whether the catalytic function of KDMs is the sole or most important role in the context of chromatin biology [5]. Most, if not all histone demethylases are transiently associated to transcriptional regulators contributing to transcriptional activation or suppression [2]. In that respect, the importance of KDM scaffolding functions is highlighted in several instances (reviewed in [30]), and it is thought that KDMs create certain chromatin states, which provide a permissive environment for distinct transcriptional complexes. We believe that investigations into scaffolding functions of KDMs will be facilitated by genetic approaches, but also by specific and potent inhibitors, which will contribute to dissect specific roles of KDMs. We also predict that system-wide analysis of non-chromatin methylomes will deliver rich sets of possible novel KDM targets, thereby extending their substrate repertoire beyond chromatin biology. For example, a recent MS-based methylome study [83] revealed that > 500 intracellular sites can be methylated, and multiple studies show the importance of this modification on their function, also suggesting that reversible methyl modifications could occur on non-histone proteins. For these reasons, the continued development and improvement of potent and specific KDM inhibitors will contribute to the understanding of chromatin biology, with the ultimate goal of understanding and treating human disease.

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HIGHLIGHTS

- histone methylation is critical in genome integrity and gene regulation
- histone lysine demethylation is accomplished by two distinct KDM enzyme families
- inhibitor development is ongoing including first clinical trials in oncology
- genetic and inhibitor approaches are needed to dissect the complex biology of KDMs
- cellular metabolic states control KDM activity and chromatin methylation

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LEGEND to FIGURES

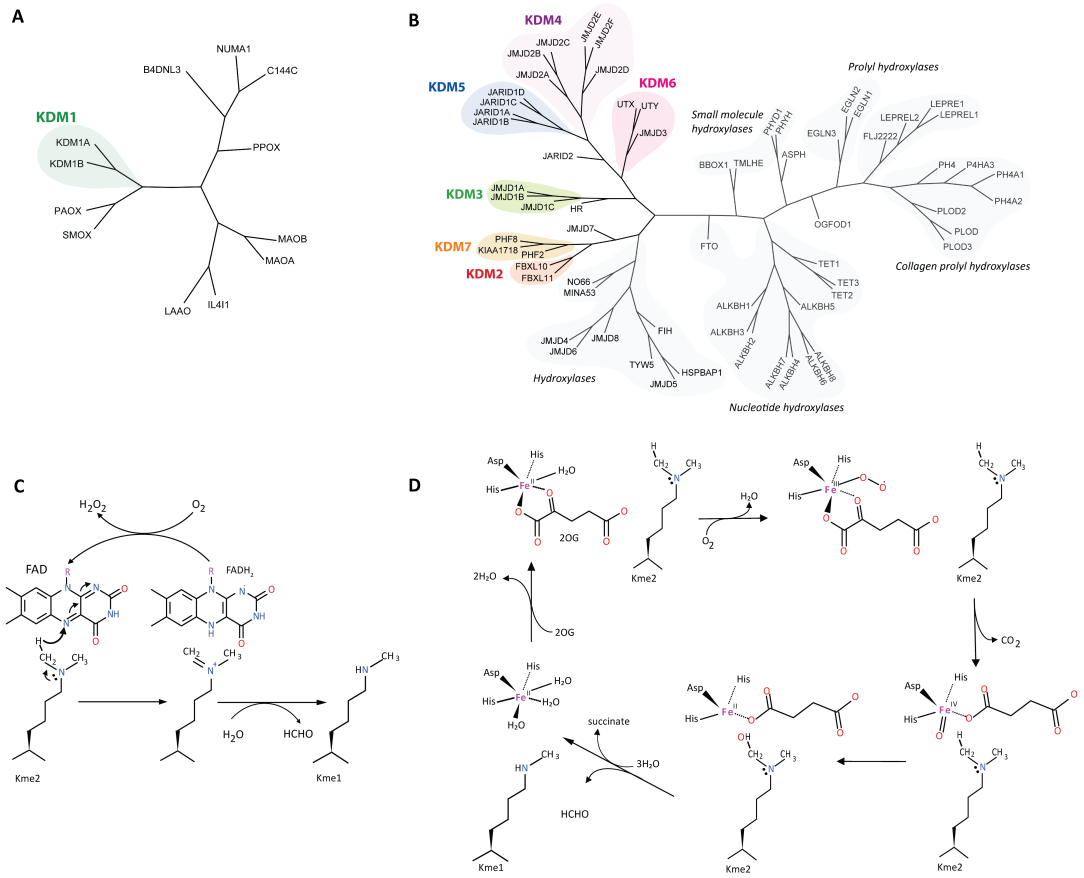
Figure 1: Histone demethylases belong to two distinct enzyme families. KDM1A and KDM1B are members of the amine oxidase family (A), utilize a FAD-dependent oxidase mechanism to generate a hemiaminal intermediate that fragments into a demethylated product and formaldehyde (C). Other histone demethylases are members of the 2-oxoglutarate oxygenase family with various identified subfamilies (KDM2-7), that utilize a Fe (IV)-oxoferryl species (generated by 2-oxoglutarate to succinate formation) to produce a hemiaminal species, leading to a demethylated residue and formaldehyde.

Figure 2: Structural overview of the two KDM enzyme families. Ribbon representation of A) amine oxidases (KDM1A, pdb 2UXN) and B) 2OG dependent di-oxygenases (KDM4A, pdb 2OQ6) showing the catalytic oxidase- and JmjC-domain coloured in blue. Cofactor and peptide binding pocket in C) KDM1A (2UXN) and D) KDM4A (2OQ6). E) Inhibitor 1-ethyl-Tranylcypromine (D70) bound in the peptide binding pocket of KDM1A (4UV9) F) Inhibitor GSK-J1 bound to the cofactor binding pocket in KDM6B (4ASK).

Figure 3: Overview on selected KDM inhibitor chemotypes.

Figure 4: KDM enzymes influence epigenomic signatures via enzymatic and scaffolding functions. The enzymatic function can be regulated through several variables, including signals such as growth factors, cytokines, metabolic state, endogenous or xenobiotic inhibitors, and oxygen concentration.

FIGURE 1


A
NUMA1
B4DNL3
C144C
KDM1
KDM1A
KDM1B
PPOX
PAOX
SMOX
MAOB
MAOA
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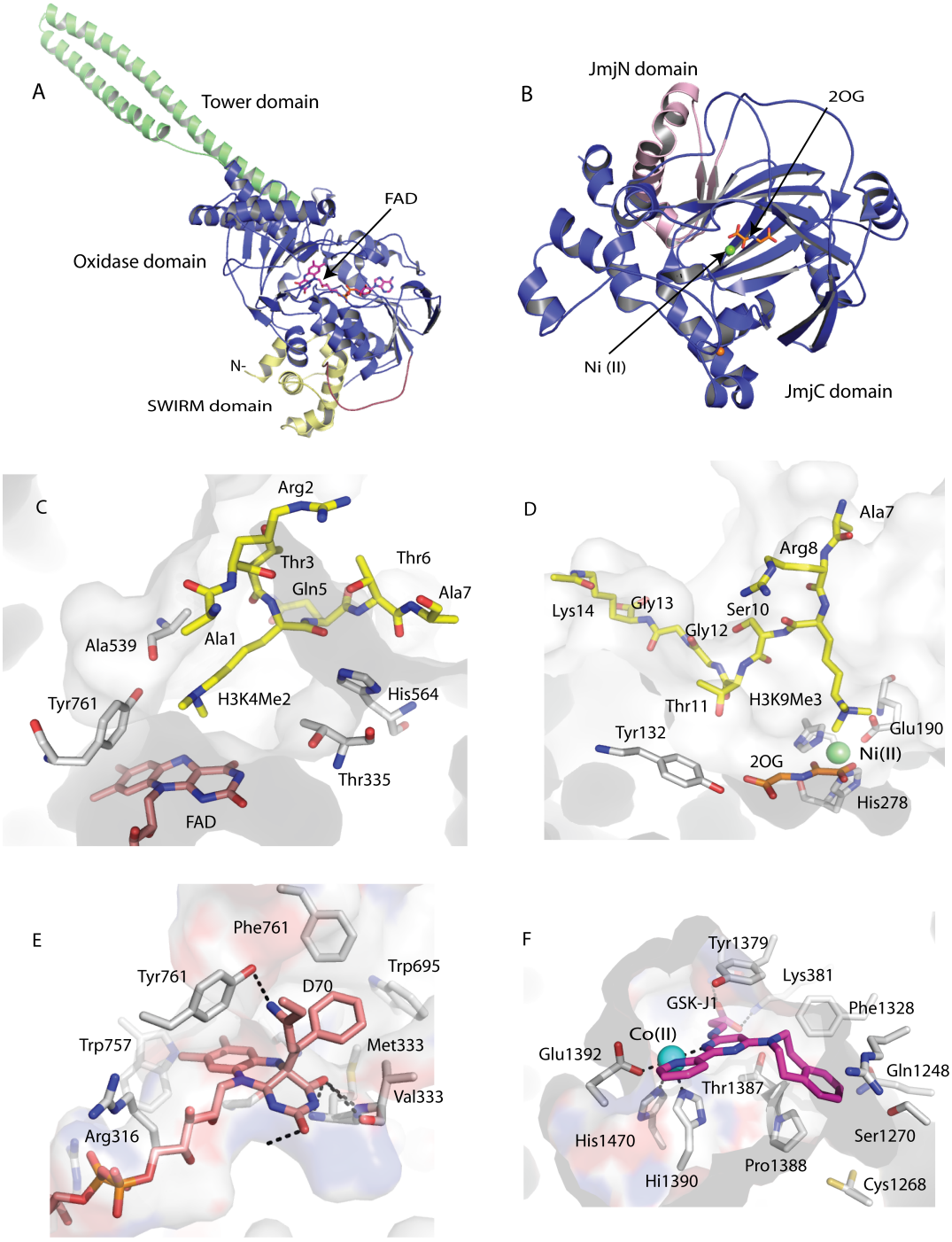
IL4I1
B
KDM4
JMJD2E
JMJD2F
JMJD2C
JMJD2B
JMJD2A
JMJD2D
KDM5
JARID1D
JARID1C
JARID1A
JARID1B
KDM6
UTX
UTY
JMJD3
JARID2
KDM3
JMJD1A
JMJD1B
JMJD1C
HR
KDM7
PHF8
KIAA1718
PHF2
KDM2
FBXL10
FBXL11
NO66
MINA53
JMJD7
FTO
Small molecule hydroxylases
PHYD1
PHYH
Prolyl hydroxylases
EGLN2
EGLN1
EGLN3
LEPRE1
LEPREL1
LEPREL2
FLJ2222
BBOX1
TMLHE
ASPH
PH4
P4HA3
PH4A1
PH4A2
PLOD2
PLOD
PLOD3
OGFOD1
Collagen prolyl hydroxylases
TET1
TET3
TET2
ALKBH5
ALKBH1
ALKBH3
ALKBH2
ALKBH7
ALKBH4
ALKBH8
ALKBH6
FIH
JMJD4
JMJD6
JMJD8
TYW5
HSPBAP1
JMJD5
Hydroxylases
Nucleotide hydroxylases
C
FAD
FADH2
H2O2
O2
H2O
HCHO
Kme2
Kme1
D
2OG
2H2O
CO2
succinate
3H2O
HCHO
Kme2
Kme1

FIGURE 2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Tower domain
FAD
Oxidase domain
N-
SWIRM domain
B
JmjN domain
2OG
Ni (II)
JmjC domain
C
Arg2
Thr3
Thr6
Gln5
Ala7
Ala539
Ala1
Tyr761
H3K4Me2
His564
Thr335
FAD
D
Ala7
Arg8
Lys14
Gly13
Ser10
Gly12
Thr11
H3K9Me3
Tyr132
Glu190
Ni(II)
2OG
His278
E
Phe761
Tyr761
Trp695
D70
Trp757
Met333
Val333
Arg316
F
Tyr1379
Lys381
GSK-J1
Phe1328
Co(II)
Glu1392
Gln1248
Thr1387
His1470
Ser1270
Pro1388
Hi1390
Cys1268

FIGURE 3

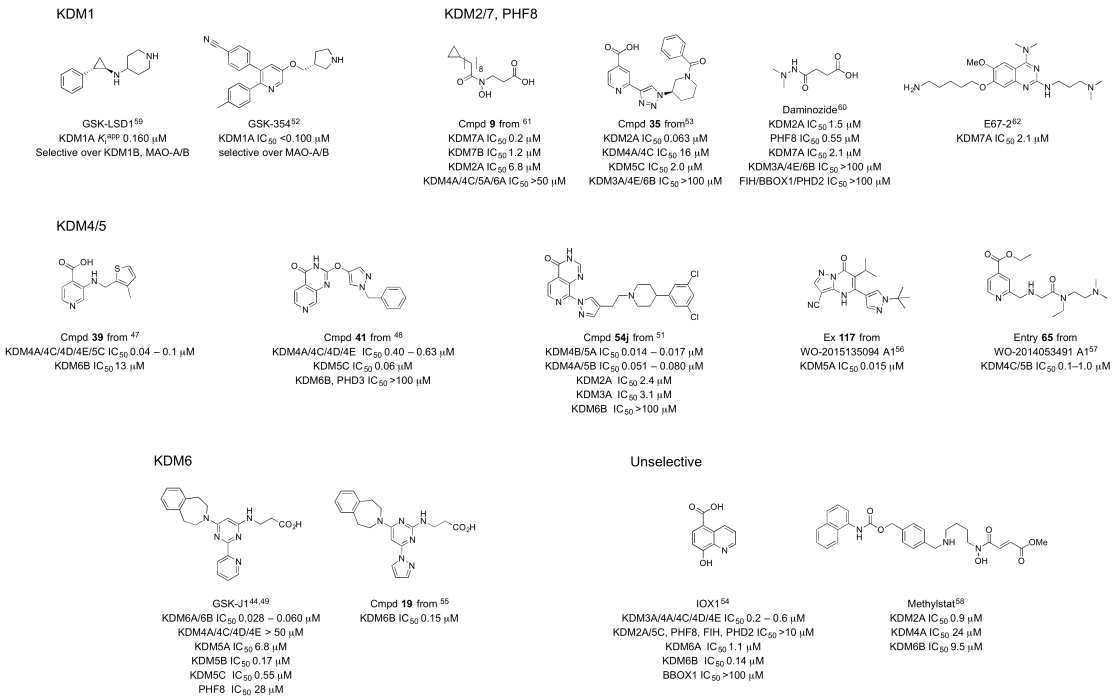
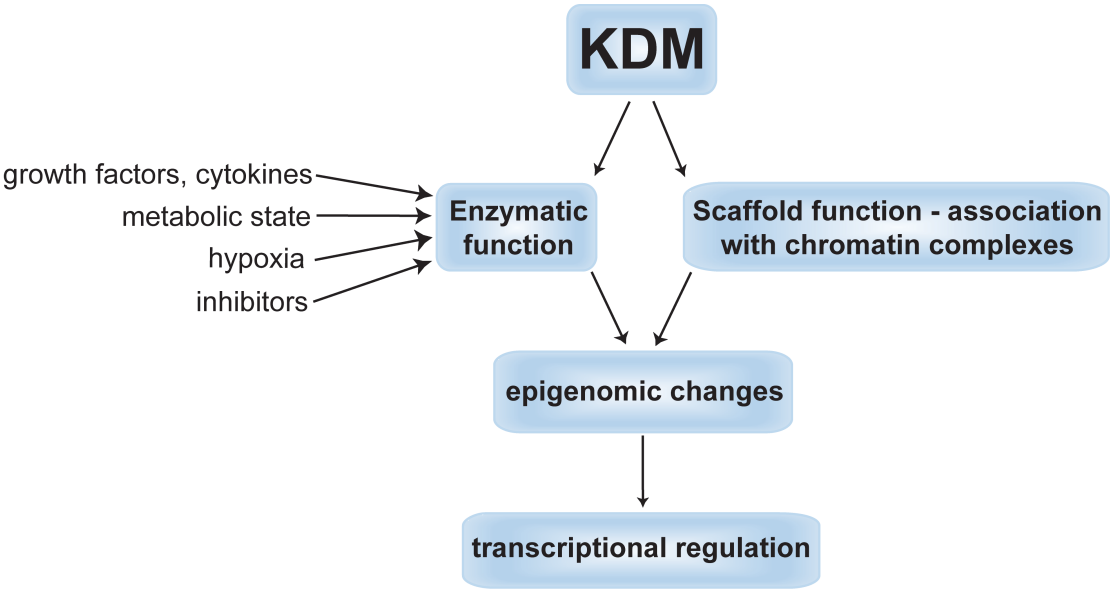

KDM1
GSK-LSD1[59]
KDM1A K_i^{app} 0.160 μM
Selective over KDM1B, MAO-A/B
GSK-354[52]
KDM1A IC_{50} <0.100 μM
selective over MAO-A/B
KDM2/7, PHF8
Cmpd 9 from [61]
KDM7A IC_{50} 0.2 μM
KDM7B IC_{50} 1.2 μM
KDM2A IC_{50} 6.8 μM
KDM4A/4C/5A/6A IC_{50} >50 μM
Cmpd 35 from[53]
KDM2A IC_{50} 0.063 μM
KDM4A/4C IC_{50} 16 μM
KDM5C IC_{50} 2.0 μM
KDM3A/4E/6B IC_{50} >100 μM
Daminozide[60]
KDM2A IC_{50} 1.5 μM
PHF8 IC_{50} 0.55 μM
KDM7A IC_{50} 2.1 μM
KDM3A/4E/6B IC_{50} >100 μM
FIH/BBOX1/PHD2 IC_{50} >100 μM
E67-2[62]
KDM7A IC_{50} 2.1 μM
KDM4/5
Cmpd 39 from [47]
KDM4A/4C/4D/4E/5C IC_{50} 0.04 – 0.1 μM
KDM6B IC_{50} 13 μM
Cmpd 41 from [48]
KDM4A/4C/4D/4E IC_{50} 0.40 – 0.63 μM
KDM5C IC_{50} 0.06 μM
KDM6B, PHD3 IC_{50} >100 μM
Cmpd 54j from [51]
KDM4B/5A IC_{50} 0.014 – 0.017 μM
KDM4A/5B IC_{50} 0.051 – 0.080 μM
KDM2A IC_{50} 2.4 μM
KDM3A IC_{50} 3.1 μM
KDM6B IC_{50} >100 μM
Ex 117 from
WO-2015135094 A1[56]
KDM5A IC_{50} 0.015 μM
Entry 65 from
WO-2014053491 A1[57]
KDM4C/5B IC_{50} 0.1–1.0 μM
KDM6
GSK-J1[44,49]
KDM6A/6B IC_{50} 0.028 – 0.060 μM
KDM4A/4C/4D/4E > 50 μM
KDM5A IC_{50} 6.8 μM
KDM5B IC_{50} 0.17 μM
KDM5C IC_{50} 0.55 μM
PHF8 IC_{50} 28 μM
Cmpd 19 from [55]
KDM6B IC_{50} 0.15 μM
Unselective
IOX1[54]
KDM3A/4A/4C/4D/4E IC_{50} 0.2 – 0.6 μM
KDM2A/5C, PHF8, FIH, PHD2 IC_{50} >10 μM
KDM6A IC_{50} 1.1 μM
KDM6B IC_{50} 0.14 μM
BBOX1 IC_{50} >100 μM
Methylstat[58]
KDM2A IC_{50} 0.9 μM
KDM4A IC_{50} 24 μM
KDM6B IC_{50} 9.5 μM

FIGURE 4


KDM
growth factors, cytokines
metabolic state
hypoxia
inhibitors
Enzymatic function
Scaffold function - association with chromatin complexes
epigenomic changes
transcriptional regulation