Histone Demethylases in Neuronal Differentiation, Plasticity, and Disease

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Abstract

For more than 40 years after its discovery, histone methylation was thought to be largely irreversible. However, the first histone demethylase (HDM) was identified in 2004, challenging this notion. Since that time, more than 20 HDMs have been identified and characterized, and many have been shown to have critical roles in organismal development, cell fate, and disease. Here, we highlight some of the recent advances in our understanding of the function of HDMs in the context of neuronal development, plasticity, and disease. We focus in particular on molecular genetic studies of LSD1, Kdm6b, and Kdm5c that have elucidated both enzymatic and non-enzymatic gene regulatory functions of these HDMs in neurons.

Introduction

Post-translational modifications to the N-terminal tails of the histone proteins play crucial roles in genome regulation [1]. These modifications (*e.g.* acetylation, methylation, phosphorylation) are deposited by so-called "writer" enzymes and dynamically removed by the action of "eraser" enzymes. Although histone methylation was long thought to be irreversible due to its chemical stability, the identification of a large group of enzymes with histone demethylase activity challenged this assumption. Histone demethylases (HDMs) can be broadly classified into two families based on their mechanisms of enzymatic action: the two amine oxidase demethylases (LSD1/KDM1A and LSD2/KDM1B) and the much larger Jumonji C domain (JmjC) family, which has more than 20 members [2]. The discovery of such a large set of HDMs, along with the observation of their specificity for demethylation

of distinct residues on the histone tails, immediately raised the possibility that these enzymes dynamically regulate histone methylation-dependent cellular processes. Indeed, HDMs have been found to play crucial roles in development and contribute to pathological processes like cancer and aging.

Neurons are long-lived postmitotic cells that continuously adapt their gene expression programs to the environment; thus, these cells serve as a particularly good substrate for discovering the function of chromatin regulators including HDMs in genome dynamics. Here we review recent hallmark studies on the functions of HDMs in neurons, focusing in particular on biological and biochemical studies of three of the best studied neuronal HDMs (summarized in Fig. 1) that offer new insight into the roles of chromatin regulation in the brain.

LSD1: protein complexes and splicing determine target specificity

Lysine-specific demethylase 1 (LSD1) was the first HDM to be discovered [3] and has been one of the most widely studied. LSD1 was initially characterized as a transcriptional repressor that interacts with the CoREST complex and specifically demethylates the transcriptional activation-associated mark histone H3 mono or dimethylated at lysine 4 (H3K4me1 and H3K4me2) [4]. However, LSD1 was also co-purified with the androgen receptor and was found to act as a coactivator of transcription via its ability to demethylate the repressive marks H3K9me1 and H3K9me2 [5]. The association of LSD1 with distinct protein complexes appears to direct its specificity toward different histone modifications, allowing this single enzyme to have opposing functions in transcriptional regulation in distinct contexts [6]. Mutations that impair the enzymatic activity of LSD1 have been associated with intellectual disability (ID), suggesting the relevance of LSD1's demethylase function in human brain development (Fig. 2A) [7].

Kdm1a knockout mice die by embryonic day 6.5, precluding the study of brain development in these animals [8]. However, knockdown or inhibition of LSD1 in neuronal progenitors has revealed that it serves as a positive regulator of neuronal differentiation in the developing brain. In cortical progenitors, LSD1 forms a complex with the transcriptional corepressor CoREST and binds to the promoter of *Hes1*, a transcriptional repressor and key effector of the Notch signaling pathway [9]. Knockdown of LSD1 or CoREST by *in utero* electroporation leads to an increase in Hes1 expression and reduced expression of its proneural downstream target, the basic helix-loop-helix transcription factor, Ngn2. These data indicate that LSD1 opposes Notch to promote cortical neuronal differentiation. Similar functions for LSD1 were observed in retinal development, where peak LSD1 expression occurs during the period of rod photoreceptor differentiation [10]. Inhibition of LSD1 in cultured retinal explants was shown to increase expression and H3K4me2 deposition on promoters of progenitor-expressed genes, including *Hes1*, and block induction of terminal rod differentiation markers despite the normal expression of rod photoreceptor transcription factors.

LSD1 also contributes to the regulation of specific programs of gene expression in postmitotic, fate-committed neurons. Perhaps its most specialized role is in the complex

series of epigenetic regulatory events that permit each olfactory sensory neuron to express one and only one of the thousands of possible olfactory receptor genes [11]. To more broadly study the function of LSD1 in the adult brain, Christopher *et al.* [12] conditionally deleted floxed *Kdm1a* in adult mice with a tamoxifen-inducible Cre transgene. Loss of LSD1 from the adult brain resulted in widespread neuronal death throughout the cortex and hippocampus. Gene expression analysis showed reactivation of stem cell genes in the hippocampus of LSD1 knockout mice and bioinformatics suggested an overall similarity of the knockout gene expression profile to that seen in cases of Alzheimer's disease and frontotemporal dementia. Whether insults to LSD1 function directly cause neurodegeneration remains unknown; however, these data suggest that continuous expression of LSD1 in the adult brain is required for maintenance of proper neuronal function.

Interestingly, LSD1 has a neuronal-specific splice variant, which comprises about half of all LSD1 in the adult brain and is the predominant form of LSD1 expressed in the early postnatal brain [13]. Referred to as NeuroLSD1 or LSD1n, this variant is characterized by the inclusion of a 12-nucleotide microexon (E8a) that adds just four amino acids to the enzymatic amine oxidase domain [14]. Nonetheless, variant-specific knockout or knockdown of LSD1n has shown that this small change in the LSD1 sequence has major effects on LSD1 function. The brains of LSD1n knockout mice are hypoexcitable and show decreased seizure susceptibility [15]. LSD1n mutant mice also display impaired spatial learning and memory in the Barnes maze [16] and reduced stress-induced anxiety-like behavior [17]. These behavioral changes correlate with impaired activity-dependent induction of immediate-early genes (IEGs), which have been suggested to play important roles in the cellular and circuit adaptations that underlie learning and memory.

How does LSD1n promote IEG induction? Phosphorylation of the LSD1n tetrapeptide has been suggested to inhibit association with CoREST and to block the repressive H3K4 demethylase activity of LSD1 [18]. Given evidence that LSD1n binds to at least some of the same target genes as LSD1, one possibility is that LSD1n functions as a dominant negative, blocking the repressive actions of LSD1 [13]. Alternatively, one group found that LSD1n binds a co-regulatory protein called supervillin, which directs the complex to demethylate H3K9me2, thus promoting gene activation [19]; another group proposed a model in which LSD1n acquires specificity to demethylate H4K20, promoting transcriptional elongation [16].

Kdm6b: Relief of polycomb-mediated repression

Kdm6b/Jmjd3 is one of a small family of two HDMs (Kdm6a/Utx is the other HDM) that has specificity for removal of the H3K27me2/3 marks laid down by the polycomb repressive complex. Over the course of neuronal differentiation, Kdm6b is important for removing H3K27me3 in the context of bivalent chromatin marks. Bivalency describes regulatory elements that are associated with both H3K4me3 and H3K27me3, which are histone modifications normally correlated with gene activation and repression, respectively [20]. During the early stages of cell fate commitment, bivalent chromatin is found at promoters of cell-type specific genes that are poised to turn either on or off depending on the fate of the

cell. For example, Kdm6b-dependent resolution of bivalency is required for proper activation of Nestin expression in embryonic stem cell-derived neural progenitor cells during commitment to the neuronal lineage [21].

In vivo, conditional knockout or knockdown of Kdm6b impairs neurogenesis in the olfactory bulb (OB) [22], the spinal cord [23], and the retina [24]. These studies have revealed diversity in the action of Kdm6b. In the spinal cord, Kdm6b is bound to a set of gene promoters that are activated during TGFβ-dependent neural differentiation, and it collaborates with Smad3 to induce promoter demethylation and transcriptional activation [23]. By contrast, although Kdm6b is required for the expression of the transcription factor Dlx2 in OB progenitors, it acts by regulating H3K27me3 at a distal enhancer of this gene rather than at the gene promoter [22]. In the retina, although Kdm6b is also pro-differentiation, Kdm6b is expressed most highly in young postmitotic neurons, and loss of Kdm6b function impairs the development of only a subset of retina cell types that undergo developmental loss of H3K27me3 at the *Bhlhb4* promoter [24]. Finally, given that substantial developmental loss of H3K27me3 during neural commitment can still occur in the absence of the Kdm6 family [25], it remains important to test which of the functions of Kdm6b in neural differentiation require the action of its histone demethylation function.

Despite these data implicating Kdm6b as a positive regulator of neuronal differentiation, and surprisingly given the evidence that germline knockouts die perinatally due to an inability to breathe, the brains of *Kdm6b* knockout mice are remarkably normal in gross morphology [26,27]. Interestingly, Kdm6b acts in postmitotic neurons to regulate synapse function. Burgold *et al.* (2012) found that Kdm6b is required to maintain functionality of the Pre-Bötzinger complex (PBC), the neural pacemaker that controls the respiratory rhythm. In the absence of Kdm6b, although the neurons of the PBC are present, the circuit fails to functionally mature into a network state that can control proper breathing by the time of birth. Clues to the potential mechanisms of these circuit defects are suggested by studies of Kdm6b-dependent gene expression in developing cerebellar granule neurons (CGNs) [27]. CGN differentiation provides an effective means to identify gene expression that changes across the full time course of differentiation including postmitotic stages of neuronal and synaptic maturation. Conditional knockout of *Kdm6b* in fate-committed CGN progenitors did not affect the expression of early neuronal marker genes and did not disrupt cerebellar morphology. However loss of Kdm6b expression did impair the late upregulation of gene products including specific GABA and glutamate receptor subunits that confer mature properties upon synaptic function [27]. These data are intriguing in light of the discovery of *de novo KDM6B* mutations in patients diagnosed with autism spectrum disorder (ASD), which is thought to be a primary disorder of synapses (Fig. 2B) [28].

Finally, like LSD1n, Kdm6b is also implicated in activity-dependent gene regulation in the mature nervous system. However, unlike LSD1n, which likely acts in a constitutive manner to set the responsiveness of IEGs to synaptic stimuli, Kdm6b itself is a direct target of regulation by synaptic activity. Among all of the HDMs, the expression of *Kdm6b* is by far the most strongly induced by synaptic activity, showing over a 20-fold increase in mRNA expression in neurons of the hippocampus following pilocarpine-induced seizures [29]. Kdm6b is also induced in neurons of the prefrontal cortex following cocaine exposure and

withdrawal [30]. Whether there are direct synaptic activity-regulated targets of Kdm6b remains to be fully understood, although one study showed that pre-treatment of hippocampal neurons with the Kdm6b inhibitor GSK-J4 impaired NMDA-receptor dependent transcription of Brain-Derived Neurotrophic Factor (*Bdnf*) [31]. In hippocampal neurons Kdm6b is required for a form of synaptic activity-dependent survival called preconditioning. Surprisingly, in this context, Kdm6b is required for the induction of a program of inflammatory gene expression rather than traditional pro-survival genes like *Bdnf* [29]. These data raise the possibility that the pervasive function of Kdm6b in the control of inflammation [32] may also contribute to its cell-type specific functions in activity-dependent neuronal adaptations.

Kdm5c: Mutations in X-linked intellectual disability

As mentioned above, targeted exome sequencing studies are rapidly revealing *de novo* mutations in a number of chromatin regulators associated with neurodevelopmental disorders including ID and ASD [33,34]. In addition, familial mutations in the H3K4-selective demethylase *KDM5C* (SMCX/JARID1C) have been identified as one of the more frequent causes of X-linked intellectual disability (XLID) (Fig. 2C)[35]. In either case, the genetic association between any chromatin regulator and disease is only the beginning of the story, and recent efforts have focused on elucidating the cellular mechanisms that link chromatin dysregulation through gene expression and circuit formation to impaired brain function.

Mice with germline deletion of *Kdm5c* recapitulate many of the cognitive, adaptive and social abnormalities seen in patients with *KDM5C* mutations and, thus, offer a model for studying the neurological effects of Kdm5c disruption [36]. Behaviorally, *Kdm5c* knockout mice exhibit increased aggression, decreased anxiety, and impaired social behaviors as well as defects in learning and memory. At the cellular level, neurons in the knockout mice have defects in dendritic branching and spine morphology. Expression of disease-associated *KDM5C* mutations ($KDM5C^{H514A}$, $KDM5C^{Y751C}$ and $KDM5C^{F642L}$) in the Neuro2A cell line suppress retinoic acid-induced neurite growth, consistent with these point mutations being *KDM5C* loss-of-function at least for this cellular phenotype [37].

In *Kdm5c* germline and forebrain conditional knockout mice, RNA sequencing showed the upregulation of a large set of genes, suggesting that Kdm5c functions primarily as a transcriptional repressor [36,38]. While no changes in global H3K4me3 levels were observed in the knockout neurons, there were thousands of differentially methylated peaks, primarily exhibiting local increases in H3K4me3. *De novo* H3K4me3 peaks were most common at intergenic regions that are marked by H3K4me1 in wildtype, suggesting that enhancers more than promoters are particularly sensitive to the loss of Kdm5c [38]. Despite the fact that Kdm5c is broadly bound at the promoters of expressed genes, genes that showed the highest induction of expression and the greatest increase in H3K4me3 at their promoters in the absence of Kdm5c were those that were lowly expressed in wildtype [36,38]. These data suggest that Kdm5c is primarily required for fine-tuning gene expression by suppressing the accumulation of H3K4me3 at active regulatory elements. Notably, although most ID-associated mutations in *KDM5C* disrupt the histone demethylase activity

of this enzyme, a point mutant was recently identified that neither disrupts protein expression or enzyme activity (Fig. 2C) [39]. Thus Kdm5c may also have non-enzymatic activities that contribute to brain development.

Other HDMs in neurodevelopmental disorders

In addition to the HDMs highlighted here, many other HDMs are expressed in neurons [29], and mutations in several of these have been found in neurodevelopmental syndromes, highlighting their importance human brain development. For example, microdeletion of 12a24.31, which is a genomic region that contains both *KDM2B* and the histone methyltransferases *SETD1B*, causes an ID syndrome [40], and multiple mutations in the H3K9 demethylase *JMJD1C* (KDM3C) were found upon targeted resequencing of a set of patients clinically diagnosed with ASD, ID, or Rett Syndrome without an identified genetic defect [41].

The most common forms of ID are X-linked, adding urgency to the study of HDMs that localize to the X chromosome. The H3K9/H4K20 demethylase *PHF8* (KDM7B) is associated with both syndromic and non-syndromic forms of X-linked ID [42]. Chen *et al.* (2018) generated *Phf8* knockout mice as an animal model of these disorders and showed the knockouts exhibited learning and memory impairments in behavioral assays and long-term potentiation (LTP) deficits in slice electrophysiology [43]. A bioinformatics approach suggested the upregulation of the mTOR pathway in these mice and, interestingly, an inhibitor of mTOR (rapamycin) was able to rescue the learning/memory as well as LTP defects in these mice. Tang et al. (2017) conducted a similar study on a different X chromosome HDM, Kdm6a (Utx) [44]. They generated forebrain-specific *Kdm6a* knockout mice, which exhibited deficits in learning and memory, as well as morphological and functional abnormalities of hippocampal synapses [44]. Among the genes downregulated in the conditional knockouts, the authors focused on the serotonin receptor *Htr5b* and showed that overexpression could rescue dendritic defects in Kdm6a knockout neurons. These findings have potential relevance for the cognitive symptoms in Kabuki Syndrome, a congenital craniofacial disorder for which *KDM6A* mutations are one genetic cause. Although conditional genetic deletion of *Kdm6a* in the neural crest of mice recapitulates dysmorphic features of Kabuki syndrome, craniofacial development is mostly independent of the demethylase activity of *Kdm6a* because mice bearing a demethylase-dead knockin mutation of *Kdm6a* have normal facial morphology [45]. These data reiterate that the connections between the biological and biochemical functions of the HDMs remain to be fully explored, and they show that understanding the relevant actions of the HDMs in neurons will be required to develop meaningful potential therapeutics.

Concluding Remarks

The discovery of HDMs not only opened the door to understanding the roles of dynamic histone methylation but also gave us new genetic tools to explore the mechanisms and consequences of gene regulation in the brain. The identification of disease-associated mutations in HDMs and the study of mouse knockout models have revealed fundamental insights about the biological requirements for these enzymes. Future studies will reveal

mechanistic insights about recruitment of HDMs to their target genes, the domains in HDMs that are critical for their function, as well as non-enzymatic functions of this family.

Acknowledgements

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Highlights

- Neuronal functions of the histone demethylases LSD1, Kdm6b, and Kdm5c

- Evidence for potential non-enzymatic functions of the histone demethylases

- Summarize neurological disease-associated mutations in HDMs
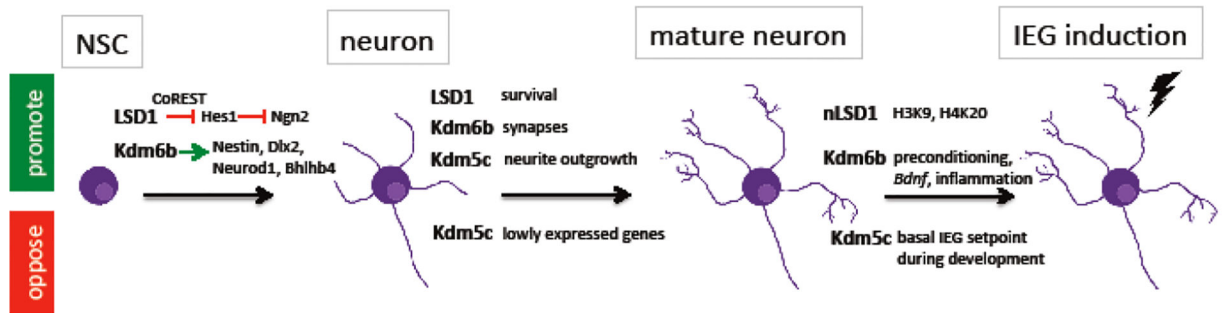
Figure 1:

Functions of HDMs in neuronal development and plasticity. Summary of the functions of LSD1, Kdm6b, and Kdm5c at different stages of neuronal development as described in the text. Green arrow or box indicates activation of a process or gene, Red bar or box indicates repression of a process or gene. NSC, neural stem cell. nLSD1, neuronal splice variant of LSD1. IEG, immediate early gene. The lightning bold represents synaptic activity and neuronal action potential firing.
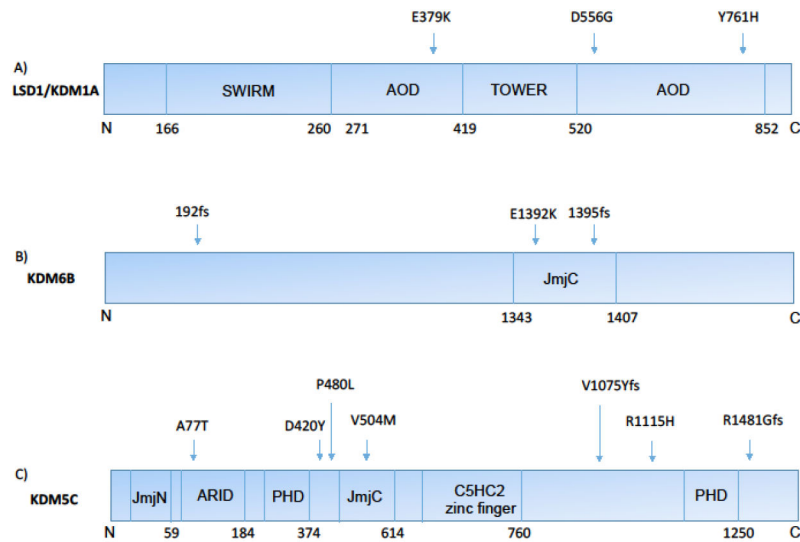
Figure 2:
Point mutations in *KDM1A*, *KDM6B*, and *KDM5C* associated with neurodevelopmental disorders. Protein domains are indicated by boxes and names, and the positions of disease associated mutations indicated by arrows. A) *KDM1A* mutations associated with cognitive impairment [7]. B) *De novo KDM6B* mutations identified in patients with ASD [28]. C) *KDM5C* mutations associated with XLID [34, 37] and one mutation (R1115H) associated with ASD/ID [39].