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## Disrupted intricacy of histone H3K4 methylation in neurodevelopmental disorders

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### Abstract

Methylation of histone H3 lysine 4 (H3K4me) is an intricately regulated posttranslational modification, which is broadly associated with enhancers and promoters of actively transcribed genomic loci. Recent advances in next-generation sequencing have identified a number of H3K4me regulators mutated in neurodevelopmental disorders including intellectual disabilities, autism spectrum disorders, and schizophrenia. Here, we aim to summarize the molecular function of H3K4me-regulating enzymes in brain development and function. We describe four H3K4me methyltransferases (*KMT2A*, *KMT2C*, *KMT2D*, *KMT2F*), four demethylases (*KDM1A*, *KDM5A*, *KDM5B*, *KDM5C*), and two reader proteins (*PHF21A*, *PHF8*) mutated in neurodevelopmental disorders. Understanding the role of these chromatin regulators in the development and maintenance of neural connections will advance therapeutic opportunities for prevention and treatment of these lifelong neurodevelopmental disorders.

### Keywords

autism spectrum disorders; brain development; chromatin; epigenetics; gene regulation; H3K4 methylation; histone methylation; intellectual disability; schizophrenia

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Chromatin remodeling is a dynamic process that regulates gene expression via changes in DNA accessibility. Posttranslational modifications on N-terminal tails of histone proteins are an integral element influencing chromatin structure. Advent of next-generation sequencing led to a rapid growth of the list of histone modifiers that are mutated in human neurodevelopmental disorders. These disorders include a plethora of intellectual disability (ID) syndromes [1] and schizophrenia [2]. Additionally, recent large-scale exome

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sequencing studies highlighted dysregulation of histone methylation as a major contributing factor of autism spectrum disorders (ASD) [3,4]. Therefore, regulation of histone modifications appears to be essential for the development and function of the central nervous system. However, the roles of these mutated histone modifiers in the brain are not well understood.

Methylation of lysine 4 of histone H3 (H3K4me) is one such modification, which is associated with gene activation. Three statuses of lysine methylation, namely mono-, di-, and tri-methyl groups (me1/2/3), confer an additional layer of complexity in chromatin remodeling events. Using technologies such as chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq), the genomic signatures of these epigenetic marks can be uncovered for specific cell types and developmental stages. Early genome-wide studies using cultured cell lines revealed that H3K4me1 is a hallmark of enhancers [5,6], while H3K4me2/3 mark promoters of actively transcribed genes [7,8]. Mono- and dimethylated H3K4 appear as broad ChIP-seq peaks, marking stretches of open chromatin throughout the genome [7]. In the human brain, similar to the observations in cell cultures, tri-methylated H3K4 is concentrated in sharp peaks 1–2 kb in length at transcription start sites of regulatory sequences such as proximal gene promoters [9]. These neuron-specific H3K4me3 peaks are enriched at promoters of genes that control synaptic function [9]. Additionally, unmethylated H3K4 (me0) also recruits a distinct set of proteins with transcriptional repressors [10,11], including *DNMT3L*, which tethers CpG DNA methylation enzymes [11].

Two families of proteins serve as primary regulators of H3K4me: histone lysine methyltransferases (KMTs) are the ‘writers’, which place the methyl marks onto histones; and histone lysine demethylases (KDMs) are the ‘erasers’, which remove them. An additional class of proteins recognizes and ‘reads’ H3K4me status, serving as effectors to recruit chromatin remodeling proteins and regulate transcriptional state. Remarkably, among other methylation sites on histones, H3K4 appears to be the most extensively targeted position by the largest number of writer and eraser enzymes. To date, in higher eukaryotes, at least six KMTs and six KDMs have been shown to regulate H3K4me with differential substrate preference to me0–3 [12,13]. This intricate balancing act at H3K4 by opposing mechanisms might have evolved to sculpt epigenetic landscapes that achieve delicate developmental and cellular processes in complex organs such as brain. Indeed, pioneering work has shown dynamic gain and loss of H3K4me3 throughout the genome in the neurons of developing human prefrontal cortex [9,14]. While H3K4me regulation has been unequivocally shown to be crucial for normal development in metazoans through *Hox* gene regulation [15], the function and regulation of this methylation mark in the developing nervous system is not fully understood. The dynamics of chromatin regulation in the brain is the focus of the emerging field of neuroepigenetics.

Among the 12 enzymes that target H3K4me in humans, mutations in four KMT and four KDM genes have been associated with neurodevelopmental disorders to date (Figure 1). An excellent review summarized the implications of H3K4 demethylases in neurodevelopmental disorders with a focus on regulatory mechanisms of the demethylases [16]. Shen *et al.* provided a comprehensive view on writer and eraser enzymes for H3K4me

in brain disorders from a clinical perspective, including data from human samples and animal models [17]. More recently, alterations of *KMT2F* in schizophrenia [2], *KDM1A* in Kabuki/KBG syndrome [18], and *KDM5A* and *KDM5B* in ID [1,19] have been reported, which were not covered by these earlier reviews. New exome sequencing of a large cohort of individuals with ASD also identified *KMT2C* and *KDM5B* [3,4]. It may only be a matter of time before alterations in the remaining four enzymes are identified in neurological disorders. Furthermore, genetic mutations in two reader proteins for H3K4me status, PHF21A and PHF8, have been associated with ID syndromes [20,21] (Figure 1). Here, we aim to summarize the recent progress in understanding the molecular and cellular consequences of mutations in H3K4me regulators. Although very limited reports described the function of H3K4me regulators at a genomic level in the brain, by reviewing the data obtained from non-brain tissues and cell types, we further discuss how intricate balancing and readout of H3K4me can be engaged in brain development genome-wide.

## The six H3K4 methyltransferases

Methylation of H3K4 is generated primarily by the lysine (K)-specific methyltransferase 2 (KMT2) family of enzymes [22,23]. This class of proteins is characterized by the presence of a catalytic Su(var), Enhancer of Zeste, Trithorax (SET) domain, conferring lysine-specific methyltransferase function. While each writer enzyme has been shown to generate all three methylation marks on H3K4 [13], specific substrate preference is often conferred by additional cofactor proteins. For example, while KMT2A itself can generate mono- and dimethyl marks on H3K4, physical contact with cofactors including RbBP5, Ash2L, and WDR5 allows tri-methylation by KMT2A [24].

The six KMT2 family members can be broken into three subclasses of protein pairs that have evolved distinct features and functions. *KMT2A* and *KMT2B* are homologues of *Trithorax (Trx)* gene found in lower organisms such as fly, whereas *KMT2C* and *KMT2D* are thought to be duplicated from the common ancestor gene *Trithorax-related (Trr)* in fly [23]. *KMT2F* and *KMT2G* are structurally distinct from the KMT2A-D enzymes. Similar to the *KMT2A/B* and *KMT2C/D* pairs, mammalian *KMT2F* has a cognate paralog, *KMT2G*, and both share a common ancestor gene *SET1* in budding yeast [23]. Four of these six KMTs have been found mutated in neurodevelopmental disorders thus far. Remarkably, evidence suggests that within each protein pair, one enzyme cannot compensate for a mutation in the other, indicating that each KMT in the duo has nonredundant functions.

## *KMT2A* in a developmental disorder with neurological symptoms

The human *KMT2A* gene (also known as *MLL*, *ALL-1*) was first discovered for its involvement in chromosomal translocations in acute leukemia [25,26]. As discussed above, *KMT2A* is responsible for generating mono-, di-, and tri-methylated H3K4 through its SET domain and by interaction with cofactors [24]. Recently, dominant *de novo* mutations in *KMT2A* have been identified in individuals with Wiedemann–Steiner syndrome (*Mendelian Inheritance in man* [MIM] no. 605130), a developmental disorder with clinical features including ID, microcephaly, and short stature [27,28]. The majority of identified mutations are heterozygous *de novo* mutations predicting a premature truncation of the protein product

(Figure 2), suggesting haploinsufficiency of *KMT2A* is responsible for these clinical phenotypes. Since the catalytic SET domain is located at the C-terminal end of the protein, the truncations most likely result in a loss of enzymatic activity. Given that H3K4me is a signature of actively transcribed chromatin, the pathogenic mechanism may be insufficient transcription of key genes in the central nervous system. However, several lines of evidence suggest the molecular etiology can be much more complex than this simplistic scenario.

*KMT2A* harbors both transcriptional activator and repressor activities. These two opposite functions appear to be located at the SET-containing C-terminal portion and the PHD-containing N-terminal segment, respectively [30]. A cluster of three plant homeodomain (PHD) fingers, located in the middle of the *KMT2A* molecule (PHD1–PHD3 in Figure 2), plays an important role in demarcating the two opposite activities. PHD fingers are one of the major modules that can recognize specifically modified histones, and thus can act as ‘readers’ of chromatin modifications [31,32]. Interestingly, *KMT2A* PHD3 binds to both H3K4me<sub>3</sub>, a reaction product of *KMT2A* itself, as well as the transcriptional corepressor protein Cyp33 [33]. While the role of PHD1 remains unknown, PHD2 appears to have ubiquitin ligase activity toward both histones and *KMT2A* itself, facilitating the degradation of *KMT2A* [34]. Thus, beyond the canonical function of PHD fingers as reader modules, the *KMT2A*-PHD cluster represents a novel regulatory hub, which defines the balance between transcriptional activator and repressor functions. Notably, some of the Wiedemann–Steiner syndrome mutations, including a missense mutation, fall within this PHD finger cluster (Figure 2). Therefore, these mutations can potentially lead to both down- and upregulation of *KMT2A*-target genes in Wiedemann–Steiner syndrome. Future studies can address these possibilities using cells harboring these mutations.

Additionally, alternative splicing events have been reported in the PHD3-encoding area [35]. Yeast two-hybrid experiments revealed that these PHD3 isoforms differ in their ability to interact with Cyp33-containing corepressor complex, suggesting an influence in the transcriptional regulatory activities of *KMT2A* [35]. Perhaps each transcript type has a unique expression pattern in the developing nervous system. It is plausible that different *KMT2A* isoforms function as either an activator or a repressor in a cell-type-dependent manner in neurons and glia cells within the central nervous system. Functional studies in developing brains are necessary to determine to what extent this alternative splicing event exists and how it contributes to molecular etiology *in vivo*.

Expression of *KMT2A* peaks in the neocortex in human fetal brains, declining slightly through early stages of postnatal development but then gradually increasing into adulthood [36]. This expression signature hints at the importance of *KMT2A* during neocortical development. Consistently, *KMT2A* plays an essential role in mouse postnatal neurogenesis [37]. Lim *et al.* demonstrated that important neurogenesis-promoting genes have bivalent chromatin domains carrying both activating H3K4me<sub>3</sub> and repressive H3K27me<sub>3</sub> marks, and that presence of *KMT2A* at these poised loci is necessary to promote an active transcription state [37]. Therefore, impaired neurogenesis by *KMT2A* mutations might be responsible for the cognitive deficits observed in Wiedemann–Steiner syndrome.

On a genomic scale, KMT2A has been shown to be required for generating 5% of the active H3K4me3 marks on promoters in mouse embryonic fibroblasts [38]. Relatively weak impact on global H3K4me3 landscape upon the loss of *KMT2A* suggests a redundant, if not completely redundant, function of paralog *KMT2B* and the four other KMT2 family members. Recent studies in mouse embryonic stem cells (mESCs) reconfirmed the functional redundancy between *KMT2A* and *KMT2B* in generating global H3K4me3, whereas removal of *KMT2B* alone lead to a 2.5-fold reduction in H3K4me levels at bivalent promoters [39]. Reconciling these findings with previous evidence of *KMT2A* at bivalent promoters in neural precursor cells [37], it is plausible that KMT2A and KMT2B are responsible for generating neural precursor- and mESC-specific bivalent promoters, respectively. Heterozygous *de novo* mutations in Wiedemann–Steiner syndrome indicate that alteration of H3K4me levels at only limited genomic loci is sufficient to attenuate proper brain development. Further studies to identify direct gene targets of KMT2A during central nervous system development will be imperative in elucidating a functional role for this writer enzyme in the brain.

### ***KMT2C*: a new gene for Kleefstra syndrome & ASD**

*KMT2C* (also known as *MLL3*, *HALR*) contributes to the implementation of mono- and dimethylation marks on H3K4 in mammalian cells [40,41]. *KMT2C* was originally discovered as a human homologue of *Drosophila* gene *Trr* in the chromosomal region lost in individuals with developmental defects of the fore-brain and myeloid leukemia [42,43]. In recent years, *KMT2C* variants have been found in two neurodevelopmental disorders. Kleefstra *et al.* identified a *KMT2C* mutation in an individual with Kleefstra syndrome (MIM no. 610253), a neurodevelopmental spectrum disorder with hallmarks including severe ID, brachy(micro)cephaly, and epileptic seizures. The female individual carries a heterozygous *de novo* nonsense mutation, p.Arg1481X [44] (Figure 2), resulting in loss of more than half of the KMT2C protein. This truncation of the C-terminal segment includes loss of the enzymatic SET domain, which likely results in the loss of histone methyltransferase activity. Additionally, five mutations were identified in recent large-scale exome sequencing of individuals with ASD (Figure 2) [3,4]. These new variants will require further studies to examine their consequences on protein function and pathogenic contribution to neurodevelopment. Clearly, disruption of *KMT2C* and its contribution to a delicate epigenetic regulatory network may underlie broad neurodevelopmental abnormalities including cognitive and social anomalies.

While neuron-specific targets of KMT2C regulation have yet to be discovered, it is known that this writer is highly expressed in the human brain [42,45]. *KMT2C* expression levels peak during human fetal development and remain steady into adulthood [36]. Notably, expression in the adult brain is stronger in the hippocampus, caudate nucleus, and substantia nigra, regions of the brain associated with learning, memory, and social behaviors [45]. These data implicate roles for *KMT2C* in the developing brain as well as in mature neuronal circuitries.

Genome-wide functional studies found that KMT2C contributes to installation of mono- and dimethylation of H3K4 at gene enhancers [40,41]. H3K4me1/2 at enhancers is required for

full activation of genes. Interestingly, KMT2C can also be found at gene promoters, where promoter-bound KMT2C functions to repress gene transcription [46]. These studies raise a possibility that KMT2C can both activate and repress gene transcription depending on localization along chromatin. Studying the chromatin regulatory network involving KMT2C will lead to a better understanding of the tight gene regulation in brain development, and can pave the way toward targeted therapeutic approaches.

### **KMT2D mutations are a major cause of Kabuki syndrome**

The human histone lysine methyltransferase *KMT2D* (also known as *ALR*, *MLL2* or *MLL4*) is partially functionally redundant to *KMT2C*, and the two enzymes are responsible for a majority of the H3K4me1/2 marks in mammalian cells [40,41]. Exome sequencing revealed *KMT2D* to be a major cause of Kabuki syndrome (*MIM* no. 147920) [47], an autosomal-dominant congenital ID disorder characterized by unique facial features. Over 100 unique mutations in *KMT2D* have been identified in Kabuki syndrome individuals, a majority of which result in premature termination of the protein product [29,47–54] (Figure 2). Though most mutations occur *de novo*, many are recurrent in unrelated individuals, indicating the importance of these repeatedly mutated amino acids in KMT2D function and/or suggesting the nature of the DNA is particularly mutable.

The large number of mutations appears to cluster into three distinct locations along the protein (Figure 2). One mutation group surrounds a triple PHD finger cluster (PHD4–PHD6 in Figure 2). These three PHD fingers bind unmethylated arginine 3 on histone H4 (H4R3me0), thereby promoting the access of the SET domain to its substrate, namely the histone H3 N-terminus [55]. This trans-tail crosstalk enables KMT2D to efficiently methylate H3K4 in a nucleosomal context [55]. Importantly, two missense mutations in Kabuki syndrome, p.Cys1430Arg and p.Cys1471Tyr in the PHD4–6 cluster, showed decreased binding affinity to H4 and reduced H3K4 methylation activity, when nucleosomes were used for the methyltransferase assays [55]. Thus, Kabuki syndrome mutations in this cluster ultimately result in loss-of-function of KMT2D methyltransferase activity. The second mutation cluster occurs in an area of the protein lacking a characterized functional domain, yet this region may be significant for the 3D structure and function of the enzyme. The third mutation group occurs around the C-terminal PHD finger, whose function remains unknown. Similar to *KMT2A* and *KMT2C* mutations, over 30 variants in *KMT2D* lead to truncation of C-terminal SET catalytic domain, likely resulting in the loss of enzymatic function.

A role for *KMT2D* in neuronal differentiation has been suggested from recent *in vitro* studies. Using human pluripotent NT2/D1 carcinoma cells, which commit to a neuronal state upon retinoic acid treatment, Dhar *et al.* showed that shRNA knockdown of *KMT2D* results in attenuation of morphological changes and impaired activation of neural differentiation hallmark genes including *HOXA1–3* and *NES-TIN* [55]. The insufficient expression of these *KMT2D* target genes was associated with decreased H3K4me levels. Thus, loss-of-function mutations in *KMT2D* in Kabuki syndrome likely result in a decrease in these methyl marks necessary to promote expression of key neuronal differentiation genes, and ultimately hinder commitment of stem cells to a neuronal state. These data suggest a critical role for *KMT2D*



in neuronal differentiation. Whether this writer enzyme also contributes to maintenance of neuronal function later in life remains to be seen.

Several lines of evidence suggest a cooperation of KMT2C and KMT2D in neuronal differentiation. First, knockdown of *KMT2C* in the NT2/D1 system resulted in downregulation of KMT2D-target genes, although to a lesser extent compared with *KMT2D* knockdown [55]. Second, KMT2C and KMT2D can be copurified in the same protein complex [56]. Third, both KMT2C and KMT2D are found at gene enhancers and are required for enhancer activation [40,41]. These results raise a possibility that deficiency in common molecular pathways, including enhancer activation of specific genes, impinge on Kabuki and Kleefstra syndromes, though they were originally described as distinct conditions.

### ***KMT2F*: a new schizophrenia susceptibility gene**

*KMT2F* (also known as *SET1*, *SETD1A*) encodes an H3K4 writer enzyme capable of generating mono-, di-, and tri-methylation marks *in vitro* [41,57]. Notably, in cells, KMT2F/G and Set1 appear to be responsible for bulk H3K4me3 [58–61].

Recently, Takata *et al.* have identified *KMT2F* as a risk gene for schizophrenia (*MIM* no. 181500), a common and severe psychiatric disorder characterized by delusions, hallucinations, and disorganized thinking [2]. It is significant that two loss-of-function mutations (Figure 2) were found in two phenotypically similar but unrelated schizophrenia individuals with a secondary diagnosis of obsessive-compulsive disorder (OCD) (*MIM* no. 164230). It is possible that individuals with *KMT2F* mutations define a new subset of schizophrenia with OCD comorbidity [2]. The first mutation is a *de novo* frameshift resulting in the introduction of a premature stop codon and likely leads to the loss of enzymatic activity carried in the C-terminal SET domain. The second individual harbors a *de novo* indel that alters a canonical splice acceptor site. This change is expected to cause a loss of exon 16 and disruption of the N-SET domain, which is critical for proper H3K4 methyltransferase function of the neighboring SET domain *in vitro* [62].

As previously discussed, KMT2F contributes to deposition of the activating H3K4me3 mark, which shows sharp peaks near transcription start sites of active genes [7]. While H3K4me1 is enriched at gene enhancers, H3K4me3 levels at enhancers appear to be low [5,6]. Hence, KMT2F acts as an H3K4me3 writer enzyme at promoters, in sharp contrast to KMT2C/D function at enhancers. Indeed, KMT2F-interacting protein Cfp1 recruits H3K4me3 activity, likely mediated by KMT2F/G, at CpG islands which are prevalent in mammalian gene promoters [63]. Recent studies found that loss of Cfp1 in mESCs leads to global loss of DNA-damage-induced accumulation of H3K4me3 at the promoters of DNA-damage-responsive genes [64]. However, induction of genes was mildly affected in Cfp1-mutant mESCs except for few dramatically affected genes, suggesting a role for KMT2F in context-dependent transcriptional activity [64]. H3K4me3 plays a crucial part in recruiting the general transcription machinery, TFIID, through an interaction between H3K4me3 and the PHD finger of TAF3, a TFIID component [65,66] Thus, the KMT2F mutations may lead

to insufficient gene transcription of important neural circuitry genes via impaired placement of H3K4me3 at their promoters.

Psychiatric disorders are often characterized by disorganization of neuronal networks, where alteration of these networks from an early time point leads to lifelong disorders. The inability to activate the correct genes at the correct time during brain development due to a loss of KMT2F function could lead to improper establishment of neuronal networks and, ultimately, impaired brain function manifested by schizophrenia. However, brain-specific roles of KMT2F or specific genomic loci that are targets of KMT2F in brain cells have yet to be defined. The aforementioned genetic studies open an avenue for interrogating chromatin regulatory mechanisms that underlie pathogenesis of psychiatric disorders.

## Mutations in H3K4 demethylases

Histone methylation was thought to be an irreversible epigenetic mark, thereby serving as memory of cellular identities. The identification and characterization of histone demethylases revealed that methylation is not a permanent event but rather a dynamic process. Four H3K4me eraser enzymes, KDM1A, KDM5A, KDM5B, and KDM5C, are mutated in neurodevelopmental disorders, evidence that the balance between methylation and demethylation is intricately regulated during brain development and function.

### ***KDM1A* in a unique neurodevelopmental syndrome**

*KDM1A* (also known as *LSD1*, *BHC110*) was the first histone lysine demethylase gene to be identified [67]. A flavin-containing amine oxidase homologue, *KDM1A* catalyzes the oxidative demethylation of H3K4me1/2. Recently, a mutation in *KDM1A* has been identified in a new neurodevelopmental disorder. Tunovic *et al.* describe two *de novo* variations in one male with mixed features of Kabuki syndrome and KBG syndrome [18]. As discussed above, Kabuki syndrome is a developmental and cognitive disorder frequently associated with mutations in other histone lysine regulators, *KMT2D* [47] or *KDM6A* [29]. KBG syndrome (MIM no. 148050) is characterized by macrodontia, craniofacial dysmorphism, and delay in brain development [68]. One identified variation was p.Tyr785His in the C-terminal amine oxidase domain of *KDM1A*. The other alteration was a deletion of three nucleotides in *ANKRD11*, which lead to the loss of an evolutionally conserved lysine residue. Haploinsufficiency of the *ANKRD11* gene, which encodes an ankyrin-repeat containing a transcription coactivator, was previously identified as a causative agent for KBG syndrome [68]. Based on the new mixed features of Kabuki and KBG syndromes found in the individual, the authors postulated that the *KDM1A* mutation might be responsible for the Kabuki-related symptoms such as ptosis and downturned mouth corners.

How could mutations in counteracting enzymes over H3K4me, namely *KMT2D* and *KDM1A*, both result in Kabuki-related syndromes? The majority of *KMT2D* mutations appear to result in loss of enzymatic activity by truncation of the catalytic SET domain [47]. One possibility is that the identified p.Tyr785His variant may be a gain-of-function mutation. In this scenario, both loss-of-function mutations in a writer and gain-of-function mutations in an eraser would lead to decreased H3K4me levels. Alternatively, developmental change in expression of key genes might require temporal coordination of



KMT2D and KDM1A at specific time points. Deletion of *Kdm1a* in mouse confers embryonic lethality [69], as *Kdm1a* is required for gastrulation and differentiation of embryonic stem cells [70,71]. Thus, if the *KDM1A* mutation is a loss-of-function one, it is likely hypomorphic. Further studies including identification of additional mutations in *KDM1A* are needed to understand this interesting observation from human genetics studies.

While only one *KDM1A* mutation has been described in a human developmental disorder thus far, studies have demonstrated pivotal roles of this eraser in neurodevelopment. *KDM1A* was originally discovered as a key subunit of the CoREST complex, which is responsible for the repression of neuronal genes such as Synapsin I (*SYN1*) in non-neuronal cells [72,73,74]. The CoREST complex is recruited to neuron-specific genes via an interaction with REST, a sequence-specific DNA binding factor [73]. Thus, it is possible that the loss of *KDM1A* function causes aberrant expression of neuron-specific genes in neural precursor and/or glia cells, thereby eventually perturbing proper brain development. Consistently, leaky expression of neuron-specific genes in HeLa cells was observed upon *KDM1A*-knockdown [67].

In addition to its involvement in neuronal gene repression, cell-autonomous functions of *KDM1A* in neurons have also been described. Neuron-specific isoforms of *KDM1A* have been shown to modulate neurite morphogenesis in zebrafish [75]. The first functional study of *KDM1A* in the mammalian nervous system demonstrated that the presence of alternatively-spliced exon E8a dynamically regulates neurite maturation. Loss of *KDM1A* upon shRNA knockdown resulted in decreased dendritic arborizations, secondary branches, and average neurite width, while overexpression lead to an increase in these morphological features in mouse cortical neurons [75]. Inclusion of exon E8a generates a small protruding loop near the catalytic site, which may provide a site for posttranslational modifications and thus confer an additional regulatory element. Taken together, current knowledge supports the idea of a significant role for *KDM1A* in neurodevelopment.

On a genomic level, ChIP studies on individual genes show that *KDM1A* localizes to active promoters in HeLa and MCF7 cells, suggesting the enzyme is a locus-specific demethylase [67,76]. Additionally, a ChIP-seq study has found that removal of *KDM1A* from enhancers of pluripotency genes influences commitment of ES cells to differentiate [71]. Thus, *KDM1A* may regulate developmental expression of neuronal genes by controlling methylation of promoters and enhancers. What factors recruit *KDM1A* in specific regulatory regions in neurons remains unanswered.

## **KDM5 family: a second class of demethylases**

The finding that *KDM1A* is unable to demethylate tri-methylated H3K4 [67] raised the possibility that additional demethylases had yet to be discovered. This led to the identification and characterization of a second class of H3K4 demethylases, the lysine (K)-specific demethylase 5 (KDM5) family of proteins (formerly JARID1 family), comprising of KDM5A, KDM5B, KDM5C, and KDM5D [77,78,79,80,81,82]. The enzymatic activity of these erasers lies in two Jumonji domains, JmjN and JmjC. This demethylation signature requires Fe(II) and  $\alpha$ -ketoglutarate as cofactors to perform the hydroxylation reaction to

remove methylation [83]. Notably, to date three of the four KDM5 family members, KDM5A, KDM5B, and KDM5C, are reported mutated in neurodevelopmental disorders, revealing nonredundant functions of this class of enzymes in the brain.

### **KDM5A: a candidate ID gene**

*KDM5A* (also known as *RBP2*, *JARID1A*) was first identified as a binding factor of retinoblastoma RB gene product [84]. Further studies revealed it to be an H3K4me3-specific demethylase from the JmjC-domain-containing family [77,80,81,82]. A mutation in *KDM5A* was recently identified in an individual with autosomal recessive ID (ARID) [1]. The homozygous *de novo* missense mutation causes a p.Arg719Gly change, substituting a nonpolar amino acid for a positively charged one. The mutation was predicted to be damaging to *KDM5A* function [1], as it is located within a zinc-finger motif likely responsible for DNA-binding and possibly contributes to enzymatic activity [85] (Figure 2). It is unknown whether this single amino acid substitution would affect overall stability, DNA-binding property, and/or enzymatic activity of *KDM5A*. Functional characterization of this mis-sense mutation is necessary to determine its effects on protein function and contribution to ID.

Little work has been done experimentally to investigate the neuron-specific functions of *KDM5A*; however, a role for this demethylase has been described in early embryonic development [86], cellular differentiation [87], senescence [88], and circadian gene regulation [89]. Genome-wide analysis in mammalian cell lines revealed *KDM5A* is bound at proximal promoter regions, about half of which are H3K4me3-positive, suggesting an inhibitory role for *KDM5A* on many genes via direct action on promoters [86]. *KDM5A* mRNA levels are high during human prenatal brain development and plateau postnatally, with cortical expression levels remaining slightly elevated throughout adulthood [35]. Considering the homozygous mutation in ARID, and the expression data showing *KDM5A* peaks at a very specific prenatal window, it is plausible that *KDM5A* serves as an ‘off’ switch for a subset of genes at significant developmental time points.

### **KDM5B mutations in ID & ASD**

*KDM5B* (also known as *PLU1*, *JARID1B*) encodes an H3K4-specific eraser enzyme that directly catalyzes the demethylation of mono-, di-, and tri-methylated H3K4 [77,78,79]. Until recently, all variants in *KDM5B* had been described in cancer. Next-generation sequencing has since revealed a *de novo* splicing mutation (c.283A>G) in *KDM5B* in an individual with nonsyndromic ID [19] (Figure 2). Six additional variants were newly identified in exome sequencing of a large cohort of individuals with ASD [3,4]. These mutations include missense, nonsense, and frameshift variants found all along the protein, many of which lie in key functional domains (Figure 2). Further studies are necessary to assess the functional effects of these mutations and the pathogenicity of mutated *KDM5B* in ID and ASD.

*KDM5B* has been shown to regulate cell fate decisions [90] in mESCs to neuronal lineage [91]. Knockdown of *KDM5B* in mESCs resulted in failure to differentiate to neurons, whereas knockout in mouse neural stem cells (mNSCs) revealed that loss of *KDM5B* at this

later stage does not affect differentiation to mature neurons [91,92]. Knockout of *KDM5B* in mouse is embryonic lethal [93], and, remarkably, overexpression also leads to impaired neural differentiation [90]. This suggests tight control of this demethylase is critical for cell fate determination. Loss of *KDM5B* may result in activation of genes that drive proliferation, while over-expression of *KDM5B* could lead to aberrant demethylation of differentiation genes in uncommitted cells to hinder neural differentiation. Therefore, either loss- or gain-of-function mutations could lead to inefficient neuronal differentiation, which can result in cognitive deficiencies. Identification of additional human mutations in neurological disorders would advance our knowledge about the significance of precise *KDM5B* regulation in neural differentiation.

How *KDM5B* regulates neuronal differentiation has become less of a mystery through recent genome-wide studies. ChIP-seq in mESCs shows *KDM5B* bound to transcription start sites of genes encoding developmental regulators [91]. *KDM5B* can be recruited to target genomic loci via association with transcription factors such as androgen receptor [79], and act as a transcriptional repressor by stopping H3K4me spreading at gene promoters [78,92,94]. Loss of *KDM5B* in mESCs results in a global increase in H3K4me3 levels and a failure to silence germ cell genes [91], suggesting that *KDM5B* is a major H3K4me3 eraser in stem cells. Additionally, *KDM5B* depletion leads to H3K4me spreading into promoters and gene bodies, revealing a role for *KDM5B* in restricting H3K4me to specific genomic areas [92,95]. Which specific genes' misregulation affects neural differentiation will be a focus of future studies.

Additionally, *KDM5B* has been suggested to regulate genomic stability by dictating the cell's response to DNA damage [96]. This critical function in DNA repair is likely a fundamental aspect of the pathogenicity of *KDM5B* mutations in cancer, however if and how this function could contribute to neurological disorders remains open to investigation.

### ***KDM5C* mutations are frequent in X-linked ID**

In 2005, the first mutations in *KDM5C* (also known as *SMCX*, *JARID1C*) were identified as a frequent cause of X-linked ID (XLID) (Figure 2) [97]. Currently, mutations in *KDM5C* are estimated to account for roughly 0.7–2.8% of all XLID [97,98,99]. The function of this gene's product remained unknown until in 2007 *KDM5C* was discovered as a specific demethylase for di- and tri-methylated H3K4 [77,100]. To date, 17 mutations have been identified in individuals with XLID, a majority of which are missense or nonsense variants (Figure 2). Of the mutations characterized thus far, functional studies indicate these human variants cause a decrease in *KDM5C* enzymatic activity, suggesting a loss-of-function pathogenic mechanism [77,100]. In addition to cognitive deficits, aggressive behaviors are frequently observed in affected individuals [97,101] and autistic features have been noted in one case [102]. Along with developmental abnormalities in males, *KDM5C* variants have also been found in females with short-term memory deficits [103].

*KDM5C* was originally identified as a gene that escapes X-inactivation [104,105], and both *KDM5C* and Y-chromosome paralog *KDM5D* are expressed in a sex-specific manner in mouse brain [106]. These findings suggest a nonredundant function for *KDM5C* and

KDM5D, and that dosage differences between males and females may underlie differential consequence of KDM5C loss between the sexes. Thus, KDM5C appears to be essential for development of a broad spectrum of cognitive and adaptive functions both in males and females.

*KDM5C* is broadly expressed, with higher levels in human brain and skeletal muscle [97]. Expression levels of *KDM5C* remain relatively unchanged throughout human brain development, from prenatal to adult stages, suggesting a lifelong critical role of this demethylase [35]. Within the mouse brain, *Kdm5c* is expressed in areas important for cognitive and emotional behaviors such as the prefrontal cortex, hippocampus, and amygdala [106,107,108]. Additionally, this eraser enzyme has been shown to affect dendritic outgrowth in rat cerebellar granule neurons and neuronal survival in developing zebrafish [77]. Together, these data may help reveal the pathogenic mechanism for mutations in *KDM5C* leading to neurodevelopmental defects.

Genetic and biochemical interactions of KDM5C with other molecules have provided insights into molecular mechanisms of ID. *KDM5C* is directly regulated by *ARX*, a homeobox gene frequently mutated in XLID and epilepsy [109]. A majority of the *ARX* variants cause a hypomorphic *ARX* allele, leading to a decrease in *KDM5C* expression, thus possibly altering the regulation of H3K4me. *KDM5C* also physically interacts with the transcriptional repressor REST [100]. As discussed above, REST represses neuronal genes in non-neuronal cells. Loss of KDM5C was shown to impair REST-mediated repression of neuronal genes, such as *SCN2A* and *SYN1* [100]. Defective repression of REST-target genes in KDM5C may be a non-cell autonomous mechanism of impaired cognitive development. XLID mutations in *KDM5C* may cause dysregulation of REST-target genes due to the impaired H3K4me demethylase activity. Further studies will be necessary to understand functions of KDM5C in different cell types in the brain.

Interestingly, KDM5C appears to have dual function depending on its localization along chromatin in mESCs, namely transcription corepressor activity at promoters and transcriptional coactivator activity at enhancers [110]. Thus, it is possible that KDM5C serves as not only a repressor of REST-target genes but also a coactivator for yet unidentified genes in the brain. Jensen *et al.* recently identified a dozen genes that are commonly dysregulated in multiple lymphocyte lines derived from individuals with *KDM5C* mutations [111]. However, it is unclear, if gene expression changes in lymphocytes can be translated to the cognitive deficits in individuals with *KDM5C* mutations. Identifying the KDM5C-target genes and mechanisms of their regulation in a genomic context, especially in relevant cell types in the brain, will greatly advance our understanding of molecular mechanisms underpinning this frequent form of ID and ultimately provide hope for therapeutic interventions.

## Recognition of H3K4me by reader molecules

Histone methylation itself does not seem to have an impact on higher-order chromatin structure. In addition to writer and eraser enzymes, reader molecules act at H3K4 by recognizing specific methylation states and recruiting effector proteins to influence

transcription. Here, we discuss two H3K4me-specific reader proteins, PHF21A and PHF8, which have been found mutated in intellectual disabilities. Both genes encode for proteins that belong to the PHD finger (PHF) family.

### ***PHF21A*: A methyl ‘zero’ reader in ID**

PHF21A (also known as BHC80) was the first reader molecule discovered to recognize unmethylated H3K4 (HeK4me0) [10], introducing unmodified H3K4 as an important addition to the histone code in regulating chromatin state. Recognition of unmodified histone H3 tail by PHF21A PHD finger is specifically inhibited by H3K4 methylations [10]. Recently, three unrelated individuals with ID and craniofacial anomalies have been identified harboring *de novo* balanced translocations disrupting *PHF21A* [20]. Two of these translocations, t(11;19)(p11.2;p13.2)dn and t(1;11)(p13;p11) dn, are predicted to lead to truncated protein products resulting in loss of the PHD finger (Figure 2) [20]. The translocation breakpoint of the third, t(X;11)(p22.2;p11.2)dn, has not been characterized, yet it is likely that loss of protein function is also the pathogenic mechanism at play [20]. Therefore, H3K4me0 recognition by PHF21A PHD finger appears to be essential for cognitive and craniofacial development. Notably, Potocki–Shaffer syndrome (MIM no. 601224) is a contiguous gene deletion syndrome of the chromosomal region where *PHF21A* is located, namely chromosome 11p11.2 [112,113]. Potocki–Shaffer syndrome has hallmark clinical features including multiple exostoses, parietal foamina, ID, and craniofacial anomalies. While *EXT2* and *ALX4* have been identified to be responsible for multiple exostoses and parietal foamina, respectively, it remained unknown which gene(s) deletion leads to ID and craniofacial anomalies. The discovery of heterozygous translocations and resulting truncations of *PHF21A* strongly suggests that haploinsufficiency of *PHF21A* is responsible for ID and craniofacial deficits in Potocki–Shaffer syndrome [20].

Transcript levels of *PHF21A* are high during early stages of human embryonic development, and remain steady throughout development into adulthood [35]. Accordingly, *in situ* hybridization and immunofluorescence experiments in mouse show *Phf21a* is expressed highly in both the central nervous system as well as in cranial bones of embryonic and adult mice [20,114], suggesting an important role for this reader protein in both development and function of nervous tissue and craniofacial bones.

To test whether a loss of *PHF21A* results in defects in neuronal and craniofacial development, Kim *et al.* studied the effects of *phf21a* knockdown by morpholino injection in zebrafish [20]. Injection of morpholino resulted in pronounced deficiencies in head development including facial dysmorphism. These abnormalities were rescued upon injection of wild-type human *PHF21A* mRNA, supporting the specificity of knockdown effect. Interestingly, *Phf21a* knockout mice show neonatal lethality with an insufficient ability to suckle [115]. *Phf21a*-null mice were not closely examined for craniofacial defects; therefore, it is also plausible that failure to suckle was a result of craniofacial anomalies, as suggested by the zebrafish studies [20]. In addition, *PHF21A* appears to be involved in pituitary function through modulation of neurosecretion, based on *in vitro* studies using mammalian cells [116]. Taken together, these functional studies in model organisms and cells support a critical role for *PHF21A* in neuro- and craniofacial development and

function. It would be informative to generate conditional knockout mice in order to assess the effects of loss of *Phf21a* specifically in neurons or cranial bones.

PHF21A is a key subunit of the CoREST complex [117], as is demethylase KDM1A, discussed above. This protein complex functions to mediate repression of neuron-specific genes [117], and thus it may not be surprising that loss of *PHF21A* leads to neurodevelopmental phenotypes. Significantly, RNAi knockdown of *PHF21A* in HeLa cells results in a derepression of neuron-specific KDM1A-target genes including *SCN1A* and *SYN1*, suggesting PHF21A occupancy at unmethylated H3K4 is required for sufficient gene repression [10,118]. A neuron-specific CoREST/KDM1A target gene, *SCN3A*, showed increased mRNA expression in the lymphoblastoid cell lines from individuals with the translocations mentioned above [20]. Additionally, ChIP results indicated decreased occupancy of KDM1A at the promoter of *SCN3A* in the PHF21A-translocation cell lines [20]. Together these findings demonstrate that PHF21A is necessary for KDM1A recruitment and the generation of a repressive chromatin state. It remains unknown what aspects of neurodevelopment are perturbed by PHF21A deficiency and whether the role of PHF21A as a neuronal gene repressor contributes to pathogenesis of ID and craniofacial abnormality.

### ***PHF8*, another XLID-associated demethylase, reads H3K4me3**

Mutations in X-linked gene *PHF8* were thought to be responsible for Siderius XLID (MIM no. 300263), a syndromic form of ID often associated with facial anomalies such as cleft palate [21,119,120,121,122]. In 2010, PHF8 was discovered to be a histone demethylase that mainly targets H3K9me1/2 and H4K20me1 [123,124,125,126,127,128]. Further studies identified that PHF8 contains a PHD finger that specifically recognizes H3K4me3 [125,126,127,129]. Thus, PHF8 represents an intriguing example of histone methylation crosstalk engaged in brain development.

All three human *PHF8* mutations in XLID are predicted to truncate the entire or large portion of the JmjC domain, which is responsible for demethylase activity (Figure 2). Therefore, loss of histone demethylase function likely underlies pathogenesis of Siderius XLID. Indeed, one missense mutation, p.Phe279Ser, which is located within the JmjC domain (Figure 2), leads to dramatic decrease in the histone demethylase activity [124,125]. Although any mutation in the PHF8 PHD finger has not yet been clinically observed, the H3K4me3 recognition by this PHD finger appears to be significant in fulfilling PHF8 function. The presence of H3K4me3 substantially enhances enzymatic activity of PHF8 via increasing affinity between the histone H3 tail and PHF8 [129]. Thus, this PHD finger domain is critical for optimal demethylase activity of PHF8. Future human genetic studies may identify loss-of-function mutations in this PHD finger.

Significantly, neuronal functions of *PHF8* have been shown in model systems. Qi *et al.* demonstrated that *PHF8* is essential for brain and craniofacial development, as well as neuronal survival in zebrafish [123]. PHF8 has also been shown to regulate neuronal differentiation in mouse carcinoma P19 cells, which differentiate into neurons after exposure to retinoic acid. Additionally, knockdown of *PHF8* in primary mouse cortical neurons



results in downregulation of cytoskeleton genes and deficient neurite outgrowth [130]. *PHF8* expression levels sharply decline during early stages of human embryonic development, and plateau at this reduced level throughout development into adulthood [35]. Together, these studies support a role for PHF8 in either proliferation of neural progenitor cells or gene expression changes during early differentiation stages in embryonic development.

Genome-wide, PHF8 has been found at the transcription start sites of more than 7000 RefSeq genes, as well as in gene bodies and intergenic regions [123,127], indicating participation of PHF8 in broad epigenome regulation. PHF8 binding sites consist of approximately one-half of total H3K4me3 peaks across the genome. The PHF8 PHD finger, whose reader function permits recognition of H3K4me2/3, likely contributes to recruitment of this reader protein to gene promoters. Additionally, PHF8 interacts with ZNF711, which is also mutated in XLID, and together the two activate reporter gene expression synergistically [127]. Since ZNF711 contains multiple C2H2 zinc-finger domains, ZNF711 may also participate in recruiting PHF8 onto target loci cooperatively with the H3K4me3/PHD interaction. Qi *et al.* showed PHF8 to be a positive regulator of gene expression, dependent on its H3K4me3-binding PHD and catalytic domains [123]. The function of PHF8 as a transcriptional activator is consistent with its enzymatic activity, which removes repressive histone methylation marks H3K9me1/2 and H4K20me1. Direct PHF8 target genes are involved in various signaling pathways including retinoic acid and Notch, both of which play pivotal roles in neural and craniofacial development [123,131,132].

Interestingly, KDM5C, an XLID-associated H3K4 demethylase discussed above, appears to be a PHF8 target gene, and consequently is downregulated upon the loss of PHF8. Moreover, PHF8 is localized at nucleoli and required for full activation of rRNA genes [126]. Given the high demand of protein synthesis in rapidly dividing stem cells, it is plausible that insufficient ribosome biogenesis by PHF8 deficiency may underlie impaired maintenance of developing brain cells observed in zebrafish [123]. These findings highlight a role for a novel network of chromatin regulators controlling expression of critical neurodevelopmental genes across the genome.

## Conclusion & future perspective

The field of neuroepigenetics is rapidly changing as we continue to define roles for chromatin-regulating proteins in the central nervous system. As the cost of DNA sequencing declines, the number of mutations discovered in neurodevelopmental disorders is dramatically increasing. We believe it is only a matter of time before many more chromatin regulators are added to the growing list of proteins implicated in neurological disorders. Functional studies of the molecular consequences of mutations, alongside thorough investigations of cellular and behavioral abnormalities in animal models of these neurodevelopmental disorders, are essential to uncovering the pathophysiology of brain dysfunction.

We mainly discussed consequences of human mutations in the context of enzymatic activity and protein–protein interactions. It is noteworthy that both writer and eraser enzymes have recently been shown to physically interact with long noncoding RNAs (lncRNAs) [133,134]. lncRNAs appear to play roles in recruitment of associated chromatin modifiers to specific genomic loci [133,134] and stability of protein complexes [135]. Thus, an important direction is to test if mutations affect physical interaction of H3K4me modifiers to lncRNAs. At the epigenomic level, pioneering comparative studies of human and primate brain samples have demonstrated that human-specific H3K4me<sub>3</sub> exist in the neurons of the cerebral cortex [136,137]. Given that neurodevelopmental disorders affect higher-order cognitive and adaptive functions, it will be highly informative to test if such human-specific H3K4me landscapes are altered by the mutations.

As summarized in earlier work [17], animal models for neurodevelopmental disorders associated with H3K4me dysregulation will be invaluable tools to dissect molecular and cellular mechanisms, as well as for drug development. Thus far, knockout mouse lines of two H3K4me enzymes, *Kmt2a* [138] and *Kmt2b* [139], have been reported to exhibit deficits in learning and memory. Harnessing the contemporary mouse genetics approach, one of the important questions that should be addressed is when the cognitive and adaptive deficits originate. One possibility is that a given chromatin modifier may play roles both in brain development and plasticity of mature circuitry. In such cases, therapeutic intervention would still hold a hope to ameliorate cognitive deficits even after affected individuals reach adulthood.

Great strides are being made with small-molecule inhibitors of writers and erasers in order to modulate H3K4me status [140,141,142]. While these strategies are currently being targeted for cancers, a therapeutic potential for these drugs in brain disorders is evident. Clearly, understanding the intricate nature of H3K4me dynamics, as well as other chromatin modifications, in the developing brain will form the groundwork necessary for targeted therapeutics and prevention for these neurodevelopmental disorders in the future.

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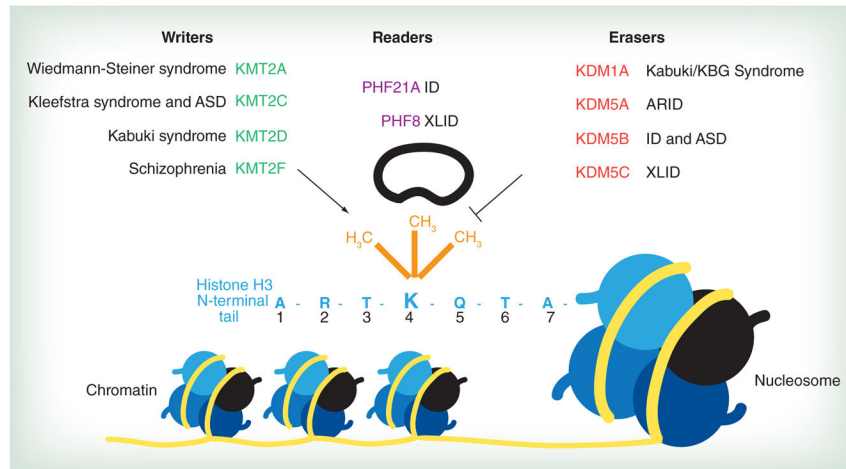
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### Executive summary

- Histone H3 lysine 4 methylation (H3K4me) is one of the most well-characterized chromatin modifications, present at enhancers and promoters of actively transcribed genes throughout the genome.
- Mutations in H3K4me regulators have recently been identified in neurodevelopmental disorders, including intellectual disability, autism spectrum disorder, and schizophrenia. These disorders lead to lifelong cognitive, emotional, and social disability, which entail a significant personal and healthcare burden worldwide.
- Here, we summarize recent progress in identification of mutations and functional investigations of H3K4me regulators in the context of neurodevelopmental disorders.
- Identification of new mutations in neurodevelopmental disorders and determining their consequences at a molecular level will allow us to decipher intricate chromatin signaling that underpins human brain development as well as basic mechanisms of gene regulation.
- Insight into the role of these H3K4me regulators in brain development and function will greatly advance therapeutic opportunities for these lifelong neurodevelopmental disorders.

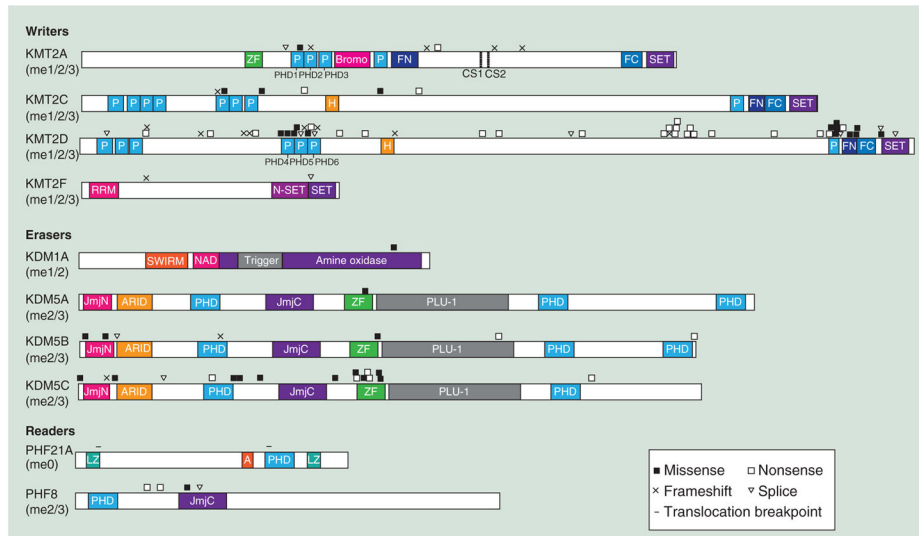


**Figure 1. H3K4me writer, eraser and reader genes in neurodevelopmental disorders**

The first seven amino acids of the N-terminal tail of histone H3 (light blue) are extending from a representative nucleosome. Lysine (K) 4 can be differentially methylated (orange) by the writer and eraser proteins shown above. Methyltransferases (green) ‘write’ methyl marks, whereas demethylases (red) ‘erase’ methyl marks. Reader enzymes (purple) recognize and ‘read’ methylation status. Mutations in each of these enzymes lead to neurodevelopmental disorders, indicated in black next to each gene name.

ARID: Autosomal recessive intellectual disability; ASD: Autism spectrum disorder; ID: Intellectual disability; XLID: X-linked ID.

For color images please see online at: [www.futuremedicine.com/doi/full/10.2217/EPI.15.1](http://www.futuremedicine.com/doi/full/10.2217/EPI.15.1)



**Figure 2. Domain organization of H3K4me regulators mutated in neurodevelopmental disorders**

Mutation types are summarized in inset. Colored blocks correspond to functional protein domains found within each gene. Substrate preference is indicated below each gene name. KMT2D mutations are limited to a subset based on the latest publication [29]. Scale is provided for each category of enzymes.

A: AT-hook; CS: Cleavage site; FC: FY-rich C-terminal domain; FN: FY-rich N-terminal domain; H: High mobility group (HMG) box; LZ: Leucine zipper; P: Plant homeodomain (PHD) finger; RRM: RNA recognition motif; SET: Su(var)3–9 Enhancer-of-zeste Trithorax methyltransferase domain; ZF: Zinc finger.