Epigenetic Regulation of the Cerebellum

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Abstract

Epigenetic regulators play fundamental roles in the control of gene transcription. Recent studies have uncovered key functions and underlying mechanisms for diverse epigenetic regulators in the development and function of the mammalian cerebellum. As powerful drivers of gene expression, epigenetic proteins recognize and alter chromatin including genomic DNA and the tightly bound histone proteins. Changes in chromatin structure reshape the local genome environment to control access of the transcriptional machinery to genes. Chromatin enzymes are highly expressed in neural precursors and postmitotic neurons in the developing cerebellum. Genetic studies have uncovered novel roles for epigenetic

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regulators in distinct phases of cerebellar circuit assembly as well as cerebellar-dependent behavior. Moreover, studies of epigenetics in the cerebellum in some cases have led to entirely new mechanistic insights of how chromatin enzymes regulate the genome. Notably, mutations of epigenetic regulators often trigger neurodevelopmental disorders including autism spectrum disorder and intellectual disability, generating wide interest in understanding how epigenetic regulators govern the development and function of brain neural circuits including in the cerebellum.

Keywords

Epigenetics · Chromatin · Histone · Histone modification · Histone acetylation · Histone methylation · Histone variant · DNA methylation · Chromatin remodeling · Gene expression · Genome · DNA · Promoter · Enhancer · Bivalent modification · Immediate early genes · Granule neuron · Purkinje cells · Precursor · Cell proliferation · Dendrite growth · Dendrite pruning · Presynaptic differentiation · Degeneration · Calcium signaling · Cerebellar-dependent behavior · CHARGE syndrome · Coffin-Siris syndrome · Rett syndrome · Intellectual disability · Autism spectrum disorders · PRC2 · Chd7 · Snf2h · Snf2l · NuRD complex · BAF complex · Kdm6a · Kdm6b · Kdm5c · HDACs · HATs · Tet enzymes · MeCP2 · Dnmt3a

A Brief Introduction to Epigenetic Mechanisms

Genomic DNA in the nucleus of eukaryotic cells is organized by histone proteins into a macromolecular structure termed chromatin. The basic building blocks of chromatin are nucleosomes, each of which comprises an octamer of histone proteins including histones H2A, H2B, H3, and H4 wrapped by ~147 base pairs of DNA (Kornberg 1974; Luger et al. 1997). Whereas transcription factors directly bind to specific DNA sequences to control gene expression, epigenetic regulators introduce changes to chromatin that alter the genome environment. The enzymatic activities of epigenetic regulators may be divided into three main categories, posttranslational modification of histones, DNA methylation, and nucleosome remodeling (Allis et al. 2015.) (Fig. 1). The first two mechanisms involve the covalent attachment of molecules to proteins or DNA, whereas the latter mechanism involves an ATP-dependent movement or alteration in histone composition of nucleosomes.

Chromatin modifications come in wide varieties, but share similar roles in genome control. First, posttranslational modification of histones, particularly on the N-terminal tail, include acetylation, methylation, phosphorylation, and ubiquitination (Li et al. 2007). Histone tail modifications strengthen or weaken DNA-histone interactions and serve as landmarks to recruit additional epigenetic regulators. Second, DNA methylation occurs at the fifth position of the pyrimidine ring of a cytosine followed by guanine (5mCG) or non-guanine nucleotide (5mCH) (Kriaucionis and Heintz 2009; Smith and Meissner 2013). Methylcytosine may be

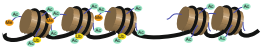
Epigenetic mechanism	Enzymes	Genes coding enzymes in cerebellar development
Histone modification 	Histone acetyltransferase Histone deacetylase Histone methyltransferase Histone demethylase	PRC2 (Ezh1/2) Kdm6b Kdm5c Hdac1/2 Hdac3 Gcn5
Nucleosome remodeling Nucleosome density  Histone variant 	ATP-dependent chromatin remodeling enzymes	NuRD complex BAF complex Chd7 Snf2h, Snf2l
DNA methylation 	DNA methyltransferase DNA dioxygenase	Tet 1/3 Dnmt3a

Fig. 1 Epigenetic mechanisms and enzymes in the cerebellum

modified to generate 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Like histone modification, DNA methylation serves as a marker to recruit or block the binding of transcription factors and epigenetic regulators that distinguish between methylated and unmodified DNA (Yin et al. 2017). Finally, nucleosome remodeling encompasses changes in nucleosome spacing, density, or subunit composition (Narlikar et al. 2013). Adjacent nucleosomes are positioned at specific genomic distances from one another in order to assemble into higher-ordered chromatin fibers. The canonical histone subunits of nucleosomes may also be exchanged for histone variants including histone H2A.x, H2A.z, and H3.3. Altering the positioning or structure of nucleosomes modulates the accessibility of transcription factors to genomic DNA sequences or may recruit additional epigenetic regulators that recognize specific histone variants. Together, these mechanisms represent a common theme in transcription, whereby groups of chromatin enzymes operate in concert to drive large-scale changes in the epigenetic landscape.

Gene expression is directly controlled by chromatin modifications at their transcriptional start site (TSS) or promoters, at neighboring regulatory genomic DNA elements termed enhancers, or along the entirety of gene bodies. At least three major transcriptional states – active, poised, or repressed – at these regulatory loci are marked by distinct chromatin modifications (Hirabayashi and Gotoh 2010). Active genes have ongoing transcription, while poised genes are silent but may be turned on upon exposure to a stimulus or with cellular differentiation (Voigt et al. 2013). Repressed genes in the brain are constitutively silenced for the long-term in

postmitotic cells (Pereira et al. 2010; Yamada et al. 2014). Chromatin features at both active and poised genes include histone H3 lysine 4 tri-, di-, and mono-methylation (H3K4me1/2/3), low nucleosome density, and the presence of mCA and 5hmC at promoters, enhancers, or gene bodies. High levels of histone H3 lysine 9, 14, and 27 acetylation (H3K9/14/27 ac) typically distinguish active genes from unstimulated, poised genes. Finally, the presence of histone H3 lysine 9 or 27 trimethylation (H3K9me3 or H3K27me3), mCG, and high nucleosome density mark constitutively repressed genes. In the next section, we will discuss how these transcriptional states are regulated in the developing cerebellum as neurons differentiate and assemble into neural circuits.

Genome-Wide Changes in the Epigenetic Landscape in the Developing Cerebellum

The mouse cerebellum undergoes significant growth in the first few postnatal weeks. From postnatal day 3 to day 30, the cerebellar vermis alone expands over fourfold in area, due to increases in cell number and neuropil (Wojcinski et al. 2017). Cerebellar precursors originate from two distinct zones in early development. Excitatory neurons including granule neurons, unipolar brush cells, and excitatory deep cerebellar nuclei neurons are generated from precursor cells that originate in the rhombic lip. Inhibitory GABAergic neurons including Purkinje cells and inhibitory deep cerebellar nuclei neurons are generated from precursor cells in the ventricular zone. Following cell cycle exit, the stereotyped pattern of differentiation and maturation of granule neurons has been well-characterized since the days of Santiago Ramón y Cajal (Ramón y Cajal 1995). Granule neurons migrate from the external granule layer to the internal granule layer, where they transiently form exuberant dendritic processes that are subsequently pruned (Palay and Chan-Palay 1974). At the final stage of differentiation, presynaptic boutons in the molecular layer and postsynaptic dendritic claws in the internal granule layer are formed, providing specialized structures for synaptic neurotransmission.

Thousands of genes encoding proteins involved in cell proliferation, neurite growth, and synaptogenesis are switched on and off during this period to support cerebellar development (Pal et al. 2011; West and Greenberg 2011; Zhu et al. 2016). The two major groups of genes important for cerebellar development include genes that are permanently turned on or off with neuronal differentiation and signaling genes that are repeatedly and transiently activated and shut off in response to extrinsic cues. Genome-wide regulation of chromatin modifications at promoters and enhancers directly control these patterns of gene expression. Because granule neurons represent the most populous cell type in the cerebellum (~70–80% of all cells) (Bandeira et al. 2009), with few exceptions, studies of epigenetics in the cerebellum have largely focused on developing granule neurons (Fig. 2).

In proliferating granule neuron precursors, cell cycle genes are maintained in an active state by the presence of H3K4me3 at their promoters, while genes necessary

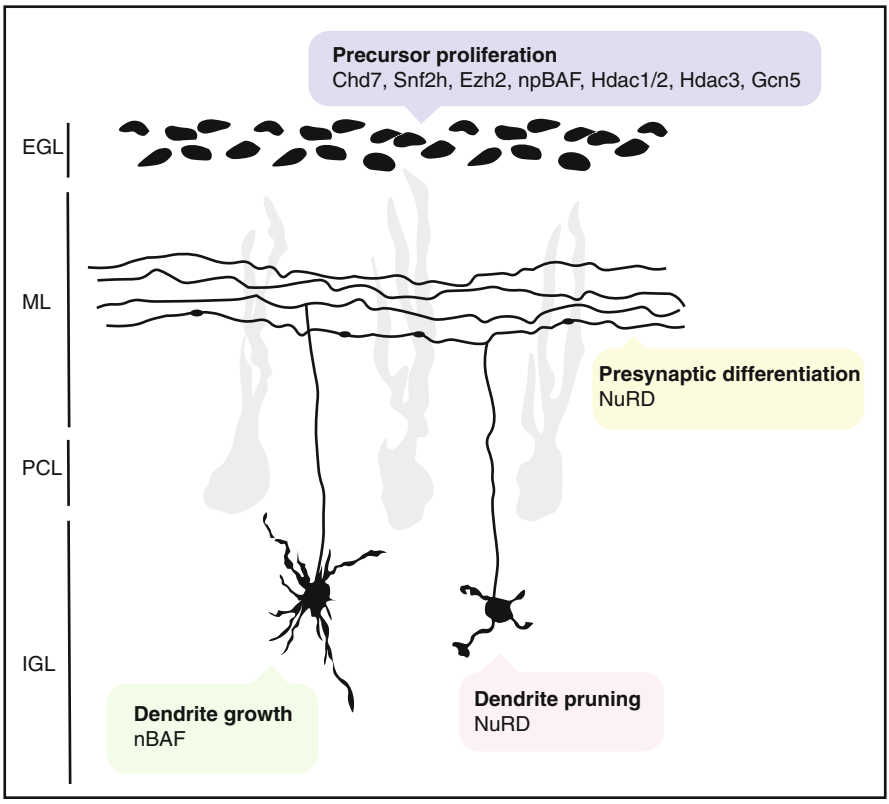


Fig. 2 Epigenetic regulation of distinct phases of cerebellar development. *EGL* external granule layer, *ML* molecular layer, *PCL* Purkinje cell layer, *IGL*, internal granule layer

for postmitotic neuron differentiation are kept silent by the bivalent modification of H3K4me3 and H3K27me3 (Bernstein et al. 2006). In newly born granule neurons, genes important for cell cycle and proliferation are constitutively repressed as their regulatory loci are marked by increases in H3K27me3, H3K9me3, 5mCG, and nucleosome density (Frank et al. 2015; Pal et al. 2011; Yang et al. 2016). Differentiating granule neurons turn on genes important for synapses, ion channels, and cell adhesion by marking promoters and enhancers with H3K4me3, H3K9/14/27 ac, 5hmCG, and 5hmCA, removing H3K27me3, and maintaining low nucleosome density (Frank et al. 2015; Pal et al. 2011; Song et al. 2011; Szulwach et al. 2011; Yang et al. 2016). The increased chromatin accessibility at these developmentally upregulated genes facilitates the recruitment of transcription factors including the Zic family to promote granule neuron maturation (Frank et al. 2015). A comprehensive analysis of histone modifications and transcriptional regulators in the adult mouse cerebellum and other tissues shows that active enhancers are expressed in a tissue-specific manner and are bound by different groups of transcription factors (Shen et al. 2012). The unique epigenetic landscape and expression patterns of active

genes in mature granule neurons define their identity by presumably determining their unique morphology and physiological properties.

A distinct class of genes are quiescent under baseline conditions but rapidly respond to extracellular signaling including growth factors and neuronal activity. These stimulus-responsive genes including immediate early genes (IEGs) enable neurons to sense changes in the local environment and consequently determine the correct time and place for differentiation or plasticity (Herschman 1989; Morgan and Curran 1989). Although genes marked by the H3K4me3/H3K27me3 bivalent histone marks may also respond to extracellular signals (Shi et al. 2014; Voigt et al. 2013), a key difference is that IEGs are activated and shut off over the lifetime of the neuron. At baseline, IEGs maintain poised promoters marked by H3K4me3, H2A.z, 5hmCG, and 5mCA and low levels of H3K9/14/27 ac (Kaas et al. 2013; Rudenko et al. 2013; Stroud et al. 2017; Yang et al. 2016). When neurons are activated, H2A.z is evicted and H3K9/14/27 ac levels increase at promoters, resulting in robust increases in gene expression (Yang et al. 2016; Zovkic et al. 2014). Networks of transcription factors including NeuroD, AP1, CREB, and MEF2 play critical roles in coupling extrinsic signals to epigenetic regulation and gene expression (Shalizi and Bonni 2005; West and Greenberg 2011). The stimuli-responsive target genes are often expressed in specific cell types or tissues (Lin et al. 2008). Together, the inducible expression of poised genes and the constitutive expression of active genes represent the major nuclear mechanisms that integrate cell-extrinsic cues with cell-intrinsic genetic programs to drive granule neuron differentiation. We will next discuss the epigenetic regulators that shape the nuclear landscape and orchestrate precise programs of gene expression in the cerebellum.

Families of Epigenetic Regulators in Mouse Cerebellar Development

To study the molecular and biological functions of epigenetic regulators in cerebellar development, mouse genetics approaches have been employed. Because chromatin enzymes are often essential for early embryogenesis, conditional knockout approaches such as the Cre-LoxP system are required to conditionally delete genes in cell type-specific populations and at specific developmental timepoints. The Nestin-Cre driver is used to knockout genes broadly in neural precursors (Tronche et al. 1999), while the Math1-Cre driver silences genes of interest in granule neurons and deep cerebellar nuclei progenitors (Machold and Fishell 2005; Wang et al. 2005). Gabra6-Cre transgenic mice are used to target postmitotic granule neurons (Funfschilling and Reichardt 2002), and Pcp2-Cre targets postmitotic Purkinje cells (Barski et al. 2000). Most studies have focused on studying the roles of epigenetic regulators in cerebellar precursors or postmitotic granule neurons because these cells are more abundant, thereby facilitating molecular characterization. However, since knockout of epigenetic regulators frequently impairs progenitor proliferation or survival in vivo, in vitro cell culture methods have also been used to study the enzymatic functions of these genes. In this section, we discuss current understanding

of how chromatin enzymes operate in the cerebellum to orchestrate gene expression and drive neuronal differentiation.

ATP-Dependent Chromatin Remodeling Enzymes

Chd7 is an ATP-dependent chromatin remodeler that is particularly highly expressed in the cerebellum (Feng et al. 2017). Within the cerebellum, Chd7 is specifically expressed in granule neuron precursors and postmitotic granule neurons (Feng et al. 2017; Whittaker et al. 2017). Chd7 maintains chromatin accessibility at thousands of regulatory loci across the genome in cultured granule neuron precursors, including at the reelin gene and long neuronal genes, though how Chd7 operates at the genomic level in these cells in vivo remains to be elucidated. Chd7 interacts with other chromatin regulators including DNA topoisomerase 1/2α/2β (Top1/2a/2b), Brg1, and Chd8 to potentially co-regulate gene expression, a feature commonly observed among chromatin enzymes. Conditional Chd7 deletion in granule neuron precursors results in impaired progenitor proliferation, differentiation, or survival, culminating in cerebellar hypoplasia (Feng et al. 2017; Whittaker et al. 2017). At earlier developmental stages, Chd7 controls the expression of the midbrain and hindbrain organizers Fgf8, Otx2, and Gbx2, but the epigenetic mechanisms by which Chd7 regulates early hindbrain patterning remain unknown (Yu et al. 2013).

The mammalian ISWI family proteins, Snf2h and Snf2l, are ATP-dependent chromatin remodeling enzymes that are highly expressed in the developing cerebellum. Whereas Snf2h is abundant in cerebellar precursors, Snf2l is expressed specifically in postmitotic neurons (Alvarez-Saavedra et al. 2014). Knockout of Snf2h in cerebellar precursors impairs chromatin organization in cerebellar precursors and Purkinje cells, resulting in global decreases in active histone modifications including H3K4me3, H3K18ac, and H3K36me2. These chromatin organization phenotypes may reflect dysregulation of the nucleosome spacing functions of ISWI (Ito et al. 1997; Varga-Weisz et al. 1997). Depletion of Snf2h in cerebellar precursors leads to cell death and reduced proliferation, culminating in cerebellar hypoplasia. Knockout of Snf2h specifically in postmitotic Purkinje cells impairs dendrite growth, leading to Purkinje cell degeneration (Alvarez-Saavedra et al. 2014).

Beyond cell proliferation and survival, ATP-dependent chromatin remodelers also have important functions in terminal granule neuron differentiation. The nucleosome remodeling and deacetylase (NuRD) complex is a multi-subunit ATP-dependent chromatin remodeling enzyme that has both histone deacetylase and nucleosome remodeling activities (Zhang et al. 1998). Subunits of the NuRD complex including the core ATPase subunit Chd4 are highly expressed in the developing cerebellum (Yamada et al. 2014). Chd4 binds genome-wide to active or poised promoters and enhancers, but the NuRD complex harbors distinct enzymatic functions at the regulatory sites of different classes of genes. At developmentally downregulated genes, the NuRD complex triggers promoter decommissioning via removal of the histone modifications H3K9/14/27 ac and H3K4me3 and increases in H3K27me3 (Yamada et al. 2014). As granule neurons mature, NuRD-

triggered promoter decommissioning leads to chronic silencing of a set of developmental genes expressed in granule neuron precursors and immature granule neurons including Nhlh1 and Elavl2. Knockout of Chd4 impairs granule neuron presynaptic differentiation and parallel fiber/Purkinje cell neurotransmission, whereas knockdown of Nhlh1 and Elavl2 in immature granule neurons triggers precocious presynaptic differentiation (Yamada et al. 2014). These results reveal important functions for the NuRD complex and developmental promoter decommissioning in releasing a break on synaptic connectivity between granule neurons and Purkinje cells.

Besides chronic silencing of a key set of developmental genes, the NuRD complex also plays a critical role in dynamic regulation of transcription in granule neurons. Neuronal activity and growth factor signaling in neurons rapidly and transiently induce the expression of immediate early genes (IEGs), which in turn mediate neuronal plasticity (Bonni and Greenberg 1997; West and Greenberg 2011). Following the cessation of extracellular signaling, IEG expression is rapidly shut down but may be quickly reactivated by new stimuli. Remarkably, the NuRD complex is required for the shutdown of IEG expression following cessation of stimulation by depositing the histone variant H2A.z at gene promoters (Yang et al. 2016). H2A.z-containing nucleosomes may serve as a scaffold for the recruitment of additional epigenetic regulators (Dann et al. 2017; Hu et al. 2013), which may mediate shutoff of IEGs. Thus, the NuRD-H2A.z pathway represents an epigenetic mechanism that actively drives the shutoff of activity-dependent transcription in the cerebellum.

Immature granule neurons require calcium signaling for dendrite growth (Gaudilliere et al. 2004; Okazawa et al. 2009). Granule neurons that have migrated to the internal granule layer undergo a ~2-day period of exuberant dendrite growth in vivo, followed by dendrite pruning (Huynh et al. 2011; Yang et al. 2016). Loss of Chd4 results in prolonged IEG expression after transient neuronal depolarization and a failure in dendrite pruning. Overexpression of the IEGs c-fos and nr4a1 phenocopy the Chd4 knockout-induced effects on dendrite patterning in vivo (Yang et al. 2016). Consistent with the abnormally exuberant dendrites, Chd4 knockout granule neurons exhibit hyperresponsivity to sensory stimuli in awake, locomoting mice (Yang et al. 2016). Together, these findings illuminate the dual epigenetic functions of the NuRD complex at stimulus-responsive genes and developmental genes to control afferent and efferent granule connectivity in the cerebellar cortex.

The BAF complex is a large multi-subunit ATP-dependent chromatin remodeling complex that interestingly exhibits antagonistic functions with the NuRD complex. The BAF complex enhances chromatin accessibility at promoters or enhancers by reducing nucleosome density (Kwon et al. 1994; Morris et al. 2014). In addition, the core ATPase subunit Brg1 directly suppresses polycomb-mediated induction of the modification H3K27me3 at promoters and thereby opposes silencing of developmentally downregulated genes (Stanton et al. 2017). At early stages of development, the neural progenitor BAF (npBAF) complex is necessary for the proliferation of neural precursors. Accordingly, conditional knockout of BAF complex subunits in granule neuron precursors results in cerebellar hypoplasia and ataxia (Moreno et al. 2014). Several BAF complex subunits are then switched with other family members

upon cell cycle exit in later development. The neuronal-specific subunits BAF53b and BAF45b/c replace the progenitor-enriched subunits BAF53a and BAF45a, respectively (Lessard et al. 2007; Wu et al. 2007). In granule neurons, the neuron-specific BAF (nBAF) complex containing BAF53b is necessary for dendrite growth (Wu et al. 2007). The BAF subunit calcium-responsive transactivator (CREST) interacts with the CREB-binding protein (CBP) to couple calcium signaling with gene activation and dendrite growth (Qiu and Ghosh 2008; Wu et al. 2007). Finally, conditional deletion of the BAF subunit Bcl7a specifically in postmitotic neurons impairs dendrite arborization in Purkinje cells (Wischhof et al. 2017). The opposing roles of BAF and NuRD complexes in activity-dependent and developmental gene activation and repression, as well as dendrite growth and pruning, reveal the critical functions of these enzymes at multiple stages of neuron maturation. Together, these findings highlight the important functions of ATP-dependent chromatin remodelers in controlling nucleosome structure in neuron progenitors and postmitotic neurons to ensure proper cerebellar development.

Histone Tail Modifiers

In addition to ATP-dependent chromatin remodeling, epigenetic regulators stimulate posttranslational histone modifications on histone tails. Among these regulators, the multi-subunit polycomb repressive complex 2 (PRC2) has emerged as a major transcriptional repressive complex that developmentally decommissions gene promoters. PRC2 stimulates the modification H3K27me3 at the promoters and distal regulatory sites of genes to permanently silence gene expression in postmitotic neurons, including in the cerebellar primordium (Feng et al. 2016). PRC2 may be recruited to gene promoters following NuRD-mediated deacetylation of H3K27 (Reynolds et al. 2012). Knockout of Ezh2, a core methyltransferase subunit of PRC2, in cerebellar precursors results in reduced precursor proliferation and Purkinje cell number, but increased interneuron cell number (Feng et al. 2016). These mice also exhibit severe cerebellar hypoplasia. Knockout of both Ezh1 and Ezh2 specifically in Purkinje cells derepresses gene expression and impairs the survival of adult Purkinje cells, resulting in cerebellar degeneration (von Schimmelmann et al. 2016).

The histone lysine demethylase enzymes Kdm6a and Kdm6b counteract polycomb activity by inducing the demethylation of H3K27. Kdm6b expression is significantly upregulated in differentiating granule neurons (Shi et al. 2014; Wijayatunge et al. 2017; Yang et al. 2016). In response to the extrinsic cues, sonic hedgehog (SHH) in the external granule layer and brain-derived neurotrophic factor (BDNF) in the internal granule layer, Kdm6b promotes the expression of granule neuron progenitor proliferation or postmitotic granule neuron terminal differentiation genes, respectively (Shi et al. 2014; Wijayatunge et al. 2017). Kdm6b activates gene expression by inducing H3K27me3 demethylation and recruiting histone H3K4 methyltransferases to gene promoters (Shi et al. 2014). Global knockout of Kdm6b impairs cerebellar growth, whereas paradoxically conditional knockout of

both Kdm6a and Kdm6b specifically in granule neuron precursors fails to alter their proliferation (Shi et al. 2014; Wijayatunge et al. 2017). These results suggest that Kdm6b may function at earlier stages in brain development or via non-cell autonomous mechanisms to regulate the expansion of cerebellar granule neuron precursors. In addition, in view of the role of Kdm6b in activation of synaptic genes necessary for terminal granule neuron differentiation (Wijayatunge et al. 2017), it will be interesting to assess the physiological properties of cerebellar cortical circuits in conditional Kdm6b knockout mice.

The histone demethylase enzyme Kdm5c induces H3K4me2/3 demethylation at promoters and enhancers of lowly expressed, but not highly expressed or promoter decommissioned genes (Iwase et al. 2007, 2016; Scandaglia et al. 2017). The lysine demethylase family members Kdm5a and Kdm5b may mediate demethylation of H3K4me3 at transcriptional start sites during promoter decommissioning (Yang et al. 2016). Lowly expressed genes that are regulated by Kdm5c include IEGs, which are inhibited under baseline, unstimulated conditions (Scandaglia et al. 2017). Kdm5c is necessary for dendrite growth in cerebellar granule neurons (Iwase et al. 2007). These findings highlight the distinct transcriptional repressive mechanisms deployed by different chromatin enzymes to regulate granule neuron differentiation.

Histone deacetylases (HDACs) remove acetyl groups from lysine residues on histone tails to repress transcription, while histone acetyltransferases (HATs) add acetyl to histone tails to stimulate gene expression (Shahbazian and Grunstein 2007). The Class I HDACs, Hdac1/2/3, associate with several transcriptional repressive complexes including the NuRD complex, Sin3a, CoREST, and NCoR. HATs such as Gnc5 and Pcaf are part of SAGA-like transcriptional activator complexes. While the genome-wide chromatin activities of HDACs and HATs in the cerebellum are poorly understood, knockout of Hdac1/2, Hdac3, or Gnc5 in neural precursors results in cerebellar hypoplasia (Martinez-Cerdeno et al. 2012; Montgomery et al. 2009; Norwood et al. 2014). By contrast, knockout of the Class II HDAC, Hdac4, in neural precursors appears to have little effect on overall cerebellar development (Price et al. 2013). It will be important in future studies to determine the epigenetic mechanisms and biological roles of distinct HDAC and HAT complexes in cerebellar precursors and postmitotic neurons during development.

DNA Methylation

Epigenetic regulators may also directly modify DNA by methylating or demethylating cytosine base pairs. In proliferating cells, 5hmC, 5fC, and 5caC are intermediate, unstable products generated during cytosine demethylation (Wu and Zhang 2017). The epigenome of postmitotic neurons in the brain including Purkinje cells and granule neurons in the cerebellum uniquely harbor high, stable levels of 5hmCG (Kriaucionis and Heintz 2009; Szulwach et al. 2011). 5hmCG is enriched, whereas 5mCG is depleted along the gene bodies of active genes (Mellen et al. 2012). The expression of ten-eleven translocation (Tet) family of dioxygenases that convert 5mC to 5hmC is upregulated in the developing cerebellum (Zhu et al. 2016).

Activation of Tet enzymes with vitamin C increases the levels of 5hmC across the body and particularly at exon start sites of axon guidance and ion channel genes in ESC-derived granule cells. The Tet enzymes Tet1/3 are necessary for the expression of these axon guidance and ion channel genes and promote dendrite arborization in developing granule neurons in ex vivo cerebellar slices.

Two common forms of 5mC are 5mCG and 5mCA. Among all tissues, 5mCA levels are found highest in postmitotic neurons in the brain, while 5mCG is present ubiquitously (He and Ecker 2015). DNA methyltransferase 3A (Dnmt3a) deposits 5mCA preferentially at lowly transcribed genes in the developing brain, including at long brain-enriched genes that span hundreds to thousands of kilobases (Gabel et al. 2015; Guo et al. 2014; Stroud et al. 2017). Dnmt3a and 5mCA maintain the low expression of these genes for the lifetime of the animal (Gabel et al. 2015; Stroud et al. 2017).

The brain-enriched methyl-CpG-binding protein 2 (MeCP2) binds to the transcriptionally repressive modifications 5mCG, 5mCA, and 5hmCA, but not the transcriptionally active mark 5hmCG in the cerebellum (Gabel et al. 2015; Mellen et al. 2012, 2017). MeCP2 may function as a transcriptional repressor or activator by interacting with HDACs or p300, respectively (Ben-Shachar et al. 2009; Chahrour et al. 2008; Ebert and Greenberg 2013). However, once bound to methylated DNA, MeCP2 appears to primarily downregulate gene expression (Gabel et al. 2015; Mellen et al. 2017). These results are consistent with the role of MeCP2 in suppressing global histone H3 acetylation in the cerebellum (Shahbazian et al. 2002). Blocking 5mCA methylation or increasing oxidation of 5mCG to 5hmCG along gene bodies releases MeCP2 from chromatin and increases gene expression (Gabel et al. 2015; Mellen et al. 2017). Despite the rich literature on the chromatin mechanisms of MeCP2 and Dnmt3a, their biological functions in the cerebellum are just beginning to be characterized. Mutant mice expressing a truncated MeCP2 gene at R308 have reduced Purkinje cell dendritic spine density (Kloth et al. 2015). These findings are consistent with dendrite arborization and spine number abnormalities in MeCP2 transgenic mice elsewhere in the brain (Jiang et al. 2013).

Perspectives on Epigenetic Regulators in the Mouse Cerebellum

Several themes have emerged from studies of epigenetic regulators in cerebellar development. In cerebellar precursors, knockout of multiple chromatin enzymes disrupts proliferation and results in cerebellar hypoplasia. Future experiments should address whether these enzymes control distinct phases of the cell cycle and how epigenetic modifications are regulated in neural precursors. In addition, it will be interesting to determine whether different chromatin enzymes exert distinct effects on subpopulations of granule neuron precursors in a temporally or spatially defined manner. In postmitotic granule neurons, different chromatin enzymes control distinct phases of dendrite morphogenesis including dendrite growth and pruning. To support these transitions in postmitotic neuron development, the expression of chromatin enzymes is subject to regulation. For example, the core BAF subunit Brg1 that

promotes dendrite growth is sharply downregulated after the first postnatal week, whereas the core NuRD subunit Chd4 that stimulates dendrite pruning persists in expression through the second postnatal week (Zhu et al. 2016). Hundreds of chromatin enzymes are highly expressed in the developing cerebellum (Yamada et al. 2014; Zhu et al. 2016), and much work still needs to be done to understand how these families of proteins collaborate to pattern the cerebellum. Finally, despite these first insights into epigenetic control of granule neuron differentiation, how chromatin enzymes regulate the epigenome of other cerebellar neurons including Purkinje cells, deep cerebellar nuclei neurons, molecular layer interneurons, unipolar brush cells, and others is virtually unknown. All of these cell types play critical and unique roles in cerebellar circuit function and organismal behavior. In the next section, we will discuss recent efforts to understand how epigenetic control of neuronal connectivity and cerebellar development influences mouse behavior.

Epigenetic Control of Cerebellar-Dependent Behavior

A major goal in neuroscience has been to tease apart the cell type and circuit-specific mechanisms that drive behavior. The cerebellum plays diverse roles in motor coordination, motor learning, and cognitive functions. A key question is how do the molecular and cellular phenotypes observed in knockout mice affect behavior. Furthermore, are there differences in behavioral outcome with major cerebellar architecture deficits as compared to more specific deficits in cerebellar cortical connectivity?

Knockout of epigenetic regulators in neural progenitors often results in brain malformation and severely impaired motor function or death. Neural progenitor knockouts of Hdac1/2 and Hdac3 have reduced brain size and are embryonically lethal, precluding assessment of motor function (Montgomery et al. 2009; Norwood et al. 2014). Induction of cerebellar hypoplasia by knockout of Chd7 or knockout of components of the BAF complex in granule neuron precursors causes deficits in motor coordination (Moreno et al. 2014; Whittaker et al. 2017). These findings are consistent with neurological symptoms observed with impaired cerebellar development in humans. Failure in dendrite arborization or progressive degeneration of Purkinje cells due to knockout of BAF, PRC2, or Hdac3 in postmitotic Purkinje cells also results in ataxia (Moreno et al. 2014; Norwood et al. 2014; von Schimmelmann et al. 2016). Paradoxically, conditional knockout of Snf2h in Purkinje cells leads to partial degeneration of these neurons in adult mice, but has no effects on motor coordination (Alvarez-Saavedra et al. 2014). It will be interesting to test if compensatory mechanisms that increase the activity of surviving Purkinje cells are at play in these knockout animals.

In the absence of gross cerebellar malformation or Purkinje cell degeneration, changes in neuronal connectivity may specifically impair cerebellar-dependent motor learning. Conditional knockout (cKO) of Chd4 in mature postmitotic granule neurons disrupts granule neuron to Purkinje cell connectivity and synaptic neurotransmission (Yamada et al. 2014). Conditional Chd4 knockout mice have delayed

and impaired associative learning in the eyeblink conditioning paradigm but have little or no changes in motor coordination or general locomotion behavior (Yang et al. 2016). Global, constitutive expression of mutant MeCP2 in mice reduces Purkinje cell dendritic spine density and also decreases the amplitude of the conditioned response in eyeblink conditioning, but not the probability of conditioned responses (Kloth et al. 2015). These studies suggest epigenetic regulators are important for the functions of cerebellar circuits that drive associative motor learning in mice.

Recently, increasing attention has been placed on functions of the cerebellum beyond motor control, including in cognition and social behavior (Stoodley et al. 2017; Wagner et al. 2017). Widespread cortico-cerebellar loops via pontine and thalamic relays connect the cerebellum and forebrain. The Purkinje cell-specific Snf2h knockout mice discussed above have reduced interactions with novel mice but have normal exploratory behavior with inanimate objects (Alvarez-Saavedra et al. 2014). Furthermore, these mice have impaired contextual fear conditioning but normal cued fear. These and other mouse genetic studies raise the intriguing hypothesis that the cerebellum may play important roles in social interaction and that perturbations of Purkinje cell activity may result in autistic phenotypes (Stoodley et al. 2017; Tsai et al. 2012). Furthermore, from gross cerebellar malformation to fine-scale changes in cerebellar circuits, epigenetic regulation has proven to be a key step in the development and behavior of mice. We next turn to the development of the human cerebellum to illustrate the devastating impact of misregulation of chromatin enzymes in human neuropathology.

Epigenetics in Human Disease

Just as in the mouse brain, epigenetic regulators play fundamental roles in orchestrating human brain development. Mutations in epigenetic regulators cause a wide range of neurological disorders, ranging from intellectual disability to autism spectrum disorders to epilepsy (RK et al. 2017; Ronan et al. 2013; Weiss et al. 2016). Neuroimaging of affected individuals has often revealed cerebellar dysplasia, hypoplasia, or agenesis in these disorders (Stevenson et al. 2013). We will highlight some of the key epigenetic regulators that are genetically linked to neurodevelopmental disease and cerebellar malformation.

Mutations in the ATP-dependent chromatin remodeler Chd7 cause CHARGE syndrome, manifesting with coloboma of the eye, heart defects, atresia of the choanae, retarded growth and development, genital anomalies and ear malformations or deafness. A key clinical feature observed in over 50% of CHARGE patients with mutations in Chd7 is cerebellar malformation, consistent with mouse genetic studies (Feng et al. 2017; Whittaker et al. 2017; Yu et al. 2013). The cerebellar vermis is often underdeveloped and mispositioned, resulting in an enlarged fourth ventricle. In addition, 3 out of 20 patients have broad or ataxic gait, consistent with gross cerebellar dysfunction.

In addition to Chd7, other ATP-dependent chromatin remodelers are commonly associated with human neurodevelopmental disorders. Mutations in Chd2 cause myoclonic encephalopathy together with intellectual disability, speech delay, and developmental regression that correlates with the severity of seizures (Thomas et al. 2015). Progressive cerebellar atrophy is observed in several severe cases, presenting with ataxia. The neurodevelopmental disorder Coffin-Siris syndrome (CSS) is caused by mutations in components of the BAF ATP-dependent chromatin remodeling complex (Tsurusaki et al. 2014). The primary clinical feature in CSS patients is intellectual disability, and a subset of affected individuals have seizures, microcephaly, and cerebellar hypoplasia. These findings reveal that neurodevelopmental syndromes linked to distinct chromatin enzymes often have shared neurological phenotypes.

Besides ATP-dependent chromatin enzymes, other epigenetic regulators are also necessary for normal human brain development. Rett syndrome is an X-linked disorder that affects primarily females and is due to mutations in the DNA methylation binding protein MeCP2. Males carrying the mutation only have one copy of the X-chromosome and rarely survive birth. Girls born with Rett syndrome initially exhibit normal behavior but regress in development starting 6 months of age. The anterior cerebellar lobules I–IV display mild hypoplasia at younger ages, and more severe cerebellar degeneration is observed in adults (Zanni and Bertini 2011). Common phenotypes are impaired motor coordination, communication, and cognitive function that persist for the lifetime of the individual. Mutations in the lysine demethylase Kdm5c cause an X-linked intellectual disability syndrome. Males are more severely affected, but females may present with milder phenotypes. Some male patients have seizures and cerebellar atrophy starting at 4 years of age (Fujita et al. 2016).

Additional mutations in epigenetic regulators have been reported in neurodevelopmental diseases. For example, missense mutations in Chd4 and Gatad2b, subunits of the NuRD ATP-dependent chromatin remodeling complex, result in general developmental delay and intellectual disability (Weiss et al. 2016). Mutations in methyl-CpG-binding domain 3 (MBD3), another subunit of the NuRD complex, are found in autism spectrum disorders (Cukier et al. 2010). The ATP-dependent chromatin remodeling enzymes Chd8 and nBAF are also implicated in autism spectrum disorders (Mahfouz et al. 2015; Yuen et al. 2017). These findings demonstrate that epigenetic regulators are powerful drivers of brain development and function in humans.

Conclusions and Future Directions

In the past decade, great strides have been made in uncovering the molecular mechanisms and biological roles of epigenetics in the development of the brain. Recent advances in next-generation sequencing have revealed that epigenetic regulators are often mutated in human neurodevelopmental diseases. Clinical diagnosis of human patients and characterization of mouse models have confirmed the pivotal

role of these enzymes in brain development and function. Understanding the in vivo functions of epigenetic regulators is necessary for improved understanding of debilitating neurodevelopmental brain disorders. Emerging new technologies in the fields of functional genomics and systems neuroscience will be required toward this goal.

Several exciting areas of epigenetics research should be anticipated in the upcoming years. Current methods require measuring bulk changes in the epigenome of millions of cells and with poor temporal resolution, i.e., on the tens of minutes to hours timescale. However, the ability to isolate and profile the epigenome of low abundance cell types is critical in the cerebellum where the functions of epigenetic regulators in rare cell types including unipolar brush cells and Lugaro cells and in inhibitory interneurons are unknown. Significant advances have been made in single-cell transcriptome profiling, and similar technological breakthroughs in single to hundred cell epigenomic profiling are anticipated. Another approach is to track the dynamics of epigenetic regulation in single cells using imaging. For example, in transcriptomics, by tagging mRNA with fluorescent tags, the real-time expression and degradation of single mRNA molecules can be visualized. Similarly, efforts are underway to detect changes in histone modifications and nucleosomes live in single cells (Stasevich et al. 2014). These tools will greatly aid our understanding of how the epigenome is regulated in cell type-specific neural circuits with temporal precision.

In the developing and adult cerebellum in vivo, neurons encounter a diverse set of extrinsic signals that encode for changes in the local cellular environment or animal behavioral state. A newly born granule neuron that migrates from the external granule layer to internal granule is exposed to at least dozens of secreted factors that act as a global positioning system (Komuro and Rakic 1992). Epigenetic regulators in the nucleus integrate these extrinsic signals to drive long-lasting changes in neuronal identity, i.e., from a migratory to post-migratory state. However, most in vivo signals and their downstream pathways remain poorly understood. Single-cell approaches together with laser capture microdissection, which retains spatial information, may permit discovery of how the local environment in the cerebellum controls specific epigenetic changes during neuronal differentiation and plasticity. By also applying pharmacological tools, we can elucidate the transmembrane receptors that couple extrinsic signals to epigenetic regulation in vivo.

In addition to epigenetic mechanisms including histone modification, chromatin remodeling, and DNA methylation, recent attention has focused on a new level of epigenetic programming, the regulation of three-dimensional genome architecture. Recent studies primarily using cell culture systems have uncovered novel mechanisms that create or suppress long-distance interchromosomal and intrachromosomal interactions to control gene expression. One such mechanism involves CTCF insulator proteins, which restrict the interactions of distal enhancers to specific gene promoters. However, how epigenetic regulators control genome architecture in the brain in vivo remains an open mystery. Understanding the relationship between gene expression and genome architecture and how these are dynamically controlled in the developing and adult cerebellum might reshape the way we think about gene regulation in neuroscience.

Finally, an essential but often underappreciated problem that must be solved is how we bridge the gap between genes and behavior. After identifying mutations in genes encoding epigenetic regulators as causative for human brain disorders, how can we understand the roles of these enzymes in learning and memory, motor functions, and social behaviors, all of which are at risk with disease? The answer to this question may lie in a suite of new systems neuroscience tools that allow characterization of functions of each and every genetically accessible neural circuit in behavior. An example is using genetically encoded calcium indicators to monitor neural circuit activity of cerebellar neurons in behaving control and mutant mice. Subsequently, the complementary approaches of optogenetics and chemogenetics may shed light on requirements for cerebellar circuits for the expression of a behavior or the acquisition of a learned response. This line of new research will reveal how a dysregulated epigenome in specific cell types of the cerebellum may lead to the pathogenesis of motor and cognitive disorders.

In summary, current research on the cerebellum has revealed the fundamental principle that epigenetic regulators control every facet of neuronal differentiation in the early postnatal brain. Rapid advances in functional genomics and neuroscience fueled by state-of-the-art molecular and genetic tools promise to further clarify the functions of neuroepigenetic programming of the cerebellum in health and disease.

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