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Epigenetics in Neurodevelopment

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Abstract

Neural development requires the orchestration of dynamic changes in gene expression to regulate cell fate decisions. This regulation is heavily influenced by epigenetics, heritable changes in gene expression not directly explained by genomic information alone. An understanding of the complexity of epigenetic regulation is rapidly emerging through the development of novel technologies that can assay various features of epigenetics and gene regulation. Here, we provide a broad overview of several commonly investigated modes of epigenetic regulation, including DNA methylation, histone modifications, non-coding RNAs, as well as epitranscriptomics that describe modifications of RNA, in neurodevelopment and diseases. Rather than functioning in isolation, it is being increasingly appreciated that these various modes of gene regulation are dynamically interactive and coordinate the complex nature of neurodevelopment along multiple axes. Future work investigating these interactions will likely utilize “multi-omic” strategies that assay cell fate dynamics in a high-dimensional and high-throughput fashion. Novel human neurodevelopmental models including iPSC and cerebral organoid systems may provide further insight into human-specific features of neurodevelopment and diseases.

Keywords

Neurogenesis; Developmental Disorder; DNA methylation; Chromatin Remodelling; Epitranscriptomics

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1. Introduction

1.1. Epigenetics of neurodevelopment: regulation of cellular identity

The development of the mammalian brain requires a complex orchestration of dynamic gene expression patterns in order to generate the diversity of cell types necessary for neural function (1). Despite all cells in the brain containing the same genetic material in the form of DNA, how cells specialize into different cell types and maintain distinct functions relies upon a complex regulation of cellular transitions from a more immature to differentiated state. During these transitions, asymmetric divisions may be necessary to maintain a more "stem-like" progenitor population while expanding more differentiated cells (2).

Cortical neurogenesis occurs through the action of radial glia, specialized cells that are able to generate cortical neurons as well as supporting glial cells. Unlike rodent cortex, the embryonic human cortex harbours a large number of outer radial glia cells, which lie outside of the ventricular zone during development and are thought to enable the increased size and complexity of the human brain (3). The adult mammalian brain contains continuous neurogenic niches in the subventricular zone, which generate new-born neurons to populate the olfactory bulb (4), and the subgranular zone of the dentate gyrus, which generate new-born granule neurons in the hippocampus (5) (Figure 1A). The degree to which continuous neurogenesis occurs in the adult human brain remains an area of active investigation and debate (6).

The spatiotemporal patterning of the mammalian brain undergoes several stages of development from embryogenesis to lifelong neurogenesis, with evidence of a common embryonic origin for these stem cells (7,8). Multipotent neural stem cells generate the diversity of cell types in the brain, including neurons, astrocytes and oligodendrocytes (9-11). While the nomenclature may vary depending on the particular neurogenic niche, neurodevelopment undergoes a common pattern to generate the cellular diversity of the mammalian brain. Specialized regions of the brain contain a population of self-renewing stem cells, which can divide to repopulate this niche (Figure 1B). These cells subsequently also undergo asymmetric division into neural progenitor cells of either neuronal or glial fate, which can further expand via cell division. These cells then migrate to their final destination and eventually mature and functionally integrate into the brain (12-14).

The mechanisms by which one cell either transitions to a new cellular identity (differentiation) or retains its original identity (self-renewal and/or proliferation) are epigenetic by nature. The central dogma of molecular biology refers to the process by which the general flow of sequence information starts as DNA, becomes RNA, and subsequently becomes protein (15). Historically, proteins are regarded as the primary functional molecules that carry out the biological processes necessary for life. However, it is now widely acknowledged that multiple levels of regulation as well as inherently functional properties also occur at the DNA and RNA levels. Epigenetics typically refers to the heritable changes in gene expression not directly explained by the DNA code (16). While originally studied in the context of heritable DNA methylation patterns, the term epigenetics now encompasses a vast network of different levels of gene regulation that spans the DNA-RNA-protein axis. Here, we provide an overview of the role of epigenetics by way of the hierarchical structure

of organization and regulation. In particular, we review the role of DNA methylation, chromatin regulation, non-coding functional RNAs, and epitranscriptomics in neural development and their potential roles in brain diseases (Figure 2).

2. Epigenetic modifications

2.1. DNA methylation

2.1.1. DNA methylation and gene regulation through writers and readers—

Historically, DNA methylation was one of the first appreciated regulators of gene transcription (17-19), including its role as a key feature in X-chromosome inactivation and genomic imprinting (20). Methylation patterns are also key features of cellular identity; single-cell methylomes have been used to identify neuronal subtypes in the mammalian cortex (21). The most robustly studied type of DNA methylation comes in the form of methylation of the fifth position of cytosine (5mC), which has profound effects on gene expression (22). This modification is classically associated with transcriptional repression through the placement of 5mC along CpG dinucleotides at promoter regions. However, genome-wide analysis of promoter occupancy with transcription factors has shown differential binding of key promoters based upon variable methylation patterns (23), with evidence of enhanced promoter binding in the presence of DNA methylation at distinct promoter sequences (24). Additionally, in contrast to the repressive nature of methylation of promoter regions, DNA methylation is also found within gene bodies of actively transcribed genes (25). Intriguingly, non-promoter regions of DNA methylation have been associated with neurogenesis, highlighting the complexity of this epigenetic regulation (26). Therefore, rather than simply a mark of gene silencing, DNA methylation varies based on context with multiple functional roles deriving from this epigenetic mark (27).

DNA methylation has traditionally been detected via three types of methods: methylation-sensitive restriction enzyme-based DNA digestion, bisulfite sequencing, and affinity purification-based sequencing techniques (28). Currently, bisulfite sequencing is the most widely used of these methods (27). Utilizing this technique, bisulfite treatment deaminates cytosine into uracil, which would subsequently be read as thymine upon sequencing. 5mC samples are resistant to deamination and therefore do not change their base composition. This is then compared to untreated samples for determination of methylated cytosine (29). Affinity purification techniques employing antibodies against 5mC (30) followed by high throughput sequencing of enriched DNA (MeDIP-seq) are also utilized (30,31). Additionally, methods have been developed to profile 5-hydroxymethylcytosine (5hmC), an alternative form of DNA methylation, including Tet-assisted bisulfite sequencing (TAB-seq) (32) and oxidative bisulfite sequencing (oxBS-seq) (33).

DNA methylation is a dynamically-regulated process with diverse functions throughout development (34). In order for this regulation to occur, there are various writers, readers, and erasers of DNA methylation. DNA Methyltransferases (DNMTs) are key “writers” of this process, with different DNMTs holding various roles. For instance, DNMT1 is a maintenance methyltransferase which is believed to act during cell division in order to maintain methylation patterns onto daughter cells (35). Alternate methyltransferases have been found to establish novel patterns of methylation during development: deletion of

DNMT3A and *DNMT3B* leads to deficits in *de novo* methylation in embryonic stem cells (ESCs) without any deficits in the maintenance of imprinted methylation patterns, indicating that these methyltransferases establish new patterns of methylation upon cell division (36).

DNA methylation regulates gene expression in part through the "readers" of DNA methylation, including methyl-CpG binding protein 2 (MeCP2) and other methyl-CpG binding domain family members, such as MBD1, MBD2, and MBD4 (37). However, recent evidence suggests that these proteins may not simply be mediators of transcriptional repression. For example, MeCP2 was originally described as a transcriptional repressor due to its ability to bind 5mC and repress transcription of methylated promoters *in vitro* (38), but *in vivo* loss of MeCP2 has been shown to cause both up-regulation and down-regulation of many genes, a finding inconsistent with a typical transcriptional repressor (39). Although there is strong *in vitro* evidence suggesting MeCP2 indeed functions to repress transcription via recruitment of the NCoR/SMRT co-repressor complex (40), the molecular function of MeCP2 *in vivo* remains elusive.

In addition to CpG methylation, non-CpG methylation is a brain-enriched DNA modification that plays critical roles in neuronal development and maturation. Profiling DNA methylation in the brain at the single-nucleotide resolution has revealed that non-CpG methylation (CpA, CpT, CpC) accumulates during postnatal brain development, reaching high levels during adulthood (1). Emphasizing the functional relevance of this modification, multiple studies have shown that MeCP2 binds non-CpG methylated DNA in addition to 5mCG (41-44). Non-CpG methylation in the brain has been shown to be written by DNMT3A during postnatal development (41,42). One study has linked the functions of DNMT3A and MeCP2 within the developing postnatal brain, demonstrating that DNMT3A binds within gene bodies of low-expression genes to establish non-CpG methylation marks that are later bound by MeCP2, influencing the expression of these genes (45).

Mutations to both the writers of DNA methylation (DNA methyltransferases) as well as the protein binders of DNA methylation (such as MeCP2) lead to developmental disorders. Gain-of-function mutations in *DNMT3A* have been associated with microcephalic dwarfism, leading to hypermethylation in regions normally regulated by Polycomb-mediated histone modification (46). Loss-of-function mutations in *DNMT3A* can also cause an overgrowth syndrome with intellectual disability (47). Additionally, mutations in *DNMT3B* have been associated with immunodeficiency, centromere instability, facial anomalies (ICF) syndrome, also leading to global histone modification changes (48). Furthermore, single nucleotide polymorphisms (SNPs) in DNMTs have been associated with autism spectrum disorder (ASD) (49). Mutations to the "readers" of DNA methylation also have neurodevelopmental consequences, particularly in the case of MeCP2: *MECP2* loss-of-function mutations have been found to cause Rett syndrome (50), a progressive childhood neurological disorder characterized by developmental stagnation and subsequent regression, in which previously-learned motor, communicative, and social skills are lost (51).

2.1.2. DNA demethylation and 5-hydroxymethylcytosine: a brain-enriched base pair modification—

"Erasing" of DNA methylation is thought to occur through both active and passive means. This process is most notable during early embryogenesis, when

global demethylation occurs following the formation of the zygote and allows for the subsequent development of the vast array of cellular subtypes from a common cell of origin (52). Passive DNA demethylation refers to the dilution of 5mC during DNA replication without maintenance by DNA methyltransferases, while active DNA demethylation refers to enzymatic alterations of 5mC paired with base excision repair, wherein methylated cytosines are actively removed and replaced by their non-methylated counterparts. The process of active DNA demethylation has been demonstrated to occur through iterative oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) via the action of TET family proteins (53-55). The active DNA demethylation process is completed either by dilution of these oxidized forms of 5mC by DNA replication (56), or by thymine DNA glycosylase-mediated excision of these modified bases, which is then coupled with base excision repair, wherein methylated cytosines are actively removed and replaced by their non-methylated counterparts (57,58).

5hmC is an alternative base pair modification that is enriched in the brain compared to other mammalian tissues (59-61). While initially found to be enriched in Purkinje cells (61), it was subsequently revealed to also be enriched in the hippocampus and cortex and increases in abundance with age (60). 5hmC is generated via conversion from 5mC through the TET family of enzymes In neurodevelopment, TET1 has been identified as a key gene in the process of DNA demethylation (62). Deficiency in *TET1* leads to impaired hippocampal neurogenesis as well as hypermethylation and downregulation of genes involved in neural progenitor proliferation (63).

The mechanism by which 5hmC leads to these phenotypic changes is thought to be regulated in part by impairing the “readers” of 5mC, such as MeCP2, and therefore allowing for gene activation. 5hmC has been found to accumulate in neurons at key genetic loci during development and to be inversely associated with MeCP2 dosage (59). MeCP2 has been subsequently found to bind to 5hmCA but not 5hmCG; therefore, the presence of 5hmCG (the predominant form of 5hmC in the brain) can result in “functional demethylation” with subsequent increases in transcription (64).

2.1.3. Genomic imprinting: DNA methylation and allele-specific inheritance in neurodevelopmental diseases—In humans, approximately three billion base pairs of DNA are organized into 23 paired chromosomes, including the X and Y sex chromosomes. One copy of each chromosome is inherited from the mother and the father, leading to allelic variability. It is increasingly recognized that preferential expression of either a paternal or maternal allele can be observed and is epigenetically regulated (65). Genomic imprinting refers to the processes by which paternal or maternal genes are preferentially expressed in an allele-specific manner. Several imprinted genes have been found to have key roles in neurodevelopment.

This allelic specificity also has elements of stochasticity and region-specific silencing. A mouse model containing a tdTomato/GFP reporter of either the maternally- or paternally-expressed *Dlk-Dio3* intergenic differentially DNA-methylated region (IG-DMR) revealed that, while many cells faithfully maintained allelic expression, changes in methylation patterns were present in a tissue-specific and cell type-specific manner (66). Intriguingly,

neurogenic regions, such as the subventricular zone and subgranular zone of the dentate gyrus, have biallelic hypermethylation of the IG-DMR region, suggesting tissue-type specificity and allowing for neuronal heterogeneity (66). DNA methylation was first thought to drive this preferential expression of genomic imprinting (67). However, it is increasingly appreciated that other mechanisms, such as transcription factors (68) and histone modifications (69), also regulate this process.

Perhaps the most apparent consequence of genomic imprinting is through its manifestation and distinct inheritance pattern (70) in human diseases (71). Two of the most commonly described diseases of genomic imprinting include Prader-Willi Syndrome (PWS) and Angelman Syndrome. In the case of PWS, which is most often due to mutations in chromosome 15, the paternal copy is preferentially expressed. Therefore, mutations to the paternal gene or the aberrant inheritance of two maternal copies lead to disease states, causing a characteristic syndrome including facial anomalies, hyperphagia and intellectual disability (72). Mechanistically, small nucleolar-long non-coding RNAs (sno-lncRNAs), a class of nuclear-enriched, intron-derived long non-coding RNAs that are processed on both ends by the small nucleolar RNA (snoRNA) machinery, have been found to be deleted in PWS (73). These sno-lncRNAs normally interact with the Fox family of splicing regulators, wherein deletion (as in PWS) leads to aberrant splicing activity and subsequent neurodevelopmental phenotypes (73). In contrast, Angelman syndrome primarily involves UBE3A, an E3 ubiquitin ligase which is also located in chromosome 15 but in a region of the chromosome where the paternal gene is typically silenced. Disease-causing states occur due to mutations on the maternal chromosome or if two paternal copies are inherited. Angelman syndrome typically manifests as cognitive disability, seizures, microcephaly, and speech impairment (74).

2.1.4. Environmental alterations of DNA methylation in neurogenesis—

Environmental factors are also known to alter epigenetic states to impact development and disease pathogenesis (75). Environmental factors can alter DNA methylation signatures and contribute significantly to the regulation of neurogenesis. *Gadd45b* was found to be upregulated following electroconvulsive therapy (ECT), with a role in promoting neural progenitor proliferation and dendritic growth of new-born neurons in the adult hippocampus. This study further showed that *Gadd45b* was required for DNA demethylation at key neurogenic promoters, indicating an epigenetic role in neurogenesis (76). Conflicting results from studies of *Gadd45b* knockdown in mice demonstrated a selective deficit in fear conditioning (77) versus enhanced memory in motor performance, aversive conditioning, and spatial navigation (78). Neuronal activity has also been linked to changes in DNA methylation (79). Intriguingly, differential DNA methylation patterns in Alzheimer's disease patients compared to controls were preferentially associated with genes involved in neurodevelopment and neurogenesis (80).

Similarly, glucocorticoids have a known effect on DNA demethylation (81). With regards to neurodevelopment, glucocorticoids have been found to alter DNA methylation in the hippocampus and prime future stress responses (82). More recently, a novel modification, N(6)-methyladenine (6mA) has been identified as another form of DNA methylation in mammalian cells (83). While its role continues to be elucidated, one study has found that

6mA methylation is elevated in the brain in a chronic stress mouse model. These methylation changes overlap with genes involved in depression, schizophrenia, and ASD (84).

2.2. Histone modifications

2.2.1. Chromatin-based regulation

2.2.1.1. Chromatin structure: Chromatin refers to the higher-ordered structure through which DNA is organized to form chromosomes. Eukaryotic DNA wraps around histone proteins to form nucleosomes, the fundamental repeating unit of chromatin (85). Nucleosomes are comprised of approximately 147 base pairs DNA wrapped around an octamer of histone proteins, with two copies of the core histones H2A, H2B, H3, and H4 (86,87). A linker sequence of DNA of approximately 53 base pairs binds to the H1 histone and further contributes to chromatin compaction (88,89). These histone proteins hold not only a structural importance, but also a functional role as accessibility of DNA for RNA transcription can be regulated by the histone state, with binding affinities to histone proteins being altered by covalent, but reversible modifications to the histone proteins. Euchromatin refers to DNA that is not tightly bound to histone proteins and is believed to be accessible for transcription, meanwhile heterochromatin refers to DNA that is compacted and is consequently transcriptionally inactive (Figure 2A). During neurodevelopment, dynamic changes are necessary in order to transition from a more stem cell-like state to more differentiated progeny. Chromatin dynamics has been found to be among the key regulators for cell-state transition.

2.2.1.2. Chromatin accessibility: A common method to analyse the general epigenetic state is through an assessment of chromatin accessibility. Fundamental to this assessment is the notion that various molecules are able to interact with open chromatin (euchromatin) over compacted chromatin (heterochromatin) and that identifying regions of open chromatin can help identify active or readily activatable genes. Multiple methods have leveraged the differential accessibility of euchromatin compared to heterochromatin in order to assess the general chromatin landscape. Formaldehyde-assisted isolation of Regulatory Elements (Faire-Seq) utilizes differential rates of crosslinking (90), while DNAse-seq relies upon differential sensitivity to DNAse I. Assay for transposase-accessible chromatin using sequencing (ATAC-seq) is an increasingly utilized method to probe DNA accessibility. Rather than relying upon differential fixation, this method utilizes a hyperactive Tn5 transposase, which inserts sequencing primers to accessible DNA (91). These segments are then amplified for high throughput sequencing and are able to identify segments of open chromatin. Such methods have led to a better understanding of the dynamic changes underlying neurodevelopment.

Utilizing ATAC-seq, neuronal activity was found to result in genome-wide changes in chromatin accessibility one hour after activation and was mediated in large part at cFos binding sites (92). Unlike CpG methylation, in which 31% of activity related changes at 4 hours were maintained at 24 hours (79), the changes seen by ATAC-seq were rather transient, such that only 5.1% of gained-open regions detected at 1 hour were seen at 24 hours (92). Using ATAC-seq, the neuronal-associated transcription factor *FoxP2* was found

to alter the chromatin accessibility during neuronal differentiation, leading to repression of non-neuronal gene regions and activation of neuronal maturation genes (93). This further supports the notion that gene regulation by transcription factors involves changes in chromatin accessibility.

2.2.1.3. Histone modifications: Among the more studied modes of epigenetic regulation are histone modifications. Modifications to the H3 histone, including methylation and acetylation of lysine residues along the histone protein, have been found to be associated with transcriptional regulation (94). Histone modifications as well as direct interactions with histone modifiers are typically assayed through chromatin immunoprecipitation (ChIP) (95,96). In this method, cells are typically fixed using formaldehyde in order to maintain DNA-protein interactions prior to shearing of chromatin into approximately 300 base pair fragments. Chromatin of interest is enriched using antibodies against histone modifications, histone modifiers, or transcription factors, and then subsequently analysed using qPCR (97) or high-throughput sequencing based methods (98). This allows for profiling of the chromatin state at key genetic loci that may regulate gene expression.

One of the more prevalent models of chromatin regulation, particularly during neurodevelopment (99-101), is the notion of histone modification “bivalency” (102). H3K4me3 is a chromatin regulation mark commonly associated with gene activation (103,104). Acetylation of H3K9 (H3K9ac) is similarly associated with gene activation, and has been found to mediate the switch from H3K4me3-mediated transcriptional initiation to elongation (105). Meanwhile, H3K27me3 is a chromatin mark associated with gene silencing. Promoter regions that contain both H3K4me3 and H3K27me3 are thought to be transcriptionally inactive, but “poised” for activation. Dynamic changes in the histone state, particularly at promoter regions, are thought to be fundamental to transcriptomic dynamics during development. Chromatin profiling of neural progenitor cells reinforces this notion that resolution of histone marks influences cellular identity (99).

2.2.1.4. Histone modifications in neurodevelopment: The regulation of these histone modifications, including H3K4me3 and H3K27me3, relies upon enzymatic genes that either place (histone methyltransferases) or remove (histone demethylases) histone marks. Trithorax group proteins represent a class of proteins that regulate histone methylation, including H3K4me3, a marker of gene activation. *Mll1-1* is a Trithorax group H3K4me3 methyltransferase that was originally found to be necessary for postnatal SVZ neurogenesis, wherein a conditional knockout of *Mll-1* leads to failure to properly activate neurogenic transcription factors including *Dlx2* (106) and *Brn4* (107). Conditional deletion of a common H3K4 methyltransferase subunit, Dpy30, leads to both neurogenic and gliogenic defects (108). Other histone H3K4 methyltransferases include the SET family of proteins, such as SET1A, SET1B and MLL2-4 (109).

Among the most commonly appreciated mechanisms of gene regulation is through the Polycomb Repressive Complex (PRC), including both PRC1 and PRC2. The core PRC2 complex is composed of EZH1/2, EED164, SUZ12, and RBBP4/7, and this can be in complex with various PRC2 accessory proteins including PCL1-PCL3, JARID2, AEBP2, EPOP, and LCOR. PRC1 contains both a canonical and non-canonical complex. The core

PRC1 is comprised of RING1A/B and PCGF-PCGF6. This core PRC1 forms a complex in the canonical PRC1 with CBX-family proteins, PHC1-PHC3, and SCMH1/2. Meanwhile, the non-canonical PRC1 includes RYBP/YAF2, KDM2B, DCAF7, and WDR5. These two complexes are thought to serve as an H3K27me3 methyltransferase and mono-ubiquitinate H2AK119, respectively (110,111). Knockout of polycomb components *Ring1b* (PRC1), *Ezh2, or Eed* (PRC2) lead to defects in neurogenic to astrocytic transitions during cortical development (112). Mechanistically, *Ring1b* was found to regulate timed termination of subcerebral projection neurons (SCPNs) via binding and subsequently decreasing expression of *Fezf2,* a fate determinant of SCPNs (113). The ubiquitin-ligase activity of *Ring1b* acts primarily in early-stage repression of neurogenic genes in neural stem cells and NPCs but does not act to repress neuronal genes in late-stage astrogliogenic cells. Meanwhile, ubiquitin-independent PRC1-mediated repression relies on histone deacetylation and *Phc2*-mediated clustering (114). Ezh2 was found to be an essential regulator of developmental cortical neurogenesis (115) as well as postnatal neurogenesis in the adult SVZ (116) and hippocampus (117). One key role of Ezh2 in the developing neocortex is the prevention of early gliogenesis through suppression of *GFAP* expression via interaction with chromodomain helicase DNA-binding protein 4 (Chd4) (118). In the postnatal SVZ, *Ezh2* promotes neurogenesis in part through suppression of the *Ink4a/Arf* locus to promote cellular proliferation, temporal-specific repression of *Olig2* during neuronal differentiation, and also through persistent suppression of non-SVZ neuronal subtypes to confer SVZ lineage specificity (116).

Removers of these histone marks, or histone demethylases, have also been shown to be essential for neurogenesis. *Jmjd3* (also known as KDM6B) is an H3K27me3 demethylase that opposes the action of the PRC2 complex by removing the H3K27me3 mark (119). It has been found to be important on a genetic level to remove repressive marks at key neurogenic loci during neuronal differentiation. Conditional deletion of *Jmjd3* results in a neurogenic defect in the subventricular zone of postnatal mice, and JMJD3 acts at both promoter and enhancer regions of *Dlx2* to promote neurogenesis (120). UTX (also known as KDM6a) is another H3K27me3 demethylase that has been associated with neurodevelopment. Deletion of *Utx* impairs hippocampal function through reductions in long-term potentiation and amplitude of miniature excitatory postsynaptic currents, aberrant dendrite development and synapse formation. This phenotype is mediated in part through reduced expression of 5-hydroxytryptamine receptor 5b *(Htr5b*) (121). In another example, PHF2, a histone demethylase of H3K9me3, regulates the cell cycle of neural progenitors during DNA damage and genome instability (122).

Histone acetylation is typically known as a marker of active gene transcription (123). Liver-driven alcohol metabolism has been associated with elevated levels of acetate that acts on brain histone acetylation (124). However, much of what is understood of histone acetylation is via the role of histone deacetylases (HDACs), which remove histone acetylation. HDAC1 and HDAC3 are key drivers of embryonic neurogenesis, mediating H3K9 acetylation (125). HDAC inhibitors, such as valproic acid, are commonly used to treat neurologic and psychiatric diseases, such as epilepsy, bipolar disorder, and depression (126).

2.2.1.5. Histone modification-based dysregulation in diseases: Mutations in histone modifiers have been identified to cause various developmental syndromes (127). Mutations in *MLL* complex genes are associated with Wiedemann-Steiner syndrome, which is characterized by hypertrichosis cubiti, short stature, intellectual disability, and a distinct facial appearance (128). Mutations and deletions of *UTX* are associated with Kabuki syndrome, a rare genetic disease that causes developmental delay, craniofacial, and limb anomalies (129,130). Mutations to the histone acetyltransferase *KAT6A* have been identified as putative pathogenic variants to a rare neurodevelopmental disorder that includes global developmental delay, impaired speech development, and facial dysmorphism (131).

Genetic variants in genes involved in chromatin remodelling have also been associated with neurological disorders. For example, large exome sequencing studies have found that a number of ASD candidate genes encode chromatin remodellers, with genetic variants in CHD8 being particularly common (132,133). CHD8 normally interacts with the transcription factor REST, with haploinsufficiency causing autism-like phenotypes (134) and altered brain development in mice (135). AUTS2, encoded by another ASD susceptibility gene, interacts with the PRC1 repressive complex to paradoxically activate gene expression (136). Interestingly, chromatin-remodelling factors were also identified as a significant set of mutated genes when analysing the somatic landscape of glioblastoma (137). In one study, 135 of 291 (46%) of patients were found to have mutations in genes associated with chromatin remodelling, suggesting a significant epigenetic contribution to glioblastoma. The PRC2 complex is also notably found to be a key component of the tumorigenic phenotype of the H3K27M mutation (138,139), which is found in diffuse intrinsic pontine gliomas (DIPGs) and other midline gliomas. Inhibition of EZH2 in a mouse model of H3K27M-associated DIPG abolished cell growth through induction of the tumor suppressor $p16^{INK4A}$, implicating PRC2 as a possible therapeutic target in H3K27M-associated DIPG (140).

2.3 Higher-order chromatin structure

While promoter elements are a key part of genetic regulation, it is now increasingly appreciated that enhancer elements are also a contributing mechanism of chromatin context-specific gene regulation (141). These enhancer elements act in the context of three-dimensional chromatin architecture, operating *in cis* to increase gene expression by binding specific transcription factors and stabilizing transcription factor-promoter interactions (142). Though typically distal to the gene promoter in a two-dimensional map, enhancers interact directly with gene promoters through chromosomal looping in a three-dimensional fashion (143). However, more recent studies of neural differentiation have suggested that the Sonic Hedgehog (*Shh*) gene can be activated through increased physical separation, suggesting that looping may not be the only mechanism involved in the function of enhancer elements (144).

Enhancers have been thought to mediate cell type-specific gene expression programs with temporal specificity by recruiting specific transcription factors and chromatin remodelling complexes (145,146). Since enhancers carry important functions, a significant amount of effort has gone into identifying genome-wide enhancer-promoter interactions with a variety of different techniques. Enhancers have traditionally been identified with reporter assays, but

the advent of next-generation sequencing technologies has allowed for genome-wide predictions of putative enhancers. Some of these techniques involve profiling epigenetic marks associated with enhancers by leveraging the association of enhancers with particular chromatin states. In contrast to promoter regions, enhancers are commonly identified by association with H3K4 monomethylation (H3K4me1), H3K27 acetylation (H3K27ac), and p300 binding at non-promoter gene regions (147-150). Intriguingly, these enhancer regions respond to neural activity to regulate transcription (149).

Some of the most powerful techniques to understand enhancer-promoter dynamics are those that allow for the study of the three-dimensional architecture of the genome. These methods typically rely upon fixation of the three-dimensional state and the capture of this interaction through DNA ligation reactions. The fundamental concept of these chromosome conformation capture (3C) assays is to capture long-range DNA interactions through fixation and subsequent DNA enrichment (151). 3C technology has been combined with next-generation DNA sequencing in a technique called Hi-C to capture genome-wide long-range DNA interactions (152). These methods have not only allowed for identification of enhancers and their targets, but also provide fundamental information about higher-order chromatin architecture and how the genome is organized in three-dimensions. It has become clear that the genome is hierarchically organized into different features at different resolutions, with chromosomal compartments (the spaces occupied by each individual chromosome in the nucleus) as the largest organizational feature.

Hi-C has revealed that, on the subchromosomal scale, the genome exists as two compartments, denoted A and B compartments (152). The A compartment is enriched for actively transcribed genes and is thought to represent open chromatin, while the B compartment is broadly inactive and is thought to represent closed chromatin (Figure 2A) (153). On a finer scale, Hi-C maps have revealed that a large proportion of the genome is organized into topologically- associated domains (TADs), which are regions of DNA that associate preferentially with other sequences in the TAD and have boundaries demarcated by CTCF binding sites (154). Sub-TADs have also been identified, representing an even finer level of genome-wide organization (155). Chromatin loops have been identified as the organizational feature at the finest level of detail and may mediate enhancer-promoter interactions (156). These high-dimensional features have been studied extensively in mouse neural development, which demonstrated dynamic changes of TADs during neuronal differentiation, including disruption of a polycomb network and the appearance of neural transcription factor interactions (157).

As many of the disease-associated SNPs in neurodevelopmental disorders are found in non-coding regions of the genome, it is thought that one mechanism of functional consequence is the aberrant regulation of enhancer elements (158). As a neural stem cell marker, *Sox2*, has been recently found to be a key transcriptional factor that is bound to both promoter and enhancer elements in order to regulate gene transcription. Deletion of *Sox2* leads to disruption of the long-range interactions between enhancer and promoter regions with a subsequent decrease in gene expression. Exogenous rescue of *Socs3*, a gene affected by *Sox2* disruption, restored the self-renewal defect of *Sox2*-deleted NSCs (159). Meanwhile, enhancer-gene interactions in neural development unique to humans are enriched in outer-

radial glia, while GWAS analysis found genetic variants for educational attainment, neuropsychiatric disease, and brain volume to be enriched within regulatory elements involved in cortical neurogenesis (160). This work further elucidates the interaction between transcription factors and enhancer elements in the maintenance of cellular function and identity.

2.4. Non-coding RNA-based regulation

2.4.1. Non-coding RNA—In addition to chromatin-based regulation, it is being increasingly appreciated that RNA-based mechanisms also influence cellular identity. Non-coding RNAs have functional roles in regulating gene transcription. Anti-sense RNAs have been found throughout the genome and are associated with regulating nearby gene transcription (161). Long non-coding RNA (lncRNAs) are polyadenylated RNAs that have been found to operative *in trans* and interact closely with epigenetic regulators, such as chromatin modifying factors. LncRNAs carry out a variety of different functions, and have been identified as key regulators of pluripotency and differentiation, with knockdown of lncRNAs having similar consequences on transcription as known ESC regulators (162).

2.4.2. Non-coding RNA in neurodevelopment—As the first functional non-coding RNA identified in neurodevelopment, *Evf2* was initially identified as a lncRNA that regulates the transcription of *Dlx5* and *Dlx6* in the developing mouse forebrain, the knockout of which leads to reduced early production of GABAergic interneurons and reduced synaptic inhibition despite a normalization of neuronal levels in the adult hippocampus (163). *Evf2* was noted to regulate transcription in *trans* through inhibition of CpG methylation of *Dlx5* and *Dlx6* enhancer regions (164). A direct interaction of chromatin remodelling inhibition was demonstrated with *Evf2* co-localizing with SWI/SNF-related chromatin remodellers Brahma-related gene 1 (*Brg1*) and Brahama-associated factor (Baf170) in the developing mouse forebrain (165). These findings implicated lncRNAs as a key functional component of various mechanisms of epigenetic regulation.

With the advent of next generation sequencing, a catalogue of lncRNA expression was generated for adult SVZ neural stem cells with some evidence of a functional role for lineage specification (166). *Pnky* was identified to have a critical role in neurogenesis wherein knockdown of *Pnky* led to an increase in neuronal progenitors primarily through direct action on the splicing regulator PTBP1 (167). Conditional deletion of *Pnky* from the developing cortex altered cortical lamination and a BAC transgene rescued this defect, indicating that *Pnky* regulates development *in trans* (168). Transcriptomic characterization of lncRNAs during embryonic development similarly demonstrated strong correlation of expression of lncRNAs with neurogeneic genes. *Miat* is a lncRNA determined to affect brain development and regulate the splicing of Wnt7b (169).

Other lncRNAs have been found to regulate cortical development in *cis* and interact with histone modifying enzymes. LncKdm2b acts in *cis* to activate Kdm2b, a H36me2 and H3K4me3 demethylase, allowing for proper differentiation and migration of cortical projection neurons (170). Single-cell RNA sequencing analysis of lncRNAs in the developing human neocortex indicated that despite being detected at low levels in bulk

sequencing, lncRNAs can be abundantly expressed in single cells suggesting cell-type specificity (171). Utilizing both primate and human cerebral organoid models and performing RNA sequencing over time, lncRNA conservation was observed across species with transient expression in a cell-type specific manner (172). While the diversity of expression has been established, the functional roles of many lncRNAs are still unclear. Traditional analysis for lncRNAs rely upon either RNA knockdown (167,173) or genetic deletion and *in vivo* characterization (163,168). However, with the advent of CRISPR-CAS9 (174) high-throughput methods have also been developed for assessing lncRNA function (175). Future work may allow for screening on specific cell types during neurodevelopment.

2.4.3. lncRNAs in diseases—LncRNAs have been implicated in the pathogenesis of various diseases normally associated with other modes of epigenetic regulation. LncRNA *FMR4*, which is located at the Fragile X mental retardation 1 (FMR1) gene, is a chromatin-associated transcript that is enriched in genes related to neural development and cellular proliferation (176). *FMR4* has also been shown to regulate methyl-CpG-binding domain protein 4 (MBD4) (177). LncRNAs have also been associated with ASD. *SHANK2-AS* is upregulated in ASD, and exogenous overexpression leads to inhibition of neuronal proliferation and promotion of apoptosis (178). *lnc-NR2F1* was recently identified as a candidate gene in a cohort of children with ASD/intellectual disability. *lnc-NR2F1* enhances neuronal cell maturation and regulates transcription of neuronal genes including ASD-associated genes (179).

2.5. Epitranscriptomics

In addition to functional roles of non-coding RNAs, modifications of RNA transcripts through various nucleotide modifications have also been found to be a key component of gene regulation. Epitranscriptomics refers to the various posttranscriptional changes in mRNA and lncRNA in order to regulate gene expression (180). Over 100 chemical modifications have been identified in RNA. These reversible modifications have multiple roles, but act in part through altering mRNA metabolism and translation. Perhaps the most well studied form of epitranscriptomic modification is the methylation of adenosine at the sixth nitrogen position (m^6A), which has a myriad of roles at various levels of gene regulation (181).

2.5.1. N^6-methyladenosine—m^6A modification to RNA has been found to be a key feature of post-transcriptional regulation of the dynamic changes necessary for neurodevelopment. Similar to histone marks, this modification has known machinery including the “writers” (METTL3, METTL14, and WTAP), “erasers” (FTO (182) and ALKBH5 (183)), and “readers”, such as FMRP, YTHDF1, and YTHDF2. m^6A modification in particular is known to have multiple roles, including the promotion of mRNA decay, translation, regulation of epigenetic histone modification, and promoting nuclear RNA export (184).

Detection of m^6A can be performed by utilizing antibody based methods against modified RNA such as MeRIP-seq (185) and m^6A-seq (186). More recent work has determined that m^6A can be detected through an analysis of increased systematic errors and decreased base-

calling qualities. This method can detect m^{6}A with approximately 90 percent accuracy in synthetic sequences and with 87 percent accuracy *in vivo* (187).

2.5.2. Multiple roles of m^{6}A in neurodevelopment—Analysis of m^{6}A signalling on cortical neurogenesis using an embryonic *METTL14* knockout model demonstrated a prolonged cell cycle of radial glia and extension of cortical neurogenesis into the postnatal stages. Furthermore, m^{6}A was found to be associated with mRNA turnover, with m^{6}A tagging promoting mRNA decay (188). Loss of Ythdf2, a reader of m^{6}A, has been associated with delayed degradation of neuron differentiation-related genes, with a failure to produce normally functioning neurites (189). m^{6}A has been found to act locally upon axons, with inhibition of m^{6}A demethylase FTO leading to increased m^{6}A levels and consequent decreased translation of GAP-43 mRNA (190). Similarly, the m^{6}A reader YTHDF1 binds to mRNA of *Robo 3.1*, an axon guidance regulator, in order to promote protein translation. Deletion of *Ythdf1* results in axon guidance defects (191).

m^{6}A was also determined to be a key feature of mRNA transport. Utilizing a *Fmr1* knockout mouse, Fragile X mental retardation protein (FMRP) was determined to bind m^{6}A and promotes nuclear transport of methylated mRNA targets during neural differentiation. *Mettl14* conditional knockout mice are unable to obtain m^{6}A modifications and have a nuclear export deficit for some mRNAs (192).

m^{6}A modification has also been shown to be involved in the regulation of histone modifying enzymes. Knockdown of *Mettl3* led to a decrease in both *Ezh2* expression and H3K27me3 levels, leading to a neurogenic defect that could be rescued by *Ezh2* overexpression (193).

In the adult brain, m^{6}A has been shown to regulate phenotypic changes in the hippocampus. Deletion of *Ythdf1* results in learning and memory deficits as well impaired synaptic transmission and long-term potentiation. Transcriptome-wide analysis of m^{6}A RNA interaction with its reader YTHDF1 showed enrichment for key neuronal plasticity genes (194).

2.5.3. Epitranscriptomics in diseases—While the role of m^{6}A is still being elucidated, mutations in genes associated with epitranscriptomic functions have been identified in diseases. Although FTO is named for its association with obesity (195), genetic associations have also noted that mutations in *FTO* are related to risk for bipolar disorder. *In silico* analysis indicated that these mutations disturb binding sites of SP1 and SP2, which are distinct from the obesity-associating binding site perturbation of FOXP1 (196). Direct mutation of the enzymatic dioxygenese-encoding portion FTO was identified in a single family, leading to an autosomal recessive lethal syndrome associated with postnatal growth retardation, microcephaly, severe psychomotor delay, functional brain deficits, and characteristic facial dysmorphism (197). A murine model of focal ischaemia found that ischaemic pathology leads to a decrease in FTO and associated increase in m^{6}A abundance. Differentially expressed m^{6}A methylated genes were related to inflammation, apoptosis, and transcriptional regulation (198). These varied phenotypes related to FTO support the notion of the vast diversity of function in epitranscriptomics.

3. Epigenetic crosstalk: systems in combination rather than isolation

As the various components of epigenetic regulation become elucidated, it becomes increasingly obvious that these various mechanisms have significant interactions. As one example, Fragile X Syndrome is classically described as an expansion of CGG nucleotide, which leads to hypermethylation of the *FMR1* promoter (199). However, recent analyses have also shown that patients with Fragile X, as well as other repeat expansion disorders, have alterations in higher-ordered chromatin structure in the form of boundaries between chromatin domains. The extent of disruption of these chromatin domains correlates with *FMR1* silencing (156). FMR5 and FMR6 are two lncRNAs that have been identified to be expressed within the *FMR1* locus, with FMR6 being silenced in patients with both full mutations and pre-mutations, indicating its potential as a predictive biomarker (200). FMR1 protein interacts directly with *TUG1*, a lncRNA distal to the *FMR1* gene, to decrease its stability. Knockdown of *TUG1*, promotes axon development in cultured hippocampal neurons, with overexpression leading to axonal growth defects during embryonic cortical development. Knockdown of *TUG1* rescues axonal developmental defects in FMRP-deficient neurons, further emphasizing this dynamic interplay of epigenetic regulation (201). FMRP also regulates m^6A-marked mRNA targets. Loss of FMRP leads to widespread changes in the m^6A landscape, and FMRP was found to normally maintain stability of m^6A mRNAs through interaction with the m^6A reader, YTHDF2 (202). Therefore, developmental diseases can have various modes of epigenetic dysfunction as both cause and consequence that encompasses DNA methylation, chromatin structure, lncRNAs, and epitranscriptomics.

4. Challenges and future directions in epigenetics in neurodevelopment

4.1. Multi-omic analysis: combining novel technologies

While many of the components of epigenetics have traditionally been investigated in isolation, emerging technologies allow for the study of multiple aspects of epigenetic regulation in a “multi-omic” fashion and on a single-cell level (203). Methods have been developed to simultaneously sequence genomes, DNA methylation and transcription (204,205), chromatin state/nucleosome positioning, DNA methylation, copy number variation and ploidy (206), and chromatin accessibility, DNA methylation, and transcription (207). Developing a single-cell understanding of the coordination of these multiple processes may shed light on the multifaceted nature of neurodevelopment.

One of the fundamental challenges of the recently appreciated role of non-coding RNAs is the need to better identify the functional roles of this newly identified class of molecules. An emerging method to identify non-coding RNA function is through the use of CRISPR/CAS9 high-throughput screening efforts (175). More recently, CRISPR/CAS9 screening has been combined with single-cell epigenomic profiling in order to understand the epigenetic effects of CRISPR perturbations (208). As lncRNAs have been associated with many elements of epigenetic regulation, combining lncRNA-targeting CRISPR screens with single-cell epigenomic profiling could provide key insights into the degree to which lncRNAs may contribute to the epigenetic landscape.

4.2. Neurodevelopmental models

4.2.1. Mouse models of neurodevelopment—*Mus musculus* models have been a predominant means to study neurodevelopment. These animal models provide an avenue for genetic manipulation, which can facilitate the understanding of neurodevelopment in the context of knock-in/knock-out phenotypes of known disease-associated genes (209). These phenotypes can then be studied across many levels including molecular, developmental, structural, and behavioural changes upon genetic alteration (209). However, one potential limitation is the applicability of such findings in humans. For instance, murine and human m^6A profiling have distinct features such that the species is a higher determinant of methylome similarity than the tissue type (210). Therefore, alternative approaches have been developed to study the mechanisms of neurodevelopment in human cells.

4.2.2. Induced pluripotent stem cells (iPSCs)—iPSCs are multipotent stem cells that are generated from the "de-differentiation" of fully differentiated cells, commonly fibroblasts (211). iPS cells allow for the study of human cells rather than other animal systems, which may not have equivalent regulatory systems. This can be particularly important in situations wherein human-specific features might need to be studied. For instance, an emerging line of work has been the use of neurons differentiated from iPS cells derived from families with known neurodevelopmental diseases to study the effects of inherited mutations in control versus affected individuals in the same family. This has been utilized in families with Rett Syndrome demonstrating impaired neuronal maturation and function (212), and in mental disorders associated with *DISC1* (disrupted-in-schizophrenia-one) wherein mutations in *DISC1* lead to impaired interaction with Activating Transcription Factor 4 (ATF4) resulting in transcriptional and synaptic dysregulation (213). However, the iPSC system lacks the capacity to model *in vivo* phenotypes. Furthermore, the three-dimensional cellular architecture that occurs in animal models is difficult to recapitulate in these cell-based monolayer culture systems.

4.2.3. Cerebral organoids: A three-dimensional culture model—Unlike traditional iPSC-derived monolayer cultures, cerebral organoids have been recently developed that grow in three-dimensional space and maintain a three-dimensional cellular architecture that in many aspects mimics human development, including cells with markers of outer radial glia (214-217). This has been leveraged to study neurodevelopmental structural disorders of the developing human brains such as lissencephaly (218) and the developmental consequences of known mutations associated with psychiatric disorders such as schizophrenia (219). One prominent example has been the use of cerebral organoids to understand the neurodevelopmental effects of Zika virus, which is associated with microcephaly following fetal exposure. Zika virus was found to preferentially affect neural progenitor cells and consequently alter cellular architecture and neuron generation (217). Future work can leverage both multi-omic strategies and these three-dimensional cerebral organoid models to elucidate the dynamic epigenetic changes occurring in the developing human brain.

5. Conclusion

Neurodevelopment relies upon multiple layers of temporal and spatial gene regulation in order to generate the diversity of cellular subtypes appreciated in the adult mammalian brain. Epigenetic mechanisms guide this diversity of gene expression through a variety of modifications to DNA, histone proteins, and subsequent RNA. While the central dogma of biology has largely held true for many biological processes, there is a growing appreciation of the complexity of the transition from DNA to RNA to protein. Regulatory roles of non-coding DNA, such as enhancer elements and functional non-coding RNA, such as lncRNAs, also further complicate this perhaps overly simplistic model. DNA methylation, histone modifications, non-coding RNA, and RNA modifications are among the various features of gene regulation that help coordinate proper cellular identity and function. These features do not act in isolation, but rather interact together in order to generate defined cellular identities during neural differentiation and subsequent maturation. Future work may utilize novel neurodevelopmental models and assay these various levels of gene regulation simultaneously and on the single cell level, in order to gain a more precise understanding of the dynamic interaction between these various players in neurodevelopment.

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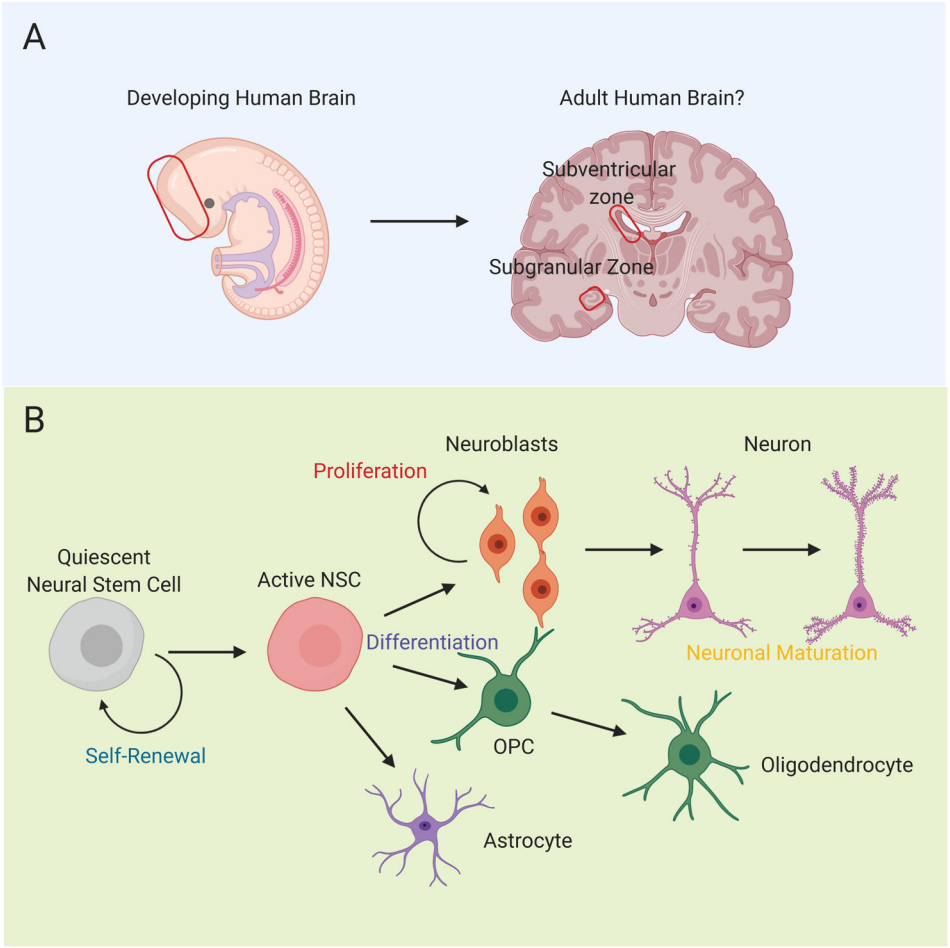

Figure 1. Regions of Mammalian Neurodevelopment.

(A) Embryonic neurodevelopment requires a complex orchestration of various cell types to generate the mammalian brain (red rectangle). As much of the brain becomes fully mature, enduring regions of adult neurogenesis have been characterized in the subventricular zone (red rectangle: top) and subgranular zone of the hippocampus (red rectangle: bottom). The degree to which this occurs in adult humans is an area of active investigation and debate. **(B)** Neural stem cells have several features including the process of self-renewal from stem cells that can become quiescent. Active neural stem cells can differentiate into multiple progeny including cells of neuronal, oligodendrocyte, and astrocyte lineages. Neuronal lineage cells (Neural progenitor cells) often undergo cellular expansion prior to neuron differentiation and subsequent maturation.

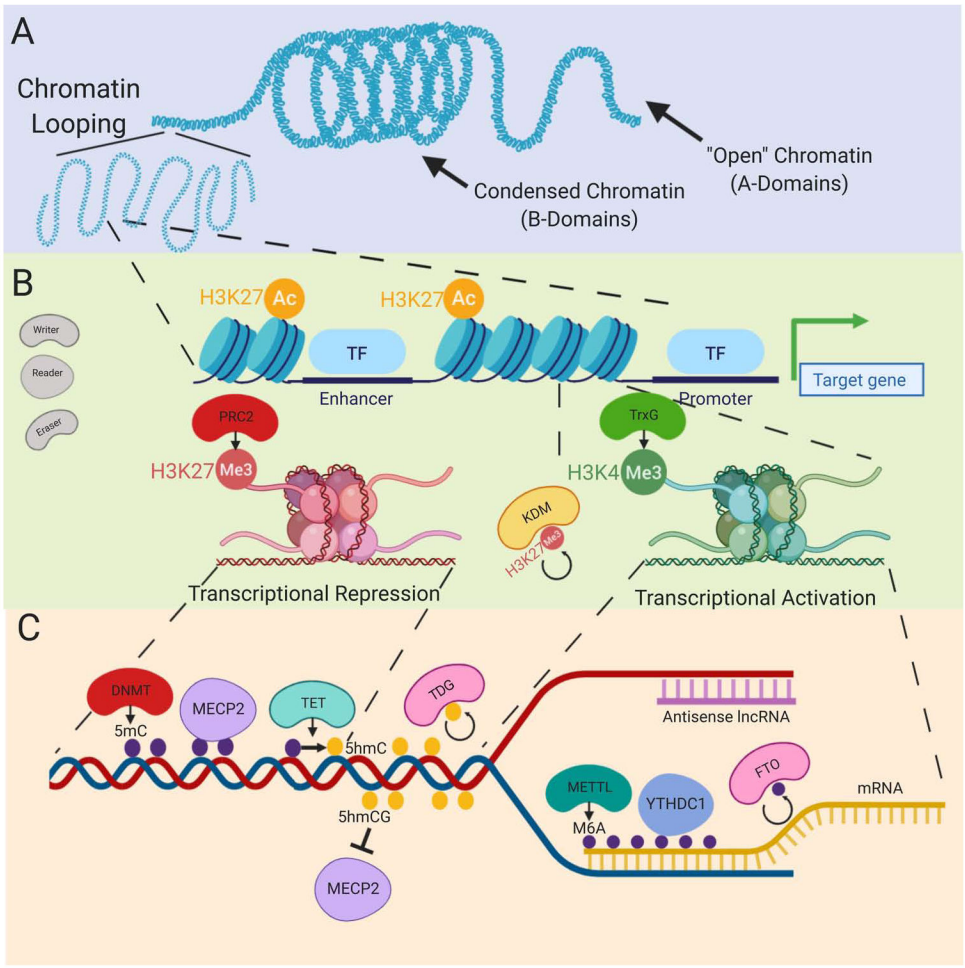

Figure 2. Epigenetic Mechanisms of Gene Regulation

(A) Epigenetic regulation of higher ordered structures begins on the level of accessibility of chromatin. A-type chromatin (euchromatin) refers to open chromatin that is accessible for transcription, while B-type (heterochromatin) chromatin refers to closed chromatin that is typically association with gene inactivity. These areas of open chromatin can form chromatin loops that generate 3-dimensional structures that allow for long-range interactions that are not directly observable from the 2-dimensional sequence. **(B)** Long range interactions generated by 3-dimensional chromatin loops can form enhancer complexes, wherein transcription factors can bind to enhancer regions that are distal to the promoter region in 2-dimensions to influence gene expression. These enhancer regions are associated with histone modifications including H3K27ac (pictured), H3K4me1, as well as the enhancer-associated p300 protein. Promoter regions can be either inactive, which is associated with the Polycomb Repressive Complex-group catalysed H3K27me3 (pictured) or active, which is associated with the Trithorax group catalysed H3K4me3 (pictured). Regions with both H3K27me3 and H3K4me3 are considered inactive but poised for transcription. Removal of H3K27me3 by histone demethylases (KDM family proteins) can lead to transcriptional activation. **(C)** DNA methylation is written by DNA methyltransferases (DNMTs) and is traditionally associated with gene silencing due to

interactions with MECP2. 5mC DNA methylation is removed through oxidation to 5hmC by TET family proteins and subsequent TDG-mediated excision, leading to base excision repair. MECP2 does not bind to 5hmCG, which is associated with regions of transcriptional activation. Transcription can be regulated by m^6A modification on the RNA strand. This modification is written by METTL family proteins, read by various readers including YTHDC1, and enzymatically removed by various erases including FTO. m^6A modification has diverse roles including regulating mRNA metabolism and translation and mediating RNA nuclear transport. Non-coding RNAs, including long-noncoding RNAs (lncRNAs), frequently occur on the anti-sense strand of mRNAs. These lncRNAs can operate locally *in cis* or distal to the area of transcription *in trans* in order to regulate gene expression.