REVIEW

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Deciphering the Epigenetic Code in Embryonic and Dental Pulp Stem Cells

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A close cooperation between chromatin states, transcriptional modulation, and epigenetic modifications is required for establishing appropriate regulatory circuits underlying self-renewal and differentiation of adult and embryonic stem cells. A growing body of research has established that the epigenome topology provides a structural framework for engaging genes in the non-random chromosomal interactions to orchestrate complex processes such as cell-matrix interactions, cell adhesion and cell migration during lineage commitment. Over the past few years, the functional dissection of the epigenetic landscape has become increasingly important for understanding gene expression dynamics in stem cells naturally found in most tissues. Adult stem cells of the human dental pulp hold great promise for tissue engineering, particularly in the skeletal and tooth regenerative medicine. It is therefore likely that progress towards pulp regeneration will have a substantial impact on the clinical research. This review summarizes the current state of knowledge regarding epigenetic cues that have evolved to regulate the pluripotent differentiation potential of embryonic stem cells and the lineage determination of developing dental pulp progenitors.

INTRODUCTION

In recent years, the field of epigenetics has grown into one of the most rapidly expanding research endeavors to date [1-4]. An increasing amount of evidence suggests that tooth development, enamel formation, and periodontal disease require tight coordination between transcriptional regulation and epigenetic modifications in stem cell precursors [5-12]. Among stem cells, dental pulp stem cells (DPSCs†) represent a unique population of precursor cells isolated from the postnatal human dental pulp capable of regenerating a reparative dentin-like complex [13,14]. DPSCs retain the ability to differentiate into different cell types including melanocytes, odontoblasts, osteoblasts, chondrocytes, adipocytes, neural progenitors, cells of blood vessels, and smooth muscle [15-18]. The following characteristics such as relative abundance, low morbidity, differentiation potential and

tolerance to biomaterials make DPSCs highly promising for tooth tissue engineering and craniomaxillofacial regeneration [19-24]. Moreover, the capacity of DPSCs to differentiate into specialized cells makes them attractive candidates for clinical applications [25-31].

DPSCs originate from the cranial neural crest (NC) and have some characteristics of neural crest progenitors, a migratory cell population that gives rise to a broad range of cell types [32-35]. It has been proposed that evolution of the NC-specification program has enabled cells at the neural plate border to acquire multipotency and migratory ability [36]. A subgroup of transcription factors termed neural plate border specifiers is required for the establishment of the neural plate border (Figure 1), as well as regulation of the downstream target factors known as neural crest specifiers, which mediate the induction of neural crest lineage [35].

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†Abbreviations: CTCF, CCCTC-binding zinc finger transcription factor; CpG, cytosine and guanine rich dinucleotides; CGIs, CpG islands; DNMT, DNA methyltransferases; DF, dental follicle; DP, dental pulp; DPSC, dental pulp stem cells; eRNA, enhancer RNA; ESC, embryonic stem cells; FRα, folate receptor alpha; HAT, histone acetyltransferase; HDACs, histone deacetyltranferases; HDACi, histone deacetylase inhibitors; HMT, histone methyltransferase; LPS, bacterial lipopolysaccharide; lncRNA, long non-coding RNA; MAPK, mitogen-activated protein kinase; MBD, methyl CpG-binding protein; miRs, microRNA; NC, neural crest; NuRD, nucleosome remodeling deacetylase; Pol II, RNA Polymerase II; PcG, polycomb; PRC, polycomb repressive complex; TAD, topologically associating domain, TDG, thymine DNA glucosylase; TET, ten-eleven translocation protein; 3D, the three-dimensional; TrxG, trithorax; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytocine; 5 caC, 5-carboxycytocine; 5-aza-CdR, 5-aza-2'-deoxycytidine.

Keywords: DNA methylation, histone modifications, chromatin topology, enhancers, embryonic stem cells, dental pulp stem cells, non-coding RNA
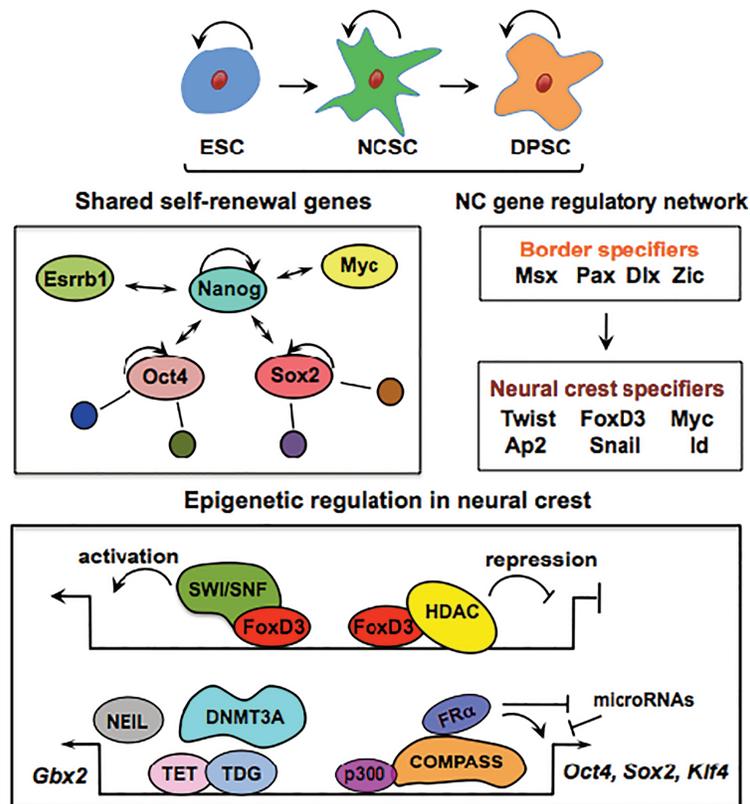
Figure 1. The self-renewal potential of embryonic stem cells (ESC), neural crest stem cells (NCSC) and dental pulp stem cells (DPSC) is dependent upon a shared gene regulatory network of self-renewal factors (left) and neural crest specifiers (right). The combinatorial activity of different epigenetic enzymes and chromatin remodeling complexes ultimately fine-turns the transcriptional output of cognate genes in neural crest in response to diverse signaling (bottom). FoxD3 binds to SWI/SNF complex to facilitate transcriptional activation, while interaction between FoxD3 and histone deacetylases (HDACs) attenuates the expression of occupied genes. The NEIL family of DNA glycosylases cooperates with thymine DNA glycosylase (TDG), TET dioxygenases, and DNA methyltransferases (DNMT) to control target genes. The expression of pluripoteny genes including *Oct4*, *Sox2,* and *Klf4* can be regulated by microRNAs. FRα, folic acid receptor, can activate pluripotency genes via microRNA inhibition.

There is growing evidence that the epigenetic landscape underlies the dynamic behavior of regulatory circuits orchestrating the NC fate specification [35,37-39]. In this regard, DNA methyltransferase DNMT3A has been shown to be critical for enabling the activation of NC specifier Gbx2 (Figure 1), an essential transcription factor for normal development of the inner ear [40]. In another study, DNA demethylation, which is mediated by the TET-TDG enzymatic complex, has been shown to regulate the formation of NC structures [41]. The NEIL family of DNA glycosylases cooperates with thymine DNA glycosylase (TDG) and TET dioxygenases in removing oxidized methylcytocines and specifying NC development. Furthermore, a recent report by Mohanty et al. identified a significant relationship between pluripotency factors and microRNAs [42]. The authors uncovered that folate receptor FRα, a mediator of folic acid biogenesis, directly activates *Oct4*, *Sox2* and *Klf4* genes in cranial NC cells. At the same time, FRα downregulates specific microRNAs, miR-138 and miR-let-7, which target pluripotency genes thus adding another dimension to gene regulatory machinery associated with the formation of NC lineage (Figure 1).

DNA methylation, post-translational modification of histone tails and non-coding RNAs are essential components of the epigenetic code, a set of fundamental principles that govern biological processes including differentiation of embryonic stem cells (ESCs), cell fate decision, and disease progression [43-48]. The scrutiny of the human epigenome in both embryonic and adult stem cells has become one of the most important strategies for elucidating the topography of regulatory circuits controlling cell-fate determination [49-52]. The spatial organization of metazoan genomes into the three-dimensional (3D) nuclear scaffold is dependent upon the contribution of several architectural protein complexes including CTCF, Mediator, and cohesin [53-56]. During cell type specification, the establishment of distinct chromatin states is facilitated by of the 3D structure that links long-range genomic interactions to the coordinated control of gene expression
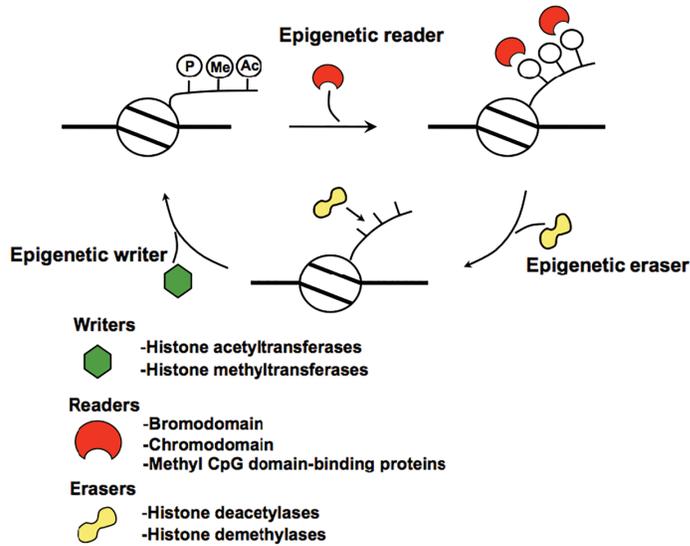
Figure 2. Epigenetic writers, readers and erasers maintain the epigenomic landscape of cells. Among epigenetic writers are histone acetyltransferases (HATs), histone methyltransferases (HMTs), and DNA methyltransferases (DNMTs) that transfer epigenetic marks to histone tails. The members of bromodomain, chromodomain and methyl-binding domain (MBD) protein families represent epigenetic readers that recognize different epigenetic modifications on nucleosomes and DNA. Epigenetic erasers such as histone deacetylases (HDACs), histone demethylases (HDMs) and members of the TET family of DNA hydroxylases remove epigenetic marks.

[49,50,57,58]. For instance, the Polycomb complex, well known for its genome-wide epigenetic repression, is involved in the structural reorganization of the 3D genome during stem cell differentiation as well as the maintenance of cellular memory [59,60].

The origin of dental pulp suggests that DPSCs have inherited a subset of NC-specific modules from the predecessor regulatory network (Figure 1). The combinatorial activity of some NC specifiers is most likely to contribute to the formation of the dental pulp-specific regulatory nodes that carry the molecular blueprint of orofacial and dental development. The common origin of osteoblasts, cementoblasts, and odontoblasts from the cranial NC is reflected in the similarity of gene expression profiles, although distinct epigenomic states delineate transcriptional programs during cell fate determination [61,62]. The regulatory genes encoding the pluripotency transcription factors and the NC specifiers including Oct4, Nanog, Rex1, Sox2 and FoxD3 have been identified in mouse DPSCs [63]. The relationship between these master regulators is complex and has been linked to self-renewal and differentiation. There is evidence that OCT4, NANOG, SOX2 and STAT3 contribute significantly to terminal differentiation of ameloblasts and odontoblasts in the tooth germ of human fetuses [64]. It was also documented that OCT4/NANOG axis maintains the mesenchymal stem cell-like property in the human DPSCs [65]. Upon differentiation of ESCs to epiblast stem cells, the forkhead transcription factor FoxD3 facilitates the simultaneous establishment of active and repressive chromatin configuration at gene targets [66]. FoxD3 is able to interact with histone deacetylases to attenuate activation of its cognate enhancers, while concurrently recruiting the SWI/SNF chromatin-remodeling complex to promote a more open chromatin via nucleosome eviction at the bound sites (Figure 1). Recently, Fujita et al. observed that during specification of mouse NC stem cells, master regulators Oct3/4, Sox2, and Nanog as well as chromatin remodeling factor

CHD7 co-bind at the H3K4me1/me3-positive regulatory elements of *FoxD3* [67].

EPIGENETIC MECHANISMS IN EMBRYONIC STEM CELLS

Although this overview highlights the latest advances in the understanding of the epigenetic processes underlying the molecular control of DPSCs, I will first summarize the current state of knowledge regarding the regulatory framework, which orchestrates ESC specification, commitment and differentiation. A more detailed discussion of the epigenetic events underlying stem cell differentiation, cellular reprogramming and development can be found in a number of excellent review articles that have been published within the last few years [68-70].

Based on their shared molecular pathways, epigenetic modulators can be divided into three functional categories [71]. Epigenetic "writer" is any protein or protein module that catalyzes the transfer of chemical groups onto N-terminal histone tails; epigenetic "eraser" is any protein or protein module that is capable to remove a chemical group and epigenetic "reader" is any protein or protein module that can decipher a specific chemical group for transmitting information into the structural changes within a discrete chromatin domain (Figure 2).

Histone Acetylation

Acetylation of the lysine residues at the N terminus of histones removes positive charges, thereby exacerbating the affinity between histones and DNA creating a more relaxed chromatin conformation permissible for transcription [72].

The genomic region surrounding transcriptionally active genes is enriched in H3K4ac, H3K27ac and H3K39ac [73-76]. Histone acetylation on H3K27 distinguishes active enhancers (Figure 3), while poised enhancers associate with H3K4me1 [73,74]. The histone acetylation

active enhancers

4 - H3K4ac
27 - H3K27ac
39 - H3K39ac

9 - H3K9ac
12 - H4K12ac
14 - H3K14ac
27 - H3K27ac

16 - H4K16ac
56 - H3K56ac
20 - H2BK20ac

27 - H3K27ac

122 - H3K122ac

repressed chromatin

NRSF/REST

20 - H4K20ac

CBP p300

MYST

GCN5

SAGA/ATAC
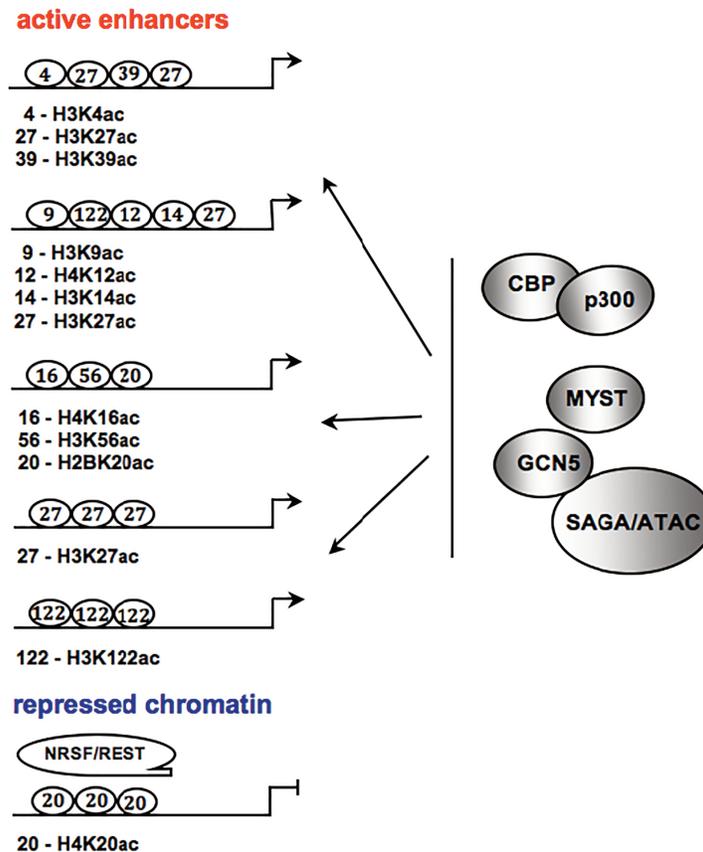
Figure 3. The combinatorial activity of different histone acetylation marks defines regulatory elements across the genome. Although nucleosome acetylation is a hallmark feature of chromatin accessibility and gene activity, in some discrete genomic regions the deposition of H4K20ac is sufficient to recruit NRSF/REST, a repressor of neuronal genes to initiate transcriptional silencing.

signature at H3K9, H4K12, H3K14, H3K27, and H3K122 defines active enhancers that are linked to CpG-rich islands and bivalent chromatin [77-79]. Interestingly, in the four-cell stage human embryos, H4K12ac is deposited in the vicinity of genes that control histone folding and DNA-dependent transcription, while in human blastocysts, the same histone mark is enriched at key developmental genes [78]. Although H3K27ac is a hallmark feature of active enhancers, a subset of active enhancers is marked with H4K16ac, H3K56ac and H2BK20ac [80-83]. Moreover, Pradeepa et al. have shown that H3K64ac and H3K122ac extend over active promoters [84]. Interestingly, this study has also revealed a novel subclass of active enhancers that lack H3K27ac, but positive for H3K122ac. In another report, hyperacetylation at H3K4, 9, 14, 18, 56 and 122 as well as acetylation at H4K5, 8, 12 and 16 (Figure 3) has been linked to the pluripotency state of human ESCs [85].

Histone acetyltransferases (HATs) mediate the transfer of an acetyl group to histones, transcription factors and other chromatin-associated proteins [86,87]. CREB-binding protein CBP and E1A-binding protein p300, two highly conserved HATs, transform condensed chromatin into a more relaxed structure by acetylating histones, tran-

scription factors and coactivator complexes associated with the basal transcription machinery [88]. The Wnt signaling pathway was shown to shift the balance between cell proliferation and cell fate specification through the CBP/β-catenin or p300/β-catenin-dependent mechanisms [89]. H3K56ac, a substrate of CBP/p300 (Table 1), marks chromatin domains with high nucleosome turnover in nuclear processes that are linked to gene transcription and DNA replication [82,83]. Rapid changes in H3K56 acetylation at the Notch-regulated enhancers require CBP binding, and reliably reflect enhancer activation [90]. In human ESCs, the recruitment of NANOG, SOX2, and OCT4 to the target promoters is associated with chromatin domains enriched in H3K56ac [91]. In addition to histone acetylation at H3K56, CBP and p300 contribute to a genome-wide acetylation on H3K18 and H3K27 [92].

The MYST2 histone acetyltransferase ensures naïve pluripotency and ESC self-renewal and has also been shown to be indispensable for early mouse post-implantation development [93,94]. Histone acetylation catalyzed by MYST2 is an intermediate step for the recruitment of Oct4 to the *Nanog* promoter indicating that MYST is an essential chromatin-remodeling enzyme required for the maintenance of pluripotency [93]. The activity of MOF is

Table 1. Epigenetic modifications in embryonic stem cells

Family	Substrate specificity	Molecular function	References
Histone acetyltransferases			
p300/CBP	H3K27, H3K18, H3K56	active enhancers	[82,83,90-92]
MYST/MOZ	H4K16, H3K9, H3K14	active enhancers	[96,98]
GCN5	H4K12, H4K16	gene activation	[99]
BRD4	H3K122	nucleosome eviction	[184]
Histone deacetylases			
HDAC1/2	H3K56ac, H3K27ac, H3K9ac	NuRD/CoREST repressor	[104,112]
Sirtuins	H4K16ac, H3K9ac	subunit of PRC4 repressor	
(SIRT1, SIPT6)	H3K56ac, H3K18ac	production of 5hmC	[113,120]
Histone methyltransferases			
EZH2	H3K27me3	subunit of PRC2	[121-124]
SUV39H1	H3K9me2/me3	repressed chromatin	[134,135]
G9A/GLP	H3K9me2, H3K27me3	repression	[136,139,140]
SETD2/7	H3K36me3	repression	[142-144]
MLL1-4	H3K4me3, H3K4me1	SET/COMPASS activator	[124,147]
Histone demethylases			
LSD1/2	H3K4me2, H3K27me3	CoREST repressor	[157,158]
KDM2B	H3K36me3	recruits RING1B/PRC1	[130-132]
KDM4B/C	H3K4me3	block H3K9m3/K36me3	[163]
KDM5B	H3K4me3	binds to PRC2 and LSD1	[165,166]
KDM6A/B	H3K27me3	β-catenin recruitment	[168-170]
PHF8	H3K9me2	removes repressive marks	[171]
Monoubiquitin ligases			
RING1B	H2AK119ub1	recruitment of PRC2	[128,174]
RNF20	H2BK120ub1	lineage commitment	[177]
DNA methyltransferases			
DNMT1, DNMT3A, DNMT3B	DNA methylation	gene repression	[69]
DNA hydroxylases			
TET1-3	convert 5mC to 5hmC, 5fC and 5caC	activation/repression	[200-211]

another MYST family member, which catalyzes the transfer of acetyl groups to H4K16 and is enriched at bivalent chromatin [95,96]. Depletion of mouse *Mof* affects the expression of *Nanog*, *Oct4*, and *Sox2* changing the potential of the pluripotent end-state [95]. MOZ, a member of the MYST family, and the Polycomb-family repressor BMI1, participate in the repression of *Hox* genes in differentiating ESCs [97]. The double PHD finger of MYST family was implicated in promoting H3K9ac and H3K14ac histone marks [98]. GCN5, a component of the SAGA and ATAC lysine acetyltransferase complexes, is important for acetylation on H4K12 and H4K16 during the stem cell reprogramming [99]. GCN5 associates with Myc to trigger the activation of a distinct alternative splicing network leading to the early acquisition of pluripotency-associated splicing state [100].

Although it is a common belief that histone acetylation is a prerequisite signature of gene activation, new data links histone acetylation to gene silencing. It has recently been documented that H4K20ac sponsors gene repression in HeLa-S3 cells [101]. What is fascinating about this discovery is the fact that transcriptional repressor NRSF/REST co-localizes with H4K20ac (Figure 3), while

transcriptional activators are excluded from the H4K20ac-enriched loci.

Histone Deacetylation

Histone deacetylases (HDACs) play a crucial role in the maintenance and differentiation of stem cells by mediating chromatin condensation and transcriptional silencing [102]. HDACs represent a class of enzymes that removes acetyl groups from the N-terminal histone tails [103]. HDAC1 and HDAC2 participate in chromatin organization and gene regulation as the core catalytic components of Sin3A, NuRD, and CoREST co-repressor complexes [102,103]. The inactivation of HDAC1, but not HDAC2, can cause elevated H3K56ac levels [104]. During early ESC differentiation, HDAC1 removes acetyl groups from key pluripotency genes [105]. Ablation of HDAC1 leads to an increase in H3K9ac and loss of DNA methylation [106].

Although the primary function of HDACs is gene silencing, several studies have linked HDACs to transcriptional activation of the pluripotent and self-renewal genes [107]. An immediate reduction in cell viability and decreased expression of *Oct4*, *Nanog*, *Esrrb*, and *Rex1* is a result of HDAC1 and HDAC2 depletion [108].

Sin3/HDAC is a corepressor complex, which is implicated in the regulation of essential biological processes including cellular proliferation, differentiation, apoptosis, and cell cycle [109]. The Nucleosome Remodeling and Deacetylase (NuRD) is another complex composed of multiple subunits, including HDAC1/2, the ATP-dependent remodeling enzymes CHD3/4, histone chaperones RbAp46/48, the CpG-binding proteins MBD2/3, p66α/β and specific DNA-binding proteins of the MTA family [110]. The balance between acetylation and methylation of histones controlled by NuRD is required for fine-turning transcription of developmental genes as well as maintaining the differentiation response of pluripotent stem cells [111,112]. Deacetylation of H3K27ac by NuRD facilitates the recruitment of Polycomb and subsequent deposition of a repressive H3K27me3 mark at the NuRD-bound promoters [112].

Sirtuins belong to a large family of deacetylases that play a major role in maintaining genome integrity, largely through targeting different histone marks, including H4K16ac, H3K9ac, H3K56ac, and H3K18ac [113]. SIRT1, a subunit of the Polycomb repressive complex PRC4, is involved in the acquisition of stress resistance, metabolism, hematopoiesis, aging, and tumor suppression [114,115]. In response to endogenous oxygen, SIRT1 blocks nuclear translocation of cytoplasmic p53 and triggers mitochondrial-dependent apoptosis in mouse ESCs [116]. SIRT1 is able to influence early cell fate decisions via the p53-mediated regulation of the *Nanog* gene [117]. In addition, SIRT1 facilitates the PTEN/JNK/FOXO1 signaling response to reactive oxygen species and regulates the induction of autophagy and mitochondrial function in

ESCs under oxidative stress [118,119]. SIRT6, another Sirtuin family member, safeguards the balance between pluripotency and differentiation through the TET-mediated production of 5-hydroxymethylcytosine (5hmC) [120]. Depletion of SIRT6 leads to augmented levels of 5hmC in the neural-specific epigenomic environment causing derepression of mouse *Oct4*, *Sox2*, and *Nanog* genes.

Histone Methylation

Histone methylation is mediated by histone methyltransferases (HMT) that catalyze the transfer of one, two, or three methyl groups to lysine and arginine residues of histone proteins. A regulated crosstalk between closed and open chromatin is mediated by the interplay of Polycomb group (PcG) and Trithorax group (TrxG) complexes [121-124]. PcG is part of the multi-subunit Polycomb Repressive Complexes PRC1 and PRC2, which are responsible for histone methylation and chromatin compaction. PRC2 contains four functional subunits EZH1/2, SUZ12, EED and RbBP4 (Figure 4A). EZH2, an essential component of PRC2, catalyzes the transfer of methyl groups on H3K27 [121-124]. In ESCs, developmental genes are poised for expression because their promoters are marked with H3K27me3/K4me3 bivalent chromatin [122,124]. Compared to ESCs, adult mouse self-renewing cells possess a relatively small number of bivalent genes [125]. The proportion of tissue-specific genes in a repressed state was shown to be significant, despite the presence of H3K4me3 at their promoters. It has been proposed that bivalent chromatin represents a major landmark to distinguish different cell types, rather than poising gene expression during differentiation [125].

PRC1 is composed of six major parts, each containing a distinct PCGF subunit, H2A monoubiquitin ligases RING1A/B and a unique set of PRC1-associated proteins [126]. PRC1 and PRC2 catalyze mono-ubiquitination on histone H2A (H2AK119ub1), which serves as a nucleation site for Polycomb to promote the subsequent deposition of H3K27me3 [127-129]. Mechanistically, PRC1 initiates ubiquitin transfer to H2AK119 (Figure 4B) leading to recruitment of PRC2 and H3K27me3 deposition [130]. Histone demethylase KDM2B interacts with RING1B to facilitate the recruitment of PRC1 to its target genes, thereby promoting H2AK119 ubiquitination in ESCs [131,132].

SUV39H1, another critical HMT, deposits H3K9me3 and HP1α to the promoter of *Oct4* in differentiating mouse ESCs [133]. To fulfill its repressive function, SUV39H1 forms a regulatory complex with a specific long non-coding RNA transcribed from the pseudogene *Oct4P4*. The genomic regions co-occupied by SUV39H1 and lncRNA are also enriched in chromatin factors DAXX and ATRX, which safeguard the genome by silencing repetitive elements [134]. Chromatin domains marked H3K9me3 are involved in attenuation of intact long interspersed nuclear
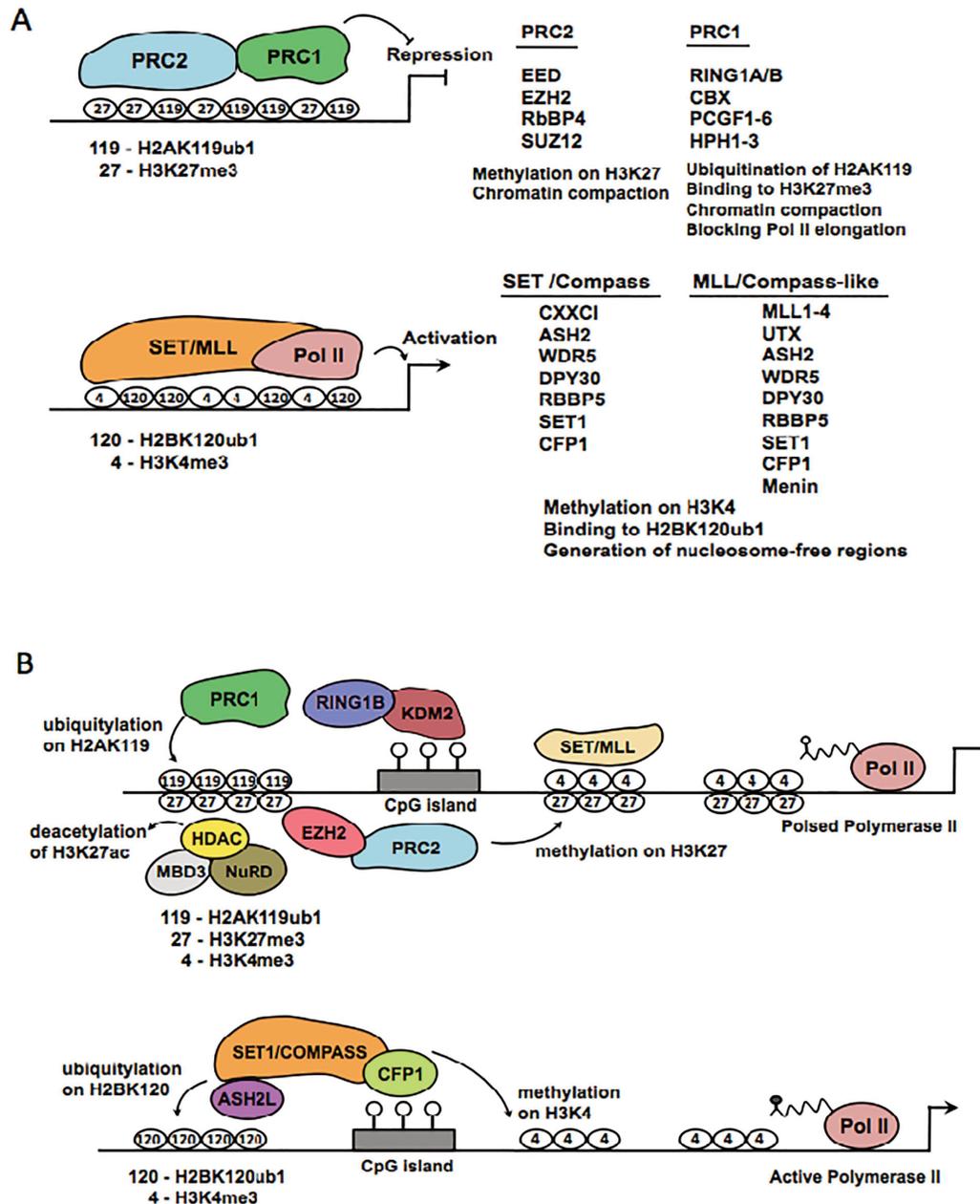
Figure 4. The composition and chromatin recognition of Polycomb (PcG) and Trithorax (TrxG) complexes in ESCs. **A.** PcG is composed of Polycomb Repressive Complexes PRC1 and PRC2, which are responsible for methylation on H3K27 and chromatin compaction. PRC2 contains four functional subunits EZH1/2, SUZ12, EED and RbBP4. PRC1 has six distinct PCGF subunits, H2A monoubiquitin ligases RING1A/B and a unique set of PRC1-associated proteins. The mammalian TrxG is composed of SET1/COMPASS and MLL/COMPASS-like complexes containing four members of MLL family, ASH2L, RbBP5, WDR5 and DPY30. RNF20 is a component of an E3 ubiquitin-protein ligase complex that mediates monoubiquitination of lysine 120 of histone H2B (H2BK120ub1). The SET1/COMPASS complex initiates a relaxed chromatin configuration by binding to H2BK120ub1. **B.** EZH2, an enzymatic subunit of PRC2, initiates trimethylation on H3K27 over the PRC2-bound regions, whereas PRC1 selectively binds to H2AK119ub1. PRC1-linked H2A monoubiquitylation is sufficient to recruit PRC2 to chromatin *in vivo*, suggesting a mechanism through which recognition of unmethylated CpG islands determines the localization of both PRC1 and PRC2 at the target sites. In the presence of PRC2, PRC1 retains the capacity to occupy the H3K27me3-enriched nucleosomes associated with poised RNA Polymerase II (Pol II), which is phosphorylated at serine 2. The TrxG-specific complex SET1/COMPASS exhibits a more robust H3K4 trimethylation activity than MLL/COMPASS-like complex. In pluripotent stem cells, CFP1 and MLL1/2 are implicated in targeting SET1/COMPASS to CpG islands, thus playing a critical role in H3K4me3 accumulation. ASH2L recognizes H2BK120ub1 to initiate the recruitment of SET1/COMPASS, which then binds activated Pol II, which is phosphorylated at serine 5, augmenting H3K4me3 domain co-transcriptionally.

elements in pluripotent stem cells [135]. G9a and GLP belong to another group of H3K9 methyltransferases that interact with the MAX repressor complex [136]. In ESCs, MAX knockdown decreases H3K9me2 over the *cis*-regulatory elements of germ cell-specific genes. G9a and GLP recruit DNA methyltransferases and LSH, a member of the SNF2 family of chromatin remodeling ATPases, to promote efficient and stable gene repression [137]. Loss of LSH significantly reduces DNA methylation across multiple genomic clusters, thereby altering transcriptional output of targeted loci [138]. The G9a-mediated deposition of H3K9me2 at active enhancers causes transcriptional silencing of developmental genes [139]. In addition to H3K9 methylation, G9a has been shown to control gene expression through the H3K27me3-dependent repressive mechanism [140]. The G9a/GLP complex protects imprinted DNA methylation by recruiting *de novo* DNA methyltransferases, which antagonize TET dioxygenase-dependent erosion of DNA methylation of imprinting control regions [141].

SETD2, the H3K36-specific HMT, guards differentiation of murine ESCs toward the primitive endoderm lineage via the Fgfr3-Erk signaling pathway [142]. The tethering of SETD2 by the antisense RNA Tsix is responsible for the H3K36me3-mediated repression of *Xist* on the X chromosome [143]. The removal of Tsix and PRC2 activities causes a rapid and profound upregulation of *Xist* and X chromosome repression in male ESCs. SETD7 is implicated in silencing delays of pluripotency genes and induction of developmental genes [144]. Methylation of the linker histone H1 by SETD7 can perturb chromatin structure via reduced deposition of H1 at the *OCT4* and *NANOG* genomic loci during human ESC differentiation.

The extended stretches of H3K4me3 are known as buffer domains, which define promoters of cell type-specific genes [145,146]. The buffer domains support transcriptional consistency, thereby ensuring transcriptional precision at key cell-identity genes. The SET1/COMPASS and COMPASS-like complexes are implicated in the establishment of the H3K4me3-riched domains to counteract the PcG-mediated gene silencing [70,124,147]. The mammalian SET1/COMPASS and MLL/COMPASS-like complexes contain four members of MLL family, namely ASH2L, RbBP5, WDR5, and DPY30 (Figure 4A). The SET1/COMPASS complex binds chromatin through H2BK120u1, a specific ubiquitinilation mark on histone H2B. Due to this epigenetic modification, SET1/COMPASS exhibits a more robust H3K4 trimethylation activity than MLL/COMPASS-like complex, an essential modulator of the long-term genomic memory implicated in cell cycle, senescence, DNA damage, and stem cell biology [148,149]. In the presence of DPY30, TrxG retains the monomethyltransferase activity but exhibits differential di- and trimethylation activities [150]. MLL2 is one of the key factors for establishing and maintaining the methylation-free state at CpG island promoters of active

genes [151]. MLL3 contributes to the maintenance of transcriptionally active genome enriched in H3K4me1/K27ac. The cooperation between MLL3 and H3K27 demethylase UTX was proposed to be important in the transition from poised to active enhancers [152]. It was demonstrated that MLL3/MLL4 are major regulators of H3K4me1 at the enhancers in human HCT116 cells and mouse ESCs [153]. Remarkably, in myoblasts, the promoter regions of muscle and inflammatory response genes, where MLL3 and MLL4 initiate H3K4me1 extension, undergo the conditional repression [154]. Interestingly, in embryonic fibroblasts, ESCs and macrophages, the genomic clusters enriched in H3K27me3 and H4K20me1 are also marked with H3K4me1. It has been proposed that epigenetic alterations of this kind are sufficient for establishing condensed chromatin and transcriptional attenuation [154].

The conserved subunit CFP1 is implicated in targeting SET1/COMPASS to CpG islands (Figure 4B). In mouse ESCs, CFP1 is instrumental for H3K4me3 accumulation, which in turn contributes to genome-wide H3K9 acetylation [155]. Therefore, deposition of H3K4me3 at gene promoters is dependent on SET1/CFP1 complex augmenting histone acetyltransferase recruitment and overall H3K9 acetylation dynamics.

Although H3K4me3/K27me3 bivalency is required for keeping developmental genes poised for transcriptional activation [122,124], a different type of bivalent signature has been described by Matsumura et al. [156]. In developing preadipocytes and upon lineage commitment of mesenchymal stem cells, H3K4me3/K9me3 bivalent structure (Figure 5A) has been shown to play an important role maintaining adipogenic regulatory genes in a poised state, prior to receiving the proper activation signal [156].

Histone Demethylation

Histone demethylase LSD1 (also known as KDM1A), a part of the CoREST repressive complex, facilitates the removal of mono- and di-methyl groups from H3K4me2 [103]. LSD1 interacts with bivalent chromatin (Figure 5B) to sustain the balance between H3K4me2 and H3K27me3 at key developmental genes [157]. Knockout of *Lsd1* in mouse ESCs leads to increased acetylation at H3K56, and deregulation of essential regulatory genes indicating that demethylation of H3K4me2 and acetylation of H3K56 are tightly regulated processes during mouse development [82,158]. LSD1 interacts with HDAC1 to facilitate deacetylation of H4K16ac, an important epigenetic mechanism to ensure the pluripotency of ESCs [159]. While LSD1 is enriched at poised promoters, LSD2/KDM1B, a closely related paralog, tends to associate with the genome undergoing active transcription [160]. LSD2 localizes specifically to euchromatin forming an active complex with G9a and NSD3 lysine methyltransferases.

KDM2B, the H3K36-specific histone demethylase, preferentially binds to CpG islands (Figure 4B). KDM2B
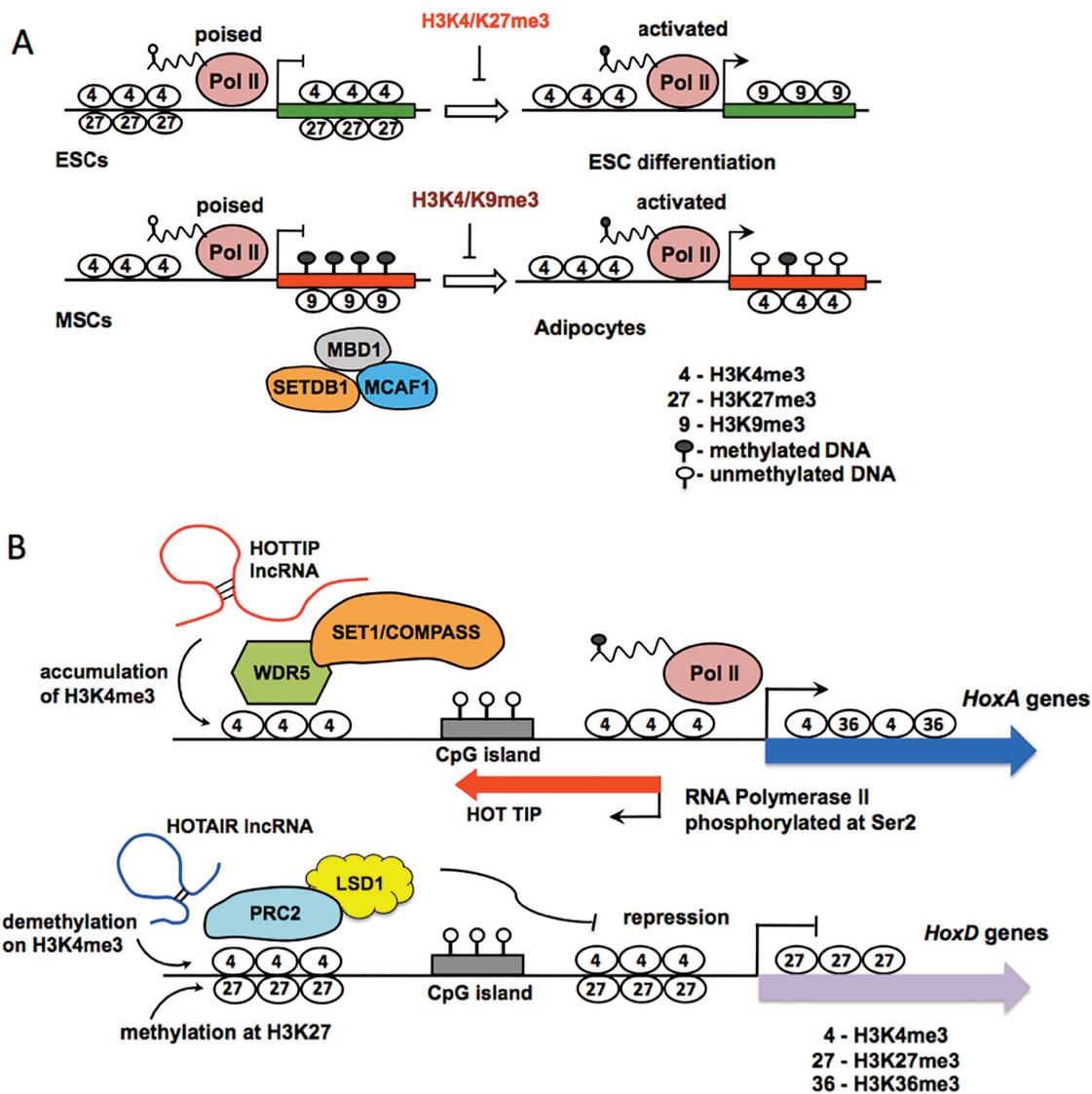
Figure 5. Bivalent chromatin and the role of non-coding RNAs in the regulation of gene expression. **A.** Two different forms of bivalent domain exist in ESCs and mesenchymal stem cells (MSCs). In pluripotent stem cells, H3K4/H3K27me3 bivalency pauses Pol II at promoters of developmental genes. In MSCs and preadipocytes, deposition of a repressive mark H3K9me3 by SETDB1 pauses Pol II at promoters. DNA methylation contributes to the formation of the H3K4/H3K9me3 bivalent domain by facilitating the recruitment of the repressive complex MDB1/MCAF1/SETDB1 over the gene bodies, thus restricting the differentiation potential of preadipocytes. Loss of H3K9me3 in adipocytes enables Pol II elongation at the developmental genes such as *Cebpa* and *Pparg*. **B.** Physical contact between lncRNAs and TrxG complex can promote transcriptional activation. WDR5, a subunit of the SET1/COMPASS complex, binds to the lncRNA HOTTIP transcribed from the *HOXA* locus. The interaction between WDR5 and HOTTIP elicits gene activation via MLL-mediated H3K4 trimethylation. As an epigenetic repressor, lncRNA HOTAIR ensures the recruitment of PRC2 and LSD1 at *HOXD* cluster to initiate accumulation of H3K27me3 and demethylation at H3K4me3, thereby enforcing a silent chromatin state.

facilitates the establishment of H2AK119u1-positive chromatin in ESCs by recruiting RING1B/PRC1 [130-132,161]. Depletion of KDM2B causes de-repression of lineage-specific genes and induction of the early stage differentiation followed by loss of H2AK119ub1. KDM2B protects the PcG-occupied promoters against ectopic *de novo* methylation; its loss leads to a progressive gain of DNA methylation [162]. Pedersen et al. [163] reported that erase of the promoter-enriched H3K9me3 by KDM4 demethylase represents a novel mechanism ensuring transcriptional competence and stability of the pluripotent cell identity. The conditional inactivation of *Kdm4a*, *Kdm4b*

and *Kdm4c* alleles in mouse ESCs revealed that while individual family members are dispensable for stem cell maintenance and embryogenesis, combined deficiency of *Kdm4a* and *Kdm4c* causes early embryonic lethality and impaired ESC self-renewal. KDM4B and KDM4C localize to the H3K4me3-positive promoters, where they have widespread and redundant roles in preventing accumulation of H3K9me3 and H3K36me3 [163]. The functional interaction between KDM5A and E2F4 underlies the spreading of a repressive chromatin structure in the cell cycle genes [164]. KDM5B can bind to PRC2 and LSD1 to coordinate reduction of H3K4me3 at active promoters, while its co-occupancy with MRG15 at the H3K36me3-rich clusters is implicated in staging the repressive state [165,166]. Depletion of KDM5B results in an increase in H3K4me3 and cryptic transcription, although transcriptional elongation of genes bound by KDM5B is prevented [166]. There has been evidence that KDM5C augments discrimination between enhancers and core promoters [167]. During neural differentiation, occupancy of KDM5C at gene promoters attenuates gene expression, while it's binding to active enhancers stimulates transcription. The reduced trimethylation of H3K27 at the *Brachyury* promoter and initiation of the Wnt-dependent mesoderm differentiation through the recruitment of β-catenin to specific regulatory elements is mediated by KDM6A and KDM6B [168-170].

Efficient differentiation of mesodermal lineage cells from mouse ESCs is dependent upon histone demethylase PHF8 [171]. Functionally, PHF8 facilitates transcription of genes critical in programmed cell death. For instance, a repressive histone mark H3K9me2, once erased with PHF8, can trigger transcriptional activation of a pro-apoptotic gene *Pmaip1*.

Histone Ubiquitylation

RNA polymerase II phosphorylated at serine 2 residue associates with genes undergoing active transcription, whereas serine 5-phosphorylated Pol II is a hallmark of poised RNA polymerase II, which binds to the silenced genes [172,173]. RING1B, the catalytic subunit of PRC1, enforces the poised Pol II configuration by histone H2A ubiquitylation [174]. H2A monoubiquitylation spreads at unmethylated CpG-rich regions (Figure 4B) and is sufficient for PRC2 recruitment *in vivo* [128]. KDM2B, functioning as a subunit of the noncanonical PRC1, is required for accumulating H2AK119ub1-positive domain in mouse ESCs [131,132]. It has been shown that KDM2B preferentially binds to CpG islands, thereby playing a key role in PRC1 recruitment to its target genes (Figure 4B). Another report suggested that the expression of lineage-specific genes is blocked by MDM2 oncoprotein, which binds to EZH2 increasing abundance of H3K27me3 and H2AK119ub1 at the PcG-bound regions [175].

Dynamic changes in histone ubiquitylation are initiated and maintained in a timely and well-coordinated manner during ESC differentiation [176]. For instance, monoubiquitylation of histone H2B on lysine 120 (H2BK120ub1), which is catalyzed by the E3 ligase RNF20, was reported to be necessary for transcriptional induction of relatively large genes [177]. H2BK120ub1 marks genes required for lineage commitment of ESCs (Figure 4B) and therefore, the reduction of H2B ubiquitylation triggers further alterations along lineage-specific pathways. There is evidence that H2BK120ub1 is critical for maintaining multipotency of human mesenchymal stem cells [178].

Upon ESC differentiation, the deubiquitinase USP44, a negative regulator of H2B ubiquitylation, is downregulated, thereby contributing to an increase in H2BK120ub1 [176]. The level of ubiquitination on H2A is controlled by USP16, another histone deubiquitinase [179].

Epigenetic Readers: Bromodomains and Chromodomains

While histone acetyltransferases are considered to be epigenetic writers, bromodomains (BRDs) serve as epigenetic readers participating in fine-tuning gene expression (Figure 2). The BET (bromodomain and extra-terminal) family of BRDs binds to an acetyl-lysine residue on histone tails [180]. Hence, BRDs occupy super-enhancers, large genomic clusters enriched in H3K27ac, p300, and Mediator, an essential epigenetic signature for dynamic and coordinated regulation of key cell identity genes [181]. The core transcription factors OCT4, SOX2, and NANOG are enriched in super-enhancers, whereas in pluripotent cells undergoing differentiation, BRD-bound super-enhancers serve as a scaffold for lineage-specific master regulators [182,183]. Recent work by Devaiah et al. have shown that BRD4 functions as histone acetyltransferase by acetylating H3K122, an intermediate step augmenting nucleosome depletion, chromatin relaxation and transcriptional activation of cognate genes [184]. H3K122 is known as a molecular target of p300/CBP and its acetylation is associated with an array of actively transcribed genes [79].

The chromodomain protein CDYL acts as an epigenetic reader by recognizing chromatin enriched in H3K9me2 and H3K27me3 as part of a repressive complex assembled at the inactive X chromosome in mouse ESCs [185]. This event, in turn, facilitates anchoring of G9a and H3K9me2 deposition at target loci. CHD1, one of nine members of the chromodomain helicase family, is also necessary for mouse ESC pluripotency [186]. CHD1 binds to active genes enriched in H3K4me3 as part of the preinitiation complex. Mediator recruits CHD1 to control the preinitiation complex assembly [186]. CHD7 associates with active enhancers to modulate ESC-specific gene expression [187]. Most of the CHD7-bound sites co-localize with p300 and H3K4me1. MRG15, another protein with chromodomain, was shown to assist KDM5B in
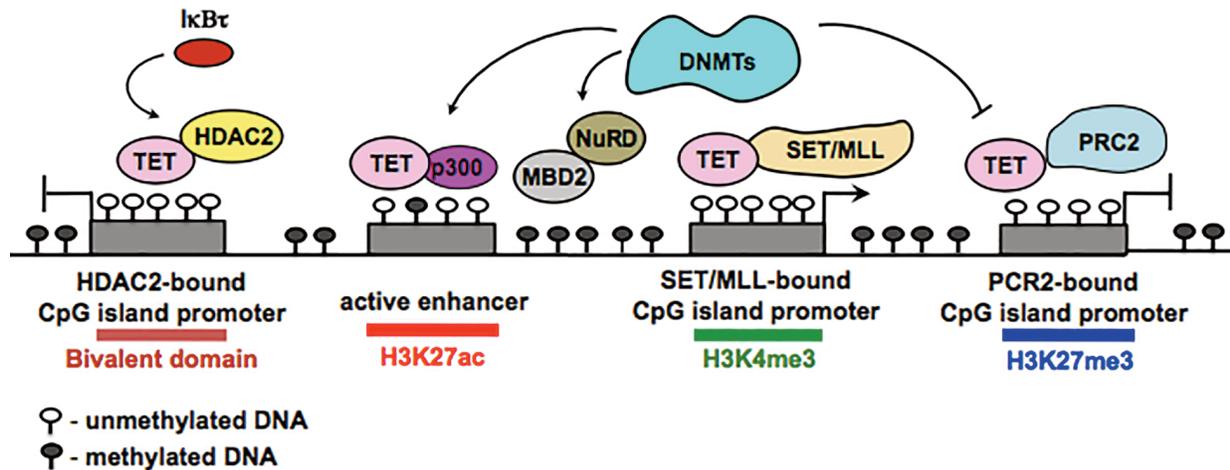
Figure 6. The close ties between DNA methylation and histone modification. Methylation of cytosine by DNA methyltransferases (DNMTs) establishes repressive chromatin, which is stabilized by PRC2 and NuRD complexes. However, in some cancer cells, DNA methylation ensures structural integrity of the H3K27ac-rich enhancers [199]. The TET-mediated DNA hypomethylation triggers the eviction of PRC2 and loss of H3K27me3 causing ectopic expression of cognate genes. Surprisingly, in innate myeloid cells, TET2 is able to recruit HDAC2 with the assistance of IκBτ factor to initiate the gene-specific transcriptional repression [206]. The TET-mediated DNA hydroxymethylation is critical for *de novo* establishment and maintenance of H3K4me3-rich active domains marking CGIs, while TET binding to PRC2 contributes to the establishment of H3K27me3-positive repressive chromatin. Methyl CpG-binding proteins (MBDs) control expression of pluripotency genes in ESCs by associating with the HDAC/NuRD repressive complex.

binding to the H3K36me3-positive nucleosomes in mouse ESCs [166].

DNA Methylation

DNA methylation, a transfer of the methyl group from S-adenosylmethionines to a cytosine, has a profound impact on chromatin structure, gene expression and maintenance of cell-type identity [188]. DNA methylation predominantly occurs at CpG islands (CGIs), one of the most prominent features of the mammalian genome [69]. CGIs retain the GC-rich base composition and high density of CpG dinucleotides. It was estimated that a great majority of human genes initiate transcription from CGIs that mark gene promoters [189,190]. The bivalent and active promoter regions are enriched in CpG, while H3K27me3-only promoters lack CpG islands [191]. Methylation of DNA at the fifth position of cytosine (5mC) in CpG dinucleotides is commonly associated with gene repression. Moreover, 5mC is enriched in exons and at intron-exon junctions, suggesting a specific role for DNA methylation in the crosstalk between elongation and RNA splicing [192].

DNMT1, DNMT3A, and DNMT3B are three conserved DNA methyltransferases that are responsible for cytosine methylation [69]. The initial stages of *de novo* methylation require the cooperative interaction between DNMT3A and DNMT3B, while completion of DNA methylation requires DNMT1 and G9a [193]. The repressed state of the non-transcribed CpG-rich genes that control cell identity is maintained by PRC2 [194]. Remarkably, the acquisition of H3K27me3 is accompanied by loss of DNA methylation [195]. Conversely, DNA hypomethylation triggers the eviction of PRC2 and loss of H3K27me3 causing ectopic expression of developmental genes [196]. The CRISPR/Cas9 genome editing was used to inactivate all three DNMTs in human ESCs [197]. Depletion of *DNMT1* resulted in cell death, whereas inactivation of *DNMT3A* or *DNMT3B* or both genes had very little effect. It has also been shown that the LSD1/NuRD-mediated histone deacetylation and demethylation triggers DNMT3A activity and DNA methylation at active enhancers (Figure 6) leading to suppression of cognate pluripotency genes during differentiation of mouse ECSs [198]. Surprisingly, in certain cancer cell lines, DNA methylation plays an unexpected role associated with the maintenance of active chromatin [199]. According to the collected evidence so far, a portion of the H3K27ac-positive enhancers co-exists with extensive DNA methylation indicating in some cases a link between DNA methylation and histone modifications as the requirement for ensuring structural integrity of enhancers in the genome of certain cell lines such as HCT116 [199].

Ten-eleven translocation (TET) proteins represent a family of DNA hydroxylases that can convert 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) through three consecutive oxidation reactions [200,201]. The TET-mediated DNA demethylation is important in pluripotent stem cells and can play an essential role in diverse biological processes, including development and disease [202,203]. Loss of 5hmC in mouse embryos lacking TET1 and TET3 is associated with a concurrent increase in 5mC at the

eight-cell stage embryos exhibiting a characteristic phenotype of holoprosencephaly [204]. Expression of genes of cholesterol biosynthesis is reduced in *Tet1/3* knockout blastocysts. Inactivation of all three *Tet* paralogs leads to a complete loss of 5hmC and impaired ESC differentiation suggesting that the TET-mediated DNA demethylation is an essential epigenetic switch to ensure proper cell function [205]. Surprisingly, TET2 is able to recruit HDAC2 to initiate the gene-specific transcriptional repression (Figure 6) using the histone deacetylation pathway [206]. The transcriptional repressor REST supports TET3 binding to chromatin, thereby influencing 5hmC generation, while the ability of TET3 to interact with NSD3 induces H3K36me3 marks in mouse retina [207]. Recently, Zeng et al. discovered that RNA-binding protein Lin28 recruits TET enzymes to genomic loci to initiate 5mC to 5hmC conversion [208]. In the vertebrate genome, a widespread DNA demethylation of enhancers occurs during the phylotypic period [209]. The recruitment of TET enzymes to 5mC and enrichment of 5hmC at enhancers implicates DNA demethylation in the activation of lineage-specific genes. Loss of TET paralogs in *Danio* leads to the reduced chromatin accessibility and an increase in DNA methylation at tissue-specific enhancers [209]. In addition, TET enzymes modulate sub-telomeric methylation levels, thereby playing a critical role in chromosomal stability of ESCs [210]. Depletion of all three Tet paralogs in mouse ESCs leads to increased frequency of telomere loss and chromosomal fusion. Mechanistically, depletion or deficiency of *Tet* genes increases DNMT3B and decreases 5hmC levels, resulting in elevated levels of DNA methylation at sub-telomeres [210]. The critical role of TETs in regulating the crosstalk between DNA methylation and histone methylation at CpG-rich bivalent promoters was recently revealed by Kong et al. [211]. Their study showed that TET2-mediated DNA demethylation is required for *de novo* establishment and maintenance of H3K4me3/K27me3 bivalency at CGIs. In human epidermal stem cells, the TET2-dependent association of DNMT3A with p63 is required for high-level maintenance of 5hmC at the center of enhancers, whereas DNMT3B sustains DNA methylation along the entire enhancer length [212]. Given the fact that active enhancers are co-occupied by DNMT3A and DNMT3B and this type of interaction is required for enhancer RNA production, this is a surprising discovery.

Methyl CpG-binding protein family (MBD) represents a set of epigenetic readers that share a conserved methyl-CpG domain [213]. Almost all MBD proteins, with the exception of MBD3, are able to bind specifically to the high-density CpG promoters [214]. MBD3, as part of the NuRD repressive complex, binds to the regulatory sequences occupied by HDAC1/NuRD (Figure 4B) controlling expression of *Oct4*, *Nanog,* and *Kfl4* in ESCs [107].

Non-coding RNAs

The discovery of a large number of non-coding RNAs (ncRNAs) forming a powerful RNA surveillance system has redefined fundamental principles of gene regulation in stem cells, including ESCs, induced pluripotent stem cells, mesenchymal stem cells, and adult stem cells [215]. Knowledge of non-coding RNAs and their function in pluripotency, self-renewal and differentiation has the potential to reveal novel molecular cues involved in induction and maintenance of the pluripotent state, as well as clinically relevant cell types and tissues [216].

Long ncRNAs (lncRNAs) refer to a heterogenic class of RNAs that includes intergenic lncRNAs, enhancer RNAs (eRNAs) and antisense transcripts [217,218]. Small ncRNAs navigate the Argonaute complex to nascent RNA templates to facilitate the recruitment of histone methyltransferases and DNA methyltransferases, while lncRNAs participate in transcriptional silencing via independent mechanisms [219]. The expression profile of lncRNAs is often tissue- or cell type-specific, including lncRNAs that have been linked to embryonic and adult stem cells [220]. Bogu et al. discovered that lncRNAs typically associate either with the promoters or enhancers in various mouse tissues [221].

The non-coding RNA Gm15055 represses *Hoxa* genes in mouse ESCs [222]. Gm15055 maintains H3K27me3 enriched chromatin over the *Hoxa* cluster by recruiting PRC2 in mouse ESCs. On the contrary, *Hottip*, a lincRNA transcribed from the 5' tip of the human *HOXA* locus, coordinates the activation of several 5' *HOXA* genes [223]. *Hottip* RNA anchors the COMPASS complex across the *HOXA* locus via binding to WDR5 (Figure 5B), an essential intermediate step for initiating H3K4me3 accumulation and transcriptional activation. In mammals, inactivation of X-chromosome is mediated by lncRNA transcript known as *Xist*, which binds along the inactive X chromosome [224]. *Xist* induces posttranslational histone modifications and DNA methylation to achieve a stable repression of all X-linked genes throughout development and adult life. In different study, ablation of mouse *Hotair* lncRNA was found to be critical in homeotic transformation of skeletal elements [225]. It has been shown that *Hotair* binding to *Hoxd* genes ensures the recruitment of PRC2 and LSD1 (Figure 5B), initiates trimethylation at H3K27 and demethylation at H3K4me3, thereby enforcing chromatin compaction. During differentiation of hematopoietic lineages, long ncRNA *HoxBlinc* binds to the mouse *Hoxb* loci to facilitate the recruitment of SETD1A/MLL1 complexes [226]. This event triggers transcriptional activation of genes in the *Hoxb* cluster through long-range chromatin interactions.

There is growing evidence that lncRNAs participate in the recruitment of PRC2 in the gene dense regions enriched in H3K27me3, H2AK119ub or H3K36me3 [227]. LncRNAs, which are derived from pseudogenes, were shown to be an integral part of the epigenetic surveillance

system [228]. It was demonstrated that the mouse *Oct4* pseudogene *Oct4P4* is critical for both ESC self-renewal and differentiation. A nuclear-restricted lncRNA, which is produced from the sense *Oct4P4* transcription, forms a complex with SUV39H1 to initiate the imposition of H3K9me3 and HP1α to the *Oct4* promoter. This regulatory cascade leads to *Oct4* silencing and reduced ESC self-renewal [228].

Divergent lncRNAs transcribe in the opposite direction to the nearby protein-coding genes and comprise almost 20 percent of total lncRNAs in the mammalian genome [229]. In pluripotent stem cells, divergent lncRNAs were shown to regulate expression of the neighbor genes. For example, binding of the divergent lncRNA Evx1as to the regulatory region of the neighbor gene *EVX1* initiates its transcription during mesendodermal differentiation [229].

MicroRNAs (miRs), a class of small non-coding RNAs 19-24 nucleotides in length, mediate post-transcriptional control of ESC pluripotency and cellular reprogramming [230,231]. MicroRNAs play a significant role in vertebrate neural crest development and facial morphogenesis [37,232-234]. A subset of miRs regulates its cognate genes during neurogenesis, chondrogenesis and hematopoiesis [235-237]. During differentiation of smooth muscle cells, miR-22 assists to anchor MeCP2 to chromatin followed by enrichment of H3K9me3 and subsequent inactivation of key developmental genes [238]. miR-24-1 has been linked to transcriptional activation of eRNAs, a regulatory event associated with the recruitment of p300 and Pol II to enhancers [239]. During osteoblast differentiation and bone formation, miR-145 plays important role in the CBFB-dependent regulation, a molecular process dependent upon the interaction between Runx2 and the core binding factor beta CBFB [240]. Surprisingly, the recruitment of EZH2 and SUZ12 to many bivalent genes requires the miR biogenesis enzyme Dicer [241]. In ESCs depleted of Dicer, miRs are linked PRC2 recruitment leading to the repression of bivalent genes. Therefore, new evidence indicates that miRs could be important regulators of the bivalency state during the early stages of ESC differentiation [241].

Furthermore, there is evidence for a coordinated crosstalk between lncRNAs and miRs [242,243]. A complex network of transcription factors, chromatin remodeling complexes, miRs and lncRNAs has been implicated in maintaining the balance between self-renewal and multilineage differentiation capacity of pluripotent stem cells [233]. The cytoplasmic lincRNA-RoR is able to trap miR-145, an inhibitor of the core pluripotency master regulators, thereby derepressing the translation of *OCT4*, *SOX2* and *NANOG* in human ESCs [244]. The primate-specific lncRNAs, such as developmental pluripotency-associated transcripts 2, 3 and 5 (*HPAT2*, *HPAT3* and *HPAT5*), are frequently clustered at transposable elements [245]. These lncRNAs modulate the function of pluripotent stem cells and the formation of the inner cell mass lineage in the preimplantation embryo. For instance, *HPAT5* acts as a key component of the pluripotency network by interacting with the let-7 microRNA [245].

EPIGENETIC MECHANISMS IN DENTAL PULP STEM CELLS

Epigenetic alterations have been documented in association with periodontal disease, hypodontia, during enamel development and odontogenic differentiation [5-12,246,247]. The paper by Duncan et al. summarizes research on functional significance of histone deacetylases and DNA methyltransferase inhibition in dental pulp as a promising potential for regenerative endodontic regimens [5]. In DPSCs committed to become mature odontoblasts, dynamic changes in the epigenetic landscape have been linked to permissive chromatin associated with transcriptional upregulation [62]. Despite some progress, relatively little is known about the epigenetic code and consequent pleiotropic effects that drive DPSCs into specialized cell lineages.

Histone Acetylation and HDAC Inhibition

Attenuation of HAT activity by garcinol, an inhibitor of histone acetyltransferase p300, can reverse histone acetylation during osteogenesis [248]. The *DSPP* gene, which is mainly expressed in odontoblasts and pre-ameloblasts, is down-regulated in the garcinol-treated DP cells. There is evidence that at the DSPP loci histone acetylation contributes to odontoblast differentiation and maturation of pulp stem cells [248]. p300 is one of the main HATs in the regulation of core pluripotency network in DPSCs [249]. The augmentation of p300 expression does not affect the ability of stem cells to proliferate, but elevates the expression of *NANOG* and *SOX2*. Although p300 exerts very little effect on the odontogenic fate, the expression levels of *DMP1*, *DSPP*, *DSP*, *OPN* and *OCN* are profoundly reduced in DP cells [249]. It has been found that p300-mediated histone acetylation can increase the extension of H3K9ac deposition on the promoters of *OCN* and *DSPP* [250]. Ablation of p300 initiates the formation of mineralized nodules and ALP activity, while the expression of *DMP1*, *DSPP*, and *DSP* (Table 2) is dramatically reduced in DPSCs undergoing odontogenic differentiation.

Histone deacetylase inhibitors (HDACi) alter the homeostatic balance between histone acetylation and deacetylation, increase the transcriptional rate and influence cell physiology suggesting that inhibition of HDACs has great potential in restorative dentistry [251]. Trichostatin A (TSA) is an organic compound that selectively inhibits the class I and class II HDACs [252]. TSA promotes proliferation and odontoblastogenesis in human DPSCs, accelerates mineral nodule formation and enhances dentin formation and odontoblast differentiation

Table 2. Epigenetic changes in human and mouse dental pulp stem cells

Family	Genomic targets	References
Epigenetic enzymes		
p300	*DMP1, DSPP, DSP, OPN, OCN*	[249,250]
EZH2	*PPAR-r, CEBP/a, Osx, BSP, IL-1b, IL-6, IL-8*	[258]
KDM6B	genes implicate in differentiation	[260]
DNMTs	*DSPP, DMP1, OSX, RUNX2, DLX5*	[262-266]
TET1	*DSPP, DMP1*	[268]
MicroRNA		
hsa-miR-218	*RUNX2*	[276]
miR-32	*DSPP*	[277,278]
miR-885-5p	*DSPP*	[277,278]
miR-586	*DSPP*	[277,278]
let-7	*DMP*	[277,278]
miR-143	*Dspp, Dmp1*	[279]
miR-145	*Dspp, Dmp1*	[279]
miR-720	*DNMTs, NANOG*	[281]
miR-433	*GRB*	[282]
has-miR-516a-3p	*WNT5A*	[283]
has-miR-7-5p	*EGFR*	[283]
miR-152	*SIRT7*	[285]
miR-146a	*IRAK1, TRAF6*	[246]

during tooth development [253]. TSA increases the expression of *PCNA*, *CCND1*, *DSPP*, *DMP1*, *BSP*, and *OCN* and activates the JNK/c-Jun-dependent pathway. The expression of *SMAD* family and *NFIC* is also elevated by TSA. The following changes in the postnatal molars such as an increased thickness of dentin and accelerated odonoblast proliferation were observed in mouse embryos treated with TSA [253]. Valproic acid (VPA), a drug primarily used to treat epilepsy and bipolar disorder, in combination with TSA accelerates the reparative processes in dental pulp [254]. HDAC inhibition triggers induction of mineralization and increases expression of *DMP1*, *BMP2/4*, *Nestin*, *p53*, and *DSPP* [255]. The suppression of histone deacetylase activity promotes expression of *OPN* and *BMP2*, thereby affecting cellular differentiation. It has been shown that VPA can reduce transcription of *OCN* in DPSCs and osteoblasts, while the expression of *OPN* and *BSP* remains elevated [256].

Histone Methylation

The potential application of EZH2 in tissue regeneration including nervous system, muscle, pancreas, and dental pulp has been recently discussed [257]. It was suggested that PRC2 has a major role during DP inflammation, proliferation, and regeneration [258]. Upon inflammatory stimuli, inhibition of the enzymatic activity of EZH2 down-regulates expression of *IL-1b*, *IL-6*, and *IL-8* in the infected pulp tissue (Table 2) and impedes DP cell proliferation by decreasing cell number, arresting the cell cycle, and increasing apoptosis. The adipogenic induction leads to the suppression of EZH2, which in turn

diminishes transcriptional activity of *PPAR-r* and *CEBP/a*, whereas alkaline phosphatase activity and expression of *Osx* and *BSP* stays elevated [258].

The research conducted by Gopinathan et al. revealed that epigenetic changes play an important role in the terminal differentiation of odontogenic lineages in dental pulp and dental follicle [62]. Compared to DF cells, the expression of pluripotency genes *OCT4*, *NANOG*, and *SOX2* is higher in DP cells. The authors also observed a substantial increase in H3K27me3 followed by repression of *DSPP* and *DMP1* in the DF, but not in the DP. The histone mark H3K4me3, associated with active genes, is enriched at the promoters of early mineralization genes *RUNX2*, *MSX2*, and *DLX5* in DF and DP progenitors, while H3K9me3 or H3K27me3, marks of repressive chromatin, are clustered in the vicinity of *OSX*, *IBSP*, and *BGLAP* [62].

Interestingly, the SUMO-specific isopeptidase SENP3 controls the level of H3K4me3 by regulating histone methyltransferase SET1/MLL in human DF stem cells [259]. Removal of SUMO from SET1/MLL by SENP3 is required for activation of key transcription factors such as *DLX3* during osteogenic differentiation of DF progenitors. This observation suggests that a similar regulatory framework could also exist in DPSCs.

In another recent study, inhibition of histone demethylase KDM6B by alcohol has been linked to the dysregulation of odontogenic/osteogenic differentiation in DPSCs [260]. The inactivation of KDM6B associates with reduced mineralization of DPSCs, while in the ethanol-treated DPSCs, KDM6B facilitates the restoration of

genes involved in odontoblast differentiation. KDM6B have already been implicated in promoting osteogenesis by erasing H3K27me3 from the promoters of odontogenic markers *SP7*, *OCN*, and *OPN* [247].

DNA Methylation

The odontogenic-specific DNA hypomethylation was suggested to be a typical feature of human dental pulp [261]. The toll-like receptors TLR-2 and TLR-4 mediate the activation of immune cells in response to bacterial infection in DP cells. The hypomethylated regions in *TLR-2* and in the *CD14* gene, which encodes TLR4 co-receptor, have been detected in healthy and inflamed human DP tissues [261].

The DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-CdR) reactivates genes repressed by DNA methylation [262-264]. The 5-Aza-CdR-triggered DNA demethylation significantly inhibits the proliferation of DP cells and up-regulates the expression of *DSPP*, *DMP1*, *OSX*, *RUNX2*, and *DLX5* (Table 2). DNA demethylation elevates the capability of odontogenic differentiation in DP cells suggesting that DNA methylation may play a fundamental role in reparative dentinogenesis in human dental pulp [265]. Similarly, the selective suppression of mouse DP cell differentiation into skeletal muscle cells by 5-Aza-CdR suggests that differential induction of DPSCs is associated with DNA demethylation [266].

The research reported by Yoshioka et al. failed to detect obvious changes in the spatial distribution of 5mC and 5hmC in odontoblasts and DP cells [267]. According to them, *DNMT1* was vigorously transcribed, while expression changes of *DNMT3A*, *DNMT3B* and members of the *TET* family were relatively modest. The only exception is *TET1*, which was shown to exhibit elevated expression in immature dental epithelial cells [267]. Interestingly, TET1 knockdown suppresses the proliferative and odontogenic capacity of human DP cells suggesting that TET1 may play an important role in dental pulp repair and regeneration [268]. The study also revealed that alkaline phosphatase activity, the formation of mineralized nodules, and expression of *DSPP* and *DMP1* were significantly reduced in the TET1-depleted DP cells undergoing odontogenesis.

Non-coding RNAs

MicroRNAs and long non-coding RNAs have an essential function in inflammation and immunity, the key processes associated with pulpal pathology [10,269-271]. Many miRs are predicted to target genes linked to multiple biological pathways including the mitogen-activated protein kinase (MAPK) and Wnt signaling, proinflammatory cytokines and other key mediators of the immune and inflammatory response [272,273]. miR-181a binds 3'UTR of *IL-8* keeping its expression at elevated levels (Table 2) [274]. Kong et al. suggested that Oct4B1, an essential functional isoform of Oct4, by interacting with miRs, con-

tributes to the inflammatory response in DP cells [275]. Upon bacterial lipopolysaccharide (LPS) stimulation, the expression dynamics of *Oct4B1* and *Oct4B* are elevated in DP cells, whereas knockdown of *Oct4B1* leads to increased apoptosis. MiRs that associate with the MAPK, Wnt and Toll-like signaling pathways are differentially regulated after *Oct4B1* knockdown [275].

In undifferentiated DP cells, hsa-miR-218 down-regulates *RUNX2* transcription, whereas during DP differentiation, decrease in hsa-miR-218 causes up-regulation of *RUNX2* [276]. The post-transcriptional control of *DSPP* is achieved via miR-32, miR-885-5p and miR-586, while *DMP* regulation is mediated by the Let-7 microRNA precursor [277,278]. miR-143 and miR-145 control the odontoblast lineage and dentin formation through the Klf4/Osx-dependent regulatory mechanisms. Down-regulation of miR-145 and miR-143 promotes odontoblast differentiation and increased expression of *Dspp* and *Dmp1* in mouse primary DP cells [279]. First, the recruitment of Klf4 and Osx to the *Dspp* and *Dmp1* promoters leads to transcriptional activation of both genes and odontoblast differentiation; second, miR-145 binds to the 3'-UTRs of *Klf4* and *Osx* to inhibit their expression; third, Klf4 recruitment to the miR-143 regulatory region suppresses miR-143 transcription; fourth, miR-143 mediates odontoblast differentiation, at least in part, through the miR-145-controlled mechanism [279]. Additionally, miR-143 together with miR-135, induces myogenic differentiation of DPSCs [280]. The expression pattern of *NANOG* and DNA methyltransferase genes is controlled by miR-720 [281]. Conversely, *GRB2* and the RAS-MAPK signaling pathways are regulated by miR-433 [282]. It was documented that the reduction of proliferative and mineralization abilities as well as increased cell death of DP cells is mediated miR-433. Vasanthan et al. reported that hsa-miR-516a-3p and hsa-miR-7-5p, two highly expressed micrRNAs in dental pulp, impose differential control on target genes [283]. Inactivation of hsa-miR-516a-3p induces *WNT5A* expression, while knockdown of hsa-miR-7-5p increases *EGFR* expression. Another microRNA, miR-424, plays a negative role in the regulation of the human pulp endothelial differentiation; the attenuation of miR-424 was proposed to contribute to dental pulp repair and regeneration [284]. During DPSC senescence, upregulation of miR-152 decreases the level of *SIRT7* expression, thus influencing the histone acetylation status in DPSCs [285]. Among developmental regulators, LPS is essential for pulpal pathogenesis; it can increase DP cell migration using the regulatory pathway controlled by miR-146a-TRAF6/IRAK1 [246]. Upon LPS induction, activated miR-146a facilitates the migration of pulp cells, whereas the expression of *IRAK1* and *TRAF6* is significantly exacerbated.

Chen et al. have documented that Wnt/β-catenin signaling can be controlled by a member of lncRNAs during DP differentiation [286]. Upregulation of lncRNA
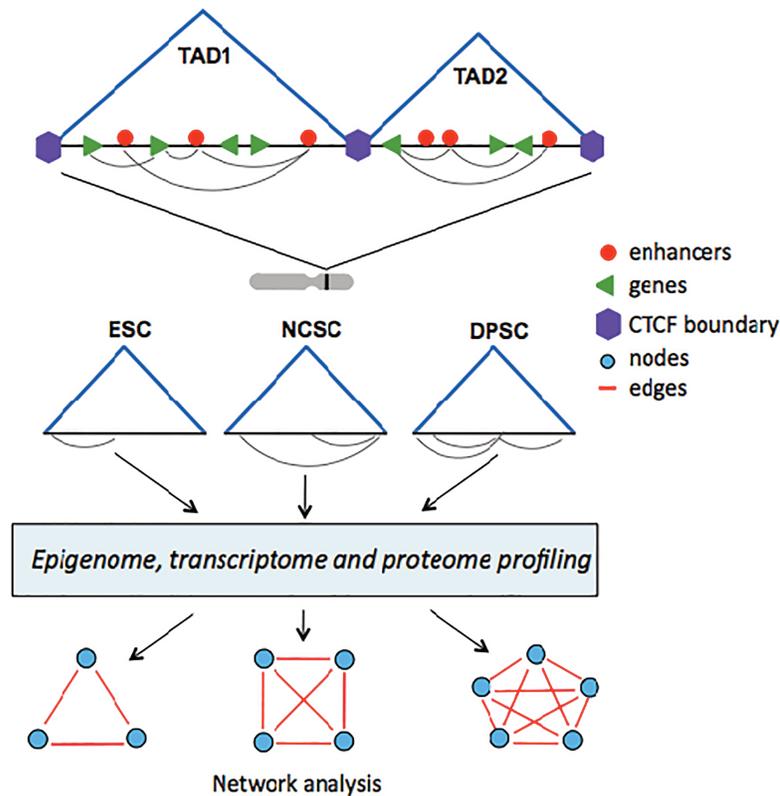
Figure 7. Long-range chromatin interactions, which are required for fine-tuning gene expression, occur predominantly inside of topologically associating domains (TADs), structural units of chromatin architecture. Insulators, the CTCF-bound genomic regions, separate TADs from each other within the 3D nuclear scaffold. Chromatin interactions are pre-determined by the epigenetic landscape and influence the unique signatures of transcriptomic and proteomic responses in living cells. The integration of network analysis and genome-wide datasets is a necessary step towards untangling the spatial and temporal dynamics of gene regulation of differentiating cells. Nodes (blue circles) represent functional units, either genes or proteins, in the network and edges (red line) represent interactions between units, whereas long-range interactions are depicted with gray curved lines.

DANCR leads to a significant decrease in the expression levels of p-GSK-3β and β-catenin indicating that lncRNA DANCR imposes inhibitory dynamics on the activation of the Wnt/β-catenin signaling pathway during odontoblast differentiation [286].

CHROMATIN TOPOLOGY

Transcription factors and chromatin remodeling complexes establish and maintain cell identity via global genomic changes. In the 3D nuclear space, the tissue-specific distal enhancers are typically located in close physical proximity to promoter regions through the formation of chromosome loops that can range from large-scale folding of whole chromosomes to smaller genomic segments [51-56]. Discrete looping interactions determine the differentiation potential of a cognate stem cell into more specialized cells such as odontoblasts. Therefore, the functional specificity of chromatin topology governing cell-state transitions should provide valuable insight into the regulatory framework underlying different phases of tooth organogenesis. It is also critical to understand how

craniofacial osteogenesis generates the phenotypic variation within a population, thereby rendering the raw material for evolution. It has been shown that variations in the highly conserved regulatory pathway controlling brain development underlie a morphological difference between the upper and lower jaws in avian species [287]. In this respect, enhancer-promoter interaction networks involved in early neurogenesis are highly sensitive to chromatin structural changes [50]. Recently, the study by Emera et al. mapped enhancers required for development of the mammalian neocortex in close proximity to genes associated with cell migration, axon guidance, and cell signaling [288]. The proposed model suggests that relatively simple *cis*-regulatory elements emerging from the genomic background are able to accumulate H3K27ac, and some elements ultimately evolve into more complex enhancers, whereas others are lost during evolution. Morphogenesis of the face and brain is intrinsically linked by the dynamics of regulatory crosstalks between key developmental genes. Signals from neural crest cells, for instance, regulate expression of Fgf8, which controls growth of the anterior forebrain, while the Shh signaling pathway is

implicated in head morphogenesis including neuroectoderm of the ventral forebrain, facial ectoderm and pharyngeal endoderm [289,290].

In fact, over past few years, the rapid progress in next-generation sequencing and advanced chromatin mapping technologies [291-294] has opened new opportunities for further interrogation of chromatin topology and assessment of proper temporal and spatial differences in the control of gene expression during orofacial morphogenesis. Integrative analysis from transcriptomics, epigenomic and proteomic data can significantly increase our ability to uncover regulatory wirings and intricate 3D communications that underlie odontogenic lineage commitment (Figure 7).

CONCLUSIONS AND OUTLOOK

The self-renewal capacity and potential to differentiate into multiple cells makes ESCs very attractive for regenerative medicine. Using advances in gene editing and 3D tissue engineering technologies, it became possible to reproduce self-organizing qualities of embryonic and adult stem cells [295]. Based on such properties of stem cells, diverse multi-cellular tissues, also known as organoids, could be re-created, although the current technology is facing some inherent limitations such as the control of the cell type, cell organization, and cell-cell/cell-matrix interactions [296]. How epigenetic changes influence chromatin features, gene expression and genome stability, and thus contribute to lineage commitment and stem cell differentiation will remain as one of the main challenges facing modern biology. Therefore, the genome-wide strategies are going to play an increasingly important role in therapies that aim to potentiate tissue regeneration and repair. The technological advances in high-resolution imaging and the development of new methods such as chromatin proteomic profiling (ChIP-MS) and quantitative interaction proteomics have established a platform for identification of protein complexes associated with regulatory sequences carrying specific histone tags [297-299]. As we become more aware of complex context-specific relationships between the epigenome and environmental fluctuations during oral development [300], the necessity to challenge long-standing paradigms is now widely acknowledged. Systems biology has the potential to unravel the essential coding principles that constitute the osteogenic capacity of different progenitor populations. A systematic approach to an in-depth understanding of epigenetic determinants in dental pulp would ultimately create important clinical implications, and can possibly reveal the causes of rare and common oral diseases.

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