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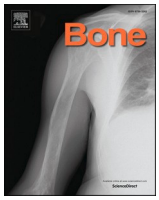

Review Article

Epigenetic regulators controlling osteogenic lineage commitment and bone formation

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ABSTRACT

Bone formation and homeostasis are controlled by environmental factors and endocrine regulatory cues that initiate intracellular signaling pathways capable of modulating gene expression in the nucleus. Bone-related gene expression is controlled by nucleosome-based chromatin architecture that limits the accessibility of lineage-specific gene regulatory DNA sequences and sequence-specific transcription factors. From a developmental perspective, bone-specific gene expression must be suppressed during the early stages of embryogenesis to prevent the premature mineralization of skeletal elements during fetal growth in utero. Hence, bone formation is initially inhibited by gene suppressive epigenetic regulators, while other epigenetic regulators actively support osteoblast differentiation. Prominent epigenetic regulators that stimulate or attenuate osteogenesis include lysine methyl transferases (e.g., EZH2, SMYD2, SUV420H2), lysine deacetylases (e.g., HDAC1, HDAC3, HDAC4, HDAC7, SIRT1, SIRT3), arginine methyl transferases (e.g., PRMT1, PRMT4/CARM1, PRMT5), dioxygenases (e.g., TET2), bromodomain proteins (e.g., BRD2, BRD4) and chromodomain proteins (e.g., CBX1, CBX2, CBX5). This narrative review provides a broad overview of the covalent modifications of DNA and histone proteins that involve hundreds of enzymes that add, read, or delete these epigenetic modifications that are relevant for self-renewal and differentiation of mesenchymal stem cells, skeletal stem cells and osteoblasts during osteogenesis.

1. Introduction

Skeletal development, bone homeostasis, fracture repair and bone regeneration each involve the formation of new bone tissue. Bone formation requires the engagement of biochemical and molecular pathways that successively control osteogenic lineage-commitment of undifferentiated mesenchymal cells or skeletal stem cells [1], maturation of osteoblasts, and extracellular matrix mineralization. The differentiation of skeletal stem cells (SSCs) into bone forming osteoblasts is controlled by genetic and *epi*genetic mechanisms that collectively mediate bone-specific gene expression. Genetic determinants have been revealed by genome-wide association studies that identified hundreds of genes with natural nucleotide variations that influence bone mineral density [2,3]. Genomic epigenetic mechanisms (epi = above or around) that directly control transcription in osteoblasts involve chemical modifications of DNA [4,5] and histone post-translational modifications (PTMs) [6,7] that are the main focus of this review. These epigenomic mechanisms are complemented by interactions of chromatin with long

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non-coding RNAs [8–12], as well as by non-genomic epigenetic mechanisms involving short non-coding microRNAs (miRNAs) that regulate mRNA levels and translation during osteoblast differentiation at post-transcriptional levels that have been covered in previous reviews [13,14]. Our understanding of the epigenetic mechanisms that control osteogenesis is fundamentally incomplete. This review provides an overview of the considerable progress that has been made to define epigenetic mechanisms during bone formation and osteoblast differentiation.

Chromatin in both bone and non-bone cells is composed of DNA assembled into nucleosomes by an octameric protein complex with two copies each of the four distinct core histone subunits (i.e., H2A, H2B, H3 and H4) (Fig. 1). The binding of histone octamers restricts DNA access and represents a key rate-limiting step in gene transcription. Covalent modifications in DNA and histone PTMs provide two closely related regulatory layers of epigenetic information [15–17]. The histone PTMs turn the nucleosome into an epigenetic signaling module [18] that converts external and metabolic signals into major PTMs such as histone acetylation [19], methylation [20], phosphorylation [16], and ubiquitination [21] (Fig. 1). Histone PTMs alter the structure and function of histones while influencing gene accessibility through alterations in (i) the topological architecture of DNA organized as nucleosomes, (ii) the positioning of nucleosomes on DNA, and (iii) formation of higher order loops that bring distant regulatory regions into proximity [22,23]. Consistent with the complexity of epigenetic control, there are a multiplicity of epigenetic regulators that control bone formation in vitro and in vivo. This review provides a structured view of the epigenetic regulators that control the generation, interpretation, alteration or removal of histone PTMs during bone formation and osteoblast differentiation.

We have organized the text to provide insights into the cell signaling pathways and gene regulatory factors that support epigenetic induction of osteogenesis by modulating DNA methylation and hydroxymethylation, as well as histone post-translational modifications during osteogenesis. We also discuss the translational relevance and limitations of studies on epigenetic enzymes and PTM recognition proteins.

2. Regulation of epigenetics by cell signaling

2.1. Developmental silencing of bone specific genes

Genes that support tissue mineralization are silenced during early stages of embryogenesis and are activated during later stages of

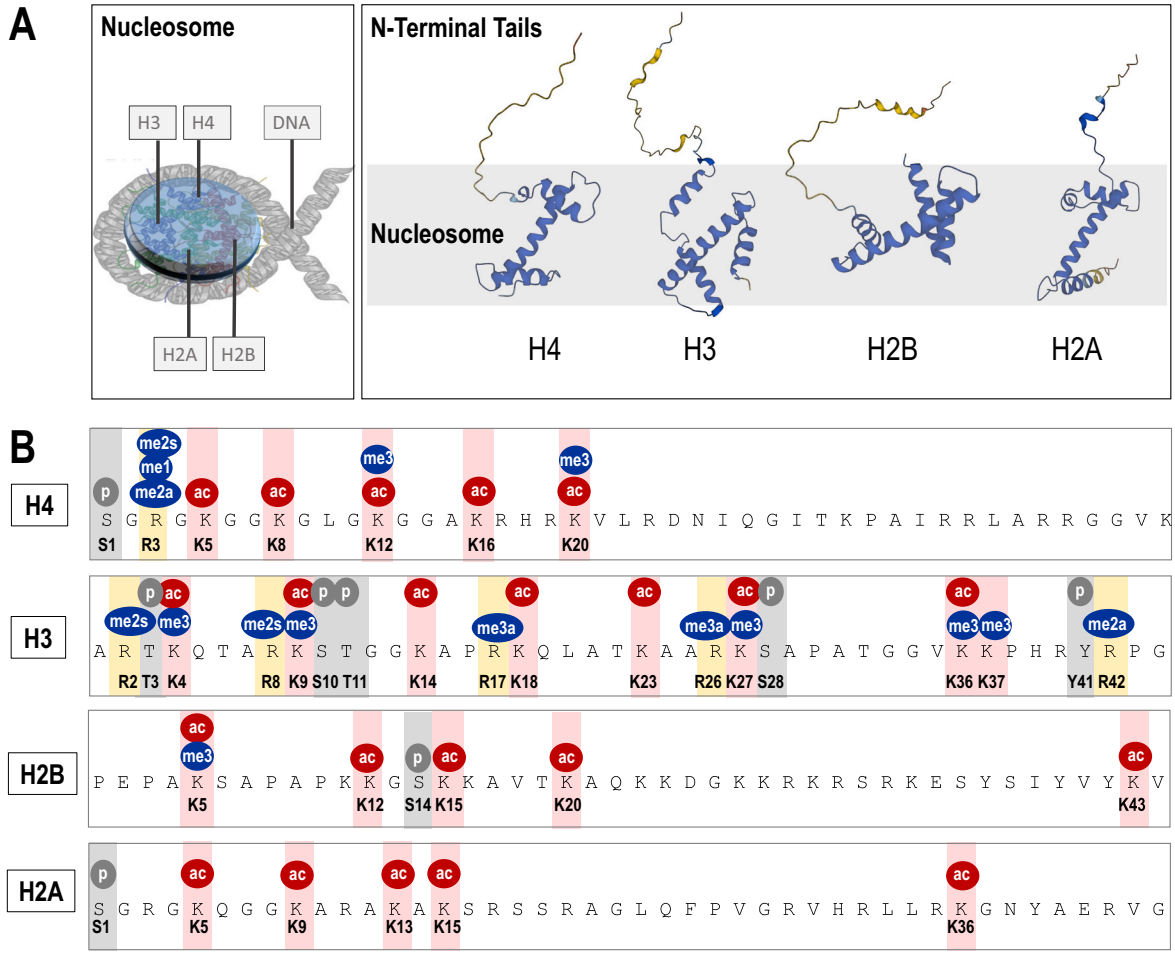


Fig. 1. Histone post-translational modifications. (A) Epigenetic control by chromatin regulates the accessibility of DNA assembled as nucleosomes that contain two copies each of four core histone proteins (H4, H3, H2B and H2A) to form a histone octamer that sequesters ~200 bp of DNA. Images of the four human core histone proteins are shown in the top right and were produced using Alphafold [285,286], with the alpha-helical regions of the histones embedded in a gray band (roughly representing the parts of each histone copy engaging in the histone octamer. The N-terminal regions of the histones which do not adopt a predicted protein structure and are thought to protrude away from the nucleosome in a manner that permits enzymatic modification. (B) Overview of post-translational modifications on the protruding parts of the N-terminal regions of each of the four histones (up to amino acid 44). Indicated are (i) methylated forms of lysines, which are typically trimethylated (me3) even though monomethyl (me1) and dimethylated (me2) forms also occur), methylated arginines, which can be dimethylated symetrically (me2s) or asymmetrically (me2a, as well as lysines that are acetylated (ac) or ubiquitinated (ub), as well as serines and threonines that are phosphorylated (p) [64]; see text for additional references).

gestation when skeletal tissues in the mammalian fetus are mineralized prior to birth. The silencing of genes proceeds by the packaging of DNA into facultative heterochromatin, a dense type of chromatin that is inaccessible to transcription factors and transcriptionally inactive. In contrast, when bone-specific genes are activated, they are converted into open transcriptionally active euchromatin. The conversion from closed to open chromatin is fundamental to gene regulation in osteogenic cells, such as skeletal stem cells (SSCs) and pre-osteoblasts (Fig. 2). The activation of bone-specific genes and the suppression of alternative cell fates (e.g., adipogenic, chondrogenic and fibroblastic programs) proceeds by the orchestrated placement, interpretation, editing and/or removal of modifications on DNA and histones. This transition is controlled by pioneering transcription factors and epigenetic regulatory complexes that produce multiple histone PTMs in enhancer or promoter regions [7,24] (Fig. 3).

Epigenetic mechanisms have been extensively studied ex vivo in a range of cell types including SSCs, mesenchymal stem/stromal cells (MSCs) derived from bone marrow or perivascular stroma, and other mesenchymal cell types (e.g., stromal fibroblasts, pre-myoblasts) [25,26]. Many immature mesenchymal cell types have the potential to differentiate into osteogenic cells ex vivo, even though osteogenic differentiation may not be their natural fate. Because omics studies traditionally require significant source material, many informative studies in the field have pragmatically used passaged or immortalized MSCs and osteoblasts from different sources (e.g., adipose tissue, bone marrow) to define key steps of osteogenic lineage commitments (see below).

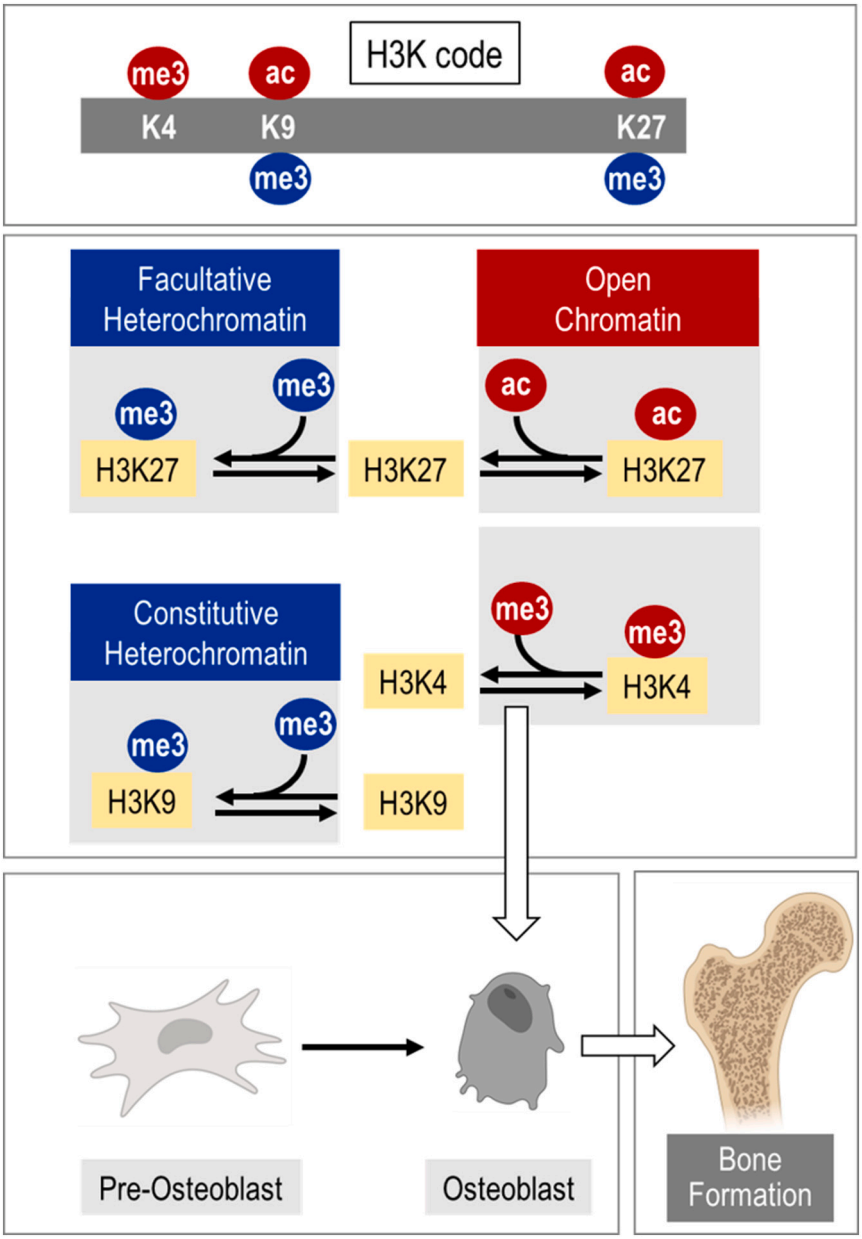


Fig. 2. The histone 3 trimethyl lysine (H3Kme3) code controls osteogenesis. The diagram shows the principal lysines (K4, K9 and K27) that undergo gene suppressive trimethylation (navy blue) or acetylation and gene activation trimethylation (burgundy red), which each are reversible (parallel arrows in opposite direction). H3K27me3 and H3K9me3 are associated with closed chromatin (heterochromatin), while H3K27ac and H3K4me3 are associated with open chromatin. Because bone specific genes are silenced during early stages of embryonic development and in non-committed stem cells, competency for bone specific gene expression requires chromatin remodeling during osteogenesis in vitro and in vivo, as indicated with the vertical open arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

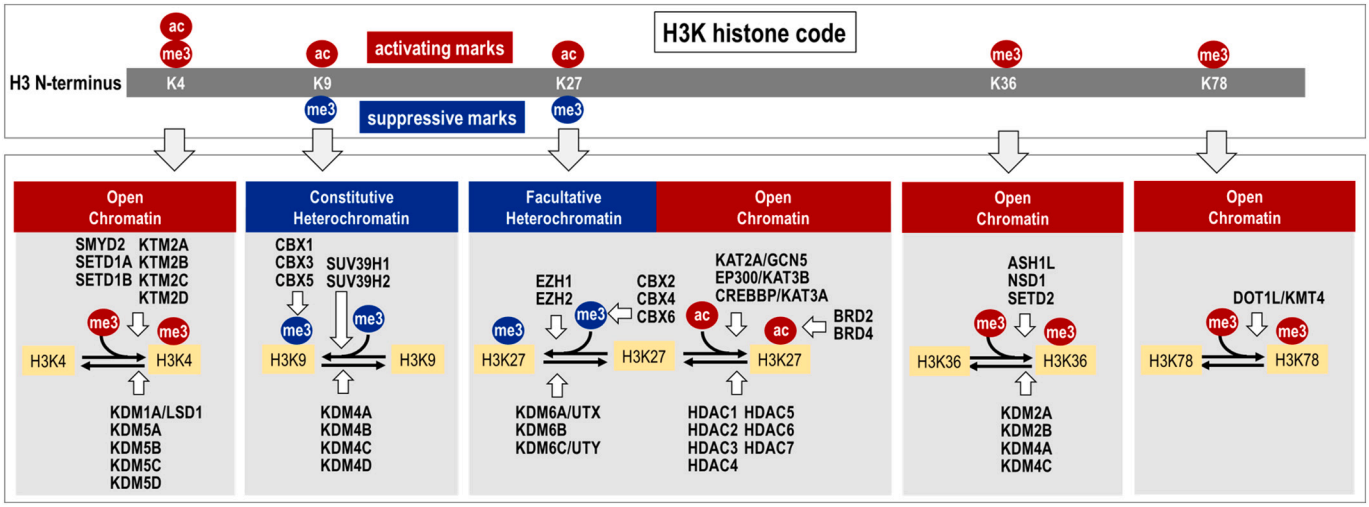


Fig. 3. Multiple enzymes and regulatory proteins control H3 methylation and function during osteogenesis. The diagram expands on the remodeling of heterochromatin into open chromatin during activation of the bone-specific gene expression program by indicating all known enzymes that control critical post-translational modifications required to convert closed chromatin into open chromatin, and vice versa using the same symbols and color schemes as shown in the previous figures.

2.2. Osteogenic induction by a cell signaling/transcription factor/epigenetic regulator axis

Epigenetic mechanisms occurring in the nucleus are controlled by external signals from osteogenic protein ligands (i.e., growth factors or morphogens; WNTs, BMPs, FGFs). These ligands bind to their cognate extracellular receptors on the cell surface. The liganded receptors activate intracellular regulatory pathways that are controlled by phosphorylation-dependent kinases (WNT-responsive GSK3β, FGF-responsive MAPKs). These kinases control secondary regulators, including transcriptional co-factors (e.g., WNT-responsive β-catenin, BMP-responsive SMAD proteins, WW-domain proteins like YAP1 and TAZ), and osteogenic transcription factors (e.g., RUNX2, SP7/Osterix, DLX5, CEBPB, ATF4 and SATB2) [27,28]. RUNX2 and SP7/Osterix are the most critical factors for osteogenic lineage progression and regulate the expression of numerous downstream target genes that encode proteins required for establishment of the osteoblast phenotype [29–31]. Early studies on key transcription factors such as RUNX2, the Vitamin D Receptor (VDR), HOX homeodomain proteins and histone acetylation established initial mechanistic models for suppression and activation of bone specific genes during osteoblast differentiation [32–37] Chromatin modifications at the RUNX2 locus itself have been examined in significant detail to understand the transcriptional regulation and autoregulation of this critical master regulator of bone formation [38,39]. Studies focusing on SP7/Osterix have revealed that this protein alters chromatin structure in bone-specific promoters in conjunction with the epigenetic regulator WRD5 [40]. The findings of these studies are consistent with a general model in which transcription factors recruit epigenetic regulators that remodel chromatin and permit RNA polymerase II mediated synthesis of osteoblast-related gene transcripts [41]. The ability of osteogenic transcription factors to interact with their recognition motifs in proximal promoters and distal enhancers depends on the extent to which nucleosome-bound DNA is rendered accessible within chromatin.

2.3. Genome-wide histone PTMs (epigenome) and transcription factor binding (TF cistrome)

The principal goal of epigenomic studies during osteogenic differentiation of MSCs or osteoblast maturation of lineage-committed pre-osteoblasts is to define the PTMs that support conversion of transcriptionally suppressive epigenetic marks (e.g., K9me3, K27me3 in H3 and 5mC in DNA) into activating marks (e.g., K4me3, K27ac, K36me3 in H3 and 5hmC in DNA). These PTMs are altered in response to the interactions with lineage-specific transcription factors controlling osteogenesis (e.g., RUNX2, SP7/Osterix) or adipogenesis (e.g., PPARG) that recruit epigenetic enzymes to distal enhancer and proximal promoter regions. A number of studies have applied high-throughput sequencing approaches to understand the epigenetic landscape in osteogenic cells, including (i) ChIP-seq using antibodies against defined panels of histone PTMs to define epigenomes, (ii) ChIP-seq to define the full complement of binding sites for key transcription factors to delineate TF cistromes, (iii) and RNA-seq (or microarrays) to assess global gene expression and define transcriptomes [29,42–44]. Within these extensive datasets, a major paradigm for epigenetic control during osteogenesis is a gene cluster that contains a series of ECM biomineralization-related genes such as IBSP (bone sialoprotein), SPP1 (osteopontin), MEPE (matrix extracellular phosphoglycoprotein), DMP1 (dental matrix protein), DSPP1 (dentin sialophosphoprotein), and SPARCL1 (SPARC/osteonectin-like protein). This gene cluster and individual osteoblast commitment markers (e.g., IBSP) are important targets for the gene regulatory effects of TFs like RUNX2 [44] and SP7 [45], as well as for derepression of the bone gene expression by EZH2 inhibition [46] and Vitamin C mediated DNA hydroxymethylation [5].

The global location of RUNX2 interactions has been studied in relation to histone PTMs and gene expression in human and mouse bone marrow-derived MSCs, as well as mouse MC3T3-E1 preosteoblasts [29,42–44]. ChIP analysis combined with microarray gene expression profiling in MC3T3 osteoblasts revealed that RUNX2 binds to ~80,000 sites within the genome [44]. This number is in far excess of the number of genes (~300–3000) that are controlled during osteoblast differentiation in different biological contexts [44,46]. Other studies have provided more conservative estimates of the number of high-confidence RUNX2 sites at ~12,000 [29] or ~9500 [42] depending of the osteoblast differentiation-stage [29]. The differences in RUNX2 binding events among these studies depend in part on the confidence thresholds that are applied for defining what constitutes a binding event. While many of RUNX2 binding sites are found in the promoters of bone-related genes, the majority of these peaks is actually found in intergenic regions [44]. RUNX2 binds at the loci for key target genes such as SP7/Osterix (activated) and EZH2 (suppressed), and may control other genes via non-promoter sites [44]. Similar to RUNX2, the RUNX2 target gene SP7/Osterix supports chromatin remodeling of its target genes during the pre-osteoblast to osteoblast transition, and is required for the deposition

of H3K4me3, H3K27ac and H3K36me3 marks in its target genes including IBSP [45]. SP7 is also necessary to support removal of the H3K9me3 binding complex HP1 (CBX1, CBX3 and CBX5), as well as the conversion of hypermethylated to hypomethylated DNA in the IBSP gene [45].

Similar to RUNX2, the VitD3 stimulated VDR has many thousands of binding sites (~7000) in MC3T3 osteoblasts [47]. A small fraction of these VDR sites (~5 %) is located in the promoters of genes and comparable to the number of genes that is modulated by VitD3, but there is considerable complexity in the VDR cistrome and global expression data in the presence or absence of VitD3 [47]. A simplified interpretation of the VDR work is that there are several hundred VitD3 responsive genes that have VDR binding sites, as well as RUNX2 and CEBPB motifs in their promoters; yet, there are also many modulated genes that lack VDR and that may be secondarily responsive to VitD3 stimulation. Similar findings were made with ChIP-seq analyses during osteogenic differentiation of mouse bone marrow MSCs where many binding sites for VDR, RUNX2 and CEBPB converge on the same genes [48]. While the TF cistrome of gene proximal binding sites is evident, understanding the functional relevance of the TF cistrome in distal regions remains a major challenge.

Osteogenic commitment of mouse bone marrow derived MSCs coincides with removal of H3K27me3 marks, even though this mark is not acquired on downregulated genes [43]. In addition, while genes can be activated by the acquisition of H3K4me3 marks, many osteoblast-specific genes do not have this modification and appear to be controlled primarily by H3K9ac and H3K27ac marks [43,47,49,50]. H3K36me3 is observed primarily in gene bodies and represents a generic marker for transcribed genes. The general model that emerges from studies in mouse bone marrow derived MSCs is that differentiation-related genes are activated by loss of H3K27me3 (due to reduced EZH2 activity) and concomitant deposition of H3K9ac and H3K27ac (mediated by histone acetyl transferases like EP300/p300), while genes are terminally suppressed by H3K9me3 accumulation in promoters [43].

Other studies have provided insight into epigenetic mechanisms of mesenchymal cell fate determination by comparing osteogenic versus adipogenic lineage commitment in bone marrow derived MSCs in mouse [48] and human [50,51]. These studies provided global genomic validation for the roles of osteogenic (e.g., RUNX2, VDR) or adipogenic (e.g., PPARG, CEBPA) lineage-specific TFs [48], that occur concomitant with epigenomic and transcriptomic changes [50,51], as well as actin-related changes in nuclear organization [52]. Differentiation of fibroblastic MSCs to either adipocytes or osteoblasts could have a default pathway where MSCs may have a greater tendency to differentiate into one of these two lineages. Systemic metabolism in lean versus obese human donors affects the lineage preference of MSCs. MSCs from obese individuals preferentially undergo adipogenic differentiation. The latter is associated with increased levels of the insulin receptor (IR) and the leptin receptor in bone marrow derived MSC, as well as perhaps epigenetic adaptation under the influence of these adipokine signaling pathways in obese patients [53]. Similarly, human adipose-derived MSCs have a different transcriptomic state that favors adipogenesis compared to bone-marrow derived MSCs that may result from local epigenetic adaptations to the tissue-environment [54].

Recent studies suggest that adipogenesis requires a greater reorganization of the epigenetic landscape of chromatin compared to osteogenesis. Osteogenesis involves utilization of enhancers that are already accessible to osteoblast-related TFs in MSCs, whereas these enhancers require major chromatin remodeling in the adipogenic lineage to render them accessible to adipocyte related TFs [50]. Both genetic and functional studies suggest an intricate balance between osteogenic and adipogenic factors that favors lineage specificity. The activation of enhancers that drive osteogenic versus adipogenic lineage commitment not only involves changes in the landscape of histone PTMs and nucleosomal organization, but also involves changes at the level of higher order chromatin and genomic contacts between distantly separated regulatory regions, as revealed by chromatin capture approaches during adipogenic differentiation of bone marrow derived MSCs. These long-distance genomic contacts at diverse enhancers appear to involve interactions of pre-established enhancers and enhancers that are newly organized at distant sites, thereby mutually stabilizing the regulatory binding events at the two interacting enhancers to form a highly integrated 3D chromatin architecture [55]. A similar mechanism likely occurs during osteogenic differentiation consistent with classic concepts of the interplay between chromatin structure and nuclear architecture [56].

2.4. Pharmacological modification of the osteoblast epigenome

The epigenetic landscape that governs lineage commitment of human bone marrow derived MSCs can be targeted by different epigenetic inhibitors, including the pan-HDAC inhibitor abexinostat [57], the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) [19] and panobinostat/LBH-589 [58], as well as the EZH2 inhibitors GSK126 [59] and tazemetostat [60] that enhance osteogenic differentiation. Other less selective epigenetic drugs (e.g., CUDC-907/Fimepinostat) with broad activity on different enzymes including HDACs enhance adipocytic differentiation [61], while the HDAC1/HDAC2 specific inhibitor romidepsin stimulates both osteogenic and adipogenic lineage commitment. Furthermore, the Class I HDAC inhibitor SAHA accelerates osteoblast maturation by increasing histone H4 acetylation [62], and it also destabilizes the multilineage potential of adipose-tissue derived MSCs [19]. Inhibitors for bromodomain proteins, which recognize histone acetylation, can block osteogenic differentiation [20], even though they can have net osteoprotective effects on bone mass in vivo [63]. Collectively, these studies show that inhibitors can change the dynamic deposition of histone PTMs in MSCs and modulate the ability of MSCs to undergo self-renewal and differentiation.

2.5. Essential nutrients that control the epigenome

The interplay between chromatin structure, cell metabolism and essential nutrients is well recognized [5,64]. Vitamin C, which is required to prevent skeletal defects observed in scurvy, has major effects related to demethylation of both DNA and histone proteins in bone and osteogenic cells [5]. Vitamin D (i.e., 1α,25-dihydroxy vitamin D3; VitD3), which is a critical clinical parameter for bone health [65], is a key ligand that controls chromatin binding and the transcriptional activity of the Vitamin D receptor (VDR). VDR is a member of the nuclear hormone receptor class of transcription factors and interacts with VitD3 responsive genes in the context of the dynamic osteoblast genome [65]. Many bone-specific genes are VitD3 responsive and bind both RUNX2 and VDR [66,67]. Importantly, VDR is not able to activate the bone specific osteocalcin (BGLAP) gene by itself unless chromatin is first opened by RUNX2 binding [68]. Early ChIP studies showed that VitD3 and VDR binding are associated with osteoblast-differentiation specific modulations in histone acetylation in bone-specific gene promoters [37,69]. The VDR binds to a large number (~7000) of genomic sites in MC3T3 osteoblasts and the expression of a comparable number of genes is modulated by VitD3 [47]. Similar to findings for RUNX2 [29,44], only a small fraction of these VDR sites (~5 %) is located in the promoters of genes. There is considerable complexity in the VDR cistrome, global transcriptome data in the presence or absence of VitD3, and co-interactions with other transcription factors [47,48]. These and other studies collectively show that there are several hundred VitD3 responsive genes with functional promoter binding sites for VDR, RUNX2 and CEBP [48], including VDR binding sites in the promoters of the key osteogenic genes RUNX2 and SP7/Osterix themselves [47]. In addition, there are many genes that do not bind VDR and may be secondarily responsive to VitD3 [48]. VDR binding to its target genes and/or tissue-specific enhancers is both lineage-specific [70], differentiation stage-dependent [47,71].

3. Enzymes mediating DNA methylation and hydroxymethylation during osteogenesis

The first layer of epigenetic control at the level of chromatin in osteoblasts involves covalent modifications of DNA that are functionally connected to the second layer of control by histone PTMs to form an integrated epigenetic code that collectively determines the organization of DNA into dense ('closed') or loose ('open') chromatin [15,17]. DNA modifications include methylation of cytosine bases at the fifth carbon position (5-methylcytosine, 5mC) and the hydroxylated products 5-hydroxymethylcytosine (5hmC), 5-formylmethylcytosine (5fC) and 5-carboxycytosine (5caC). This methylome code encompasses marks for inactive genes (5mC), transcriptionally active genes (5hmC), and methyl marks that are in different stages of demethylation (e.g., 5fC, 5caC). Formation of 5mC marks is an ancient epigenetic mark on DNA [72] that occurs primarily on palindromic CpG dinucleotides but can also occur on other cytosines in specific biological contexts (e.g., oocytes, embryonic stem cells, and neurons) [73,74]. DNA methylation is regulated in a developmental stage-specific manner and mediates long-term gene silencing [75]. The majority of gene promoters contain CpG-rich sequences [CpG islands (CGIs)] near the transcriptional start site (TSS) [76] and can be targeted by DNA methyltransferases (DNMTs) [76]. 5mC marks silence gene expression directly by inhibiting binding of transcription factors to their cognate DNA motifs or indirectly by supporting binding of methyl-DNA binding domain (MBD) proteins that interact with suppressive chromatin-modifying enzymes (e.g., HDACs) [76,77].

3.1. DNA methylation

Epigenetic 5mC marks in osteoblasts are generated by three distinct DNA methyltransferases: DNMT1, DNMT3A, and DNMT3B; the homologous protein DNMT2 was misidentified and has been renamed RNA Aspartic Acid Methyltransferase 1 (TRDMT1). DNMT1 deposits 5mC marks during DNA replication in proliferating cells to maintain DNA methylation during cell division using two distinct molecular pathways [78,79]. These two pathways involve recruitment of DNMT1 to hemimethylated DNA at replication forks via interactions with either mono-ubiquitylated histone H3 (H3ub2) or PCNA-associated factor 15 (PAF15-ub). The latter proteins are each covalently modified by the E3 ubiquitin-protein ligase activity of UHRF1 which recognizes hemimethylated CpG doubles (mCG:GC) using its conserved 'SET and RING finger-associated' (SRA) domain. The recruitment of DNMT1 to hemimethylated DNA generates symmetrical 5mC marks on newly replicated DNA to ensure stable maintenance of DNA methylation during mitosis.

Beyond S phase related DNA methylation to ensure propagation of epigenetic marks to daughter cells during mitosis during skeletal development, epigenetic control of gene expression by 5mC marks also occurs in post-proliferative mesenchymal cells independent of DNA replication. This active de novo methylation involves DNMT3A and DNMT3B that mediate gene inactivation at a select subset of promoters during lineage commitment. Formation of 5mC marks occurs concomitant with the recruitment of histone deacetylases (HDACs) that remove active acetyl groups and methyltransferases that generate gene suppressive H3K9me3 or H3K27me3 marks [79].

Symmetrical 5mC marks in CpG doublets (mCG:GCm) are recognized by methyl-CpG-binding domains (MBD) in proteins such as MECP2, MBD1, MBD2, and MBD4 [80]. MBD3 is a similar protein but is not capable of binding to methylated DNA. Other proteins that contain zinc finger-CxxC domains recognize non-methylated DNA (CG:GC). This class of proteins recruits lysine demethylases such as KDM2A/FBXL11 and KDM2B/FBX10 that remove active H3K4me3 and H3K36me2 marks [81–83], as well as the H3 lysine 4 methyltransferase KMT2A/MML1 at CpG islands [80].

3.2. DNA hydroxymethylation

Active DNA demethylation of 5mC marks is mediated by Ten-Eleven-Translocation (TET1, TET2 and TET3) proteins that are Vitamin C (VitC) and α-ketoglutarate dependent dioxygenases (αKGDD enzymes). TET enzymes successively convert 5-methylcytosine to 5-hydroxymethylcytosine (5hmc), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). In some cases, the formation of 5hmC from 5mC may trigger this pathway and result in nucleotide excision repair [75]. However, 5hmC also represents an independent and critically important epigenetic modification that stably marks osteoblast specific genes that are activated in a VitC dependent manner during bone formation [5]. Consistent with the relative importance of DNA methylation and DNA hydroxymethylation during VitC dependent differentiation of osteoblasts and bone formation in vivo [5,84], analysis of genome-wide DNA-methylation signatures of mouse bone marrow-derived MSCs differentiated to osteoblasts and adipocytes revealed that during osteoblastogenesis, there are >2000 changes in 5mC marks that are significantly enriched in enhancer regions, but no significant changes in 5mC during adipogenesis [85]. Recent studies corroborate the concept that TET-mediated DNA hydroxylation regulates osteoblast differentiation [5,86]. The potency of combined modulations in DNA methylation and histone acetylation can drive plasticity in lineage-commitment and support osteogenic transdifferentiation of adipocytes into osteoblasts [87].

Other studies investigated 5mC marks at related gene promoters during osteogenic lineage commitment of MSCs, indicating that DNA methylation results in inactivation of stemness related genes [88]. Promoter specific modulations in 5mC levels have been observed near genes involved in Erα (ESR1) signaling [89] and near the bone-specific BGLAP/osteocalcin gene during differentiation and mechanical stimulation [90,91]. While the regulatory interplay between TET proteins and DNMT3A and DNMT3B is critical for osteoblast differentiation, DNMT1 activity and 5mC marks are linked to MSC self-renewal and senescence [92–94], as well as modulated in response to inflammatory, apoptotic and biochemical cues [15,95–99]. From a therapeutic intervention perspective, the epigenetic landscape of 5mC and 5hmC marks is sensitive to natural compounds derived from fruits and vegetables. One clear example is the role of VitC, which is abundant in citrus fruits, as a co-factor in the formation of 5hmC marks by TET proteins in bone and its requirement for stimulating osteoblast differentiation [5]. A less well known but equally interesting finding is that certain isothiocyanates (e.g., sulforaphane) from cruciform vegetables (e.g., cabbage, broccoli) influence 5hmC levels in osteoblasts. Sulforaphane has both bone stimulatory and antiresorptive effects in vivo to increase bone mineralization parameters [100]. Hence, nutrients found in common diets may have direct effects on the epigenetic state of the osteoblast genome and potentially support bone health.

4. Enzymology of histone post-translational modifications during osteogenesis

Three principal classes of epigenetic regulators are known to functionally contribute to bone formation and these proteins (i) add new post-translational modifications of histones, (ii) read, edit and/or interpret existing modifications, and/or (iii) remove modifications. Most histone modifications occur on the N-terminal sequences of histones which are thought to point away from the central nucleosome core (*trans* orientation). Several hundred epigenetic regulators control many tens of chemical modifications on lysines (K), arginines (R) and serines (S) [101]. Lysines can be modified by acetylation via histone/lysine acetyl transferases (HATs or KATs) or histone/lysine deacetylases (HDACs), amino-groups in arginines are controlled by symmetric or asymmetric protein arginine methyl transferases (PRMTs), while kinases and phosphatases control phosphorylation of serines or theonines (e.g., MSK1, MSK2, PP1, PP2A), and yet other enzymes mediate mono-ubiquitylation of H2A (e.g., PRC1 complex) [102] and mono-ubiquitylation of H2B (e.

g. RNF20/40) [103,104].

The functional effects of histone PTMs in osteogenesis are perhaps best understood for histone acetylation (ac) and methylation (i.e., mono-, di, and trimethylation, respectively, me, me2 and me3). Histone acetylation on H4 and H3 correlates with open chromatin and/or active transcription. Histone methylation on H3 lysines 9 and 27 (H3K9me3 and H3K27me3) is associated with gene suppression, while H3K4me2, H3K4me3 and H3K79me3 are linked to open chromatin and gene activation. The function of H4 lysine 20 (H4K20me2) is more ambivalent, because H4K20me2 is associated with both repressed and expressed chromatin, because most H4 proteins in chromatin have the H4K20me2 modification [105]. Conversion of H3K9me3 and H3K27me3 marks by demethylation and acetylation into H3K9ac or H3K27ac marks represents a typical epigenetic switch during development and cellular differentiation that induces expression of repressed genes.

Beyond methylation, acetylation and phosphorylation of core histone proteins H2A, H2B, H3 and H4, many other types of histone modifications occur on lysines, arginines and/or prolines including mono-ubiquitination (Ub), ADP-ribosylation, hydroxylation, biotinylation, O-GlcNAcylation, glutathionylation, sumoylation, neddylation, citrullination, crotonylation, succinylation, and malonylation [101,106–111]. Of the four types of core histones proteins, H3 has the largest number of PTMs [111]. The combination of these modifications provides an intricate histone code that reflects the epigenetic and metabolic state of the cell, resulting in a staggering complexity in the combined information that emerges from multiple histone modifications that may influence regulatory interactions on the same or adjacent nucleosomes [112]. Different histone marks may either stimulate or suppress chromatin opening and gene activation, and bivalent genes can have either activating H3K4me3 or repressive H3K27me3 marks [113]. Furthermore, some histone-modifying enzymes represent chromatin editors that recognize one histone mark while adding or removing other marks. One important example of the latter are multifunctional integrated complexes (e.g., COMPASS-like) that support H3K27me3 demethylation, H3K4 methylation, as well as acetylation of H3K9 and H4K16 through several distinct subunits (e.g., UTX, MLL3/4 and WDR5) [114].

5. Enzymes regulation histone lysine acetylation and acylation

5.1. Histone lysine acetyltransferases

A number of skeletal disorders have been linked to histone or lysine acetyltransferases (HAT/KATs), which add acetyl groups to multiple different lysine residues in H3 and H4. The lysine-acetyl modification is recognized by bromodomain and YEATS proteins and eliminated by histone deacetylases (HDACs). Deposition of acetyl groups in osteoblasts involves CREBBP (CBP/KAT3A) and p300 (EP300/KAT3B), which are two key enzymes that have functionally evolved in higher eukaryotes [115]. Nucleotide variation in CREBBP is genetically associated with dysplasia of skeletal and facial bones (Rubinstein-Taybi syndrome) [116–118]. Both CREBBP (CBP) and p300 (EP300/KAT3B) act as transcriptional co-factors that stimulate promoters (and enhancers) of osteoblast-related genes [32,33,119–124]. In addition, two classes of HAT/KATs have essential cellular functions that each acetylate H3 and H4 proteins. The first class is represented by several Gcn5-related *N*-acetyltransferases, which include KAT2A (GCN5) and KAT2B (PCAF/GNC5-like). The second class comprises MOZ/YBF2/SAS2/TIP60 (MYST) proteins, including MYST1 (KAT8), MYST2 (KAT7), as well as MYST3 (KAT6A) and MYST4 (KAT6B). Biological roles in osteogenesis have been reported for KAT2B (PCAF) [122,125], as well as for KAT6A and KAT6B (MYST3 and MYST4) [126,127]. Because KATs are generally required for transcriptional activation, similar to RNA polymerase II and associated general transcription factors [128], these proteins are collectively important for osteogenesis. However, pharmacological agents that influence the activity of these regulatory enzymes have not yet been studied specifically in the context of bone biology.

5.2. Recognition of histone lysine acetylation

Bromodomain (BRD) proteins recognize lysine acetylation and different BRD subtypes perform distinct roles in bone. The best studied subclass of BRD proteins is 'bromodomains and extraterminal Domains' (BET) proteins. BET proteins are acetyl-lysine binding chromatin readers that contribute to normal skeletal development and bone homeostasis [63]. Expression surveys for ~40 bromodomain proteins showed that proteins BRD2 and BRD4, two members of BET protein subclass, are both prominently expressed in MC3T3 osteoblasts [20].

BRD2 has been characterized in vitro using human osteoblastic cells as a mechanosensitive regulator that is associated with RUNX2 transcription [129] and participates in an epigenetic feedback loop that responds to mechanical forces. BRD2 interacts with the RUNX2 promoter and its expression requires RUNX2, yet BRD2 levels are suppressed by mechanical loading of osteoblastic cells in vitro [129]. Decreased BRD2 expression correlates with reduced interactions at the promoter of the RANKL/TNFSF11 gene consistent with the idea that it may be involved in the coupling between osteoblast and osteoclast activities [129]. Functional studies using siRNA depletion in cultured immortalized mouse osteoblasts show that both BRD2 and BRD4 contribute to osteoblast differentiation, but BRD4 inactivation has stronger effects [20]. Similarly, depletion of BRD2 (or BRD3) impairs osteogenic differentiation of human MSCs [130]. BRD2 mutation in mice causes embryonic lethality during mid-gestation (around embryonic day 11.5), which precludes assessment of effects on bone formation [131]. Beyond on human and mouse studies, a genome-wide association study (GWAS) in chicken examined bone-related genotype/phenotype correlations and revealed that the BRD2 gene represents a quantitative trait locus (QTL) with nucleotide variation associated with bone fractures [132]. Although several studies indicate that BRD2 contributes to osteoblast differentiation [20,129,130], this bromodomain protein has remained under-explored during osteoblastogenesis.

BRD4 is the best-studied member of the BRD family, and this isoform controls bone development and homeostasis by dual effects on both osteoblast and osteoclast differentiation [130,133]. Genetic knock-out in mesenchymal tissues (PRRX1-Cre/BRD4$^{fl/fl}$ genotype) has distinct effects on murine endochondral bone formation in vivo, including delayed formation of long bones and secondary ossification defects [133]. Mesenchymal loss of BRD4 reduces SOX9 (chondrogenic) and RUNX2 (osteogenic) gene expression, possibly indicating a requirement for BRD4 to support both self-renewal and differentiation potential of MSCs [133]. Consistent with this idea, BRD4 depletion accelerates osteogenic lineage-commitment of human MSCs [130], while BRD4 loss inhibits late stages of differentiation in lineage-committed MC3T3 osteoblasts osteoblast [20].

Mechanistically, BRD4 may control bone formation by regulating interactions between enhancers and promoters marked by acetylated histones (e.g., H3K9ac and H3K27ac) in bone specific promoters [20,134,135] to drive osteogenic lineage commitment and progression. This molecular function of BRD4 is controlled by phosphorylation, because dephosphorylation of BRD4 by protein phosphatase 2A (PP2A) favors osteogenesis over adipogenesis in leptin receptor-expressing skeletal stem cells [136]. BRD4 can be directed to the RUNX2 promoter via H3K9ac marks to control other bone-specific genes; BRD4 can also be targeted to the FOXP1 and PPARG2 promoters to support the adipogenic cell fate in immortalized mouse bone marrow-derived MSCs [135]. Interestingly, glucocorticoids (e.g., methylprednisolone) can alter the distribution of H3K9ac marks in the osteogenic RUNX2 versus adipogenic PPARG promoters in bone marrow derived MSCs, thus supporting lineage switching from osteogenic to adipogenic cell fates in MSCs [135]. In addition, BRD4 is known to interact with H3K27ac [134,137], which is enhanced upon EZH2 inhibition [20,138]. Chromatin immunoprecipitation studies indicate that BRD4 positively regulates osteoblast maturation by genomic interactions with promoters and enhancers of osteoblast-related genes. For example, BRD4 binds in the

vicinity of CEBP, AP1 (FOSL2/JUND) and YAP-signaling related TEAD1 protein [134], as well as near RUNX2 binding sites in chromatin [20]. Taken together, the expression and regulatory properties of BRD4 suggests that it is an important reader of acetylated histone marks in both MSCs and osteoblasts, as well as a positive epigenetic regulator of osteogenesis.

5.3. Inhibition of bromodomain proteins

Consistent with the importance of BRD4 in osteoblast differentiation, several studies have used pharmacological agents that target the BET proteins BRD2 and BRD4 (i.e., BET inhibitors; JQ1, I-BET151). These studies consistently show that BET inhibitors reduce osteogenic differentiation of human MSCs, as well as maturation of immortalized fetal osteoblasts (hFOB), mouse MC3T3 osteoblasts and IDG-SW3 mouse osteocytes [20,130,134,139]. Hence, BET inhibitors block osteogenesis independent of the differentiation-stage. Nevertheless, BET inhibitors appear to be effective as a molecular strategy to reverse low bone mass conditions, including degenerative conditions due to estrogen loss or inflammation [63]. BET inhibitors reduce bone loss due to ovariectomy [130], glucocorticoid treatment [135], TNF-induced inflammatory osteolysis [140] and inflammation-impaired bone healing [141]. This bone-protective effect of BET inhibitors is primarily due to a blockade of osteoclastogenesis (e.g., BRD4/NFATC1/RANKL axis) [130,139,140]. Consistent with anti-resorptive effects of BET inhibitors, these compounds have been applied to reduced alveolar bone loss due to periodontal inflammation [142–145]. The spatiotemporal contributions of BRD2 and BRD4, as well as the effects of BET inhibitors on differentiation in the osteoblast and osteoclast lineages need to be further examined. A more refined mechanistic understanding of the effects of BET inhibitors on the physiological and molecular interplay between osteoblasts and osteoclasts is required to obtain a sound rationale for treatment of bone-related bone degenerative disorders like osteoporosis.

Beyond biochemically selective BET inhibitors, non-specific and low affinity bromodomain inhibitors like the generic organic solvents *N*-methyl pyrrolidone (NMP), *N*,*N*-dimethylacetamide (DMA) and N-Vinyl-2-Pyrrolidone (NVP), have also been investigated for bone protective effects. These solvents may have effects on bone homeostasis by both anabolic and catabolic pathways. NMP and DMA block the negative effects of the inflammatory mediator TNFα on mineralized bone healing [141]. Other studies showed that NMP blocks both induction of Sclerostin (SOST) mRNA and protein expression by BMP2 or estrogen-deficiency (due to ovariectomy) in osteocytes, as well as inhibiting osteoclast differentiation and bone resorption in mice [146,147]. Similarly, NVP has been considered for bone regenerative applications based on non-specific interactions with bromodomain proteins. NVP enhances bone regeneration and BMP2 signaling, while blocking osteoclastogenesis in vitro [148]. However, applying generic solvents such as NMP, DMA and NVP as therapeutics for osteoporosis or bone regeneration [146–148] appears to have a very low utility due to the lack of specificity for BET proteins, as well as the potential for biological toxicity and off-target effects.

5.4. Histone lysine deacetylation

Bone phenotypes observed in mice with mutations in enzymes that mediate histone lysine deacetylation suggests that these proteins are important for osteogenesis [149,150]. Lysine deacetylation is mediated by several distinct classes of HDACs and sirtuins (SIRTs) that are highly conserved in eukaryotes and have homologs in yeast (e.g., RPD3, HAD1 or SIR2) [24,151]. Several HDACs are known to control osteoblast-specific gene expression and osteoblast differentiation [152–158], as well as principal osteogenic cell signaling pathways [19,62], including PTH mediated suppression of the WNT antagonist SOST by HDAC4 and HDAC5 [158–160]. HDACs also remove acetyl moieties from non-histone proteins including the bone-related transcription factor RUNX2 [157,161,162]. Because HDACs do not have DNA binding potential, they act as transcriptional co-repressors for several key transcription factors (e.g., RUNX2, NFATC1, ZFP521 and PBX1) [32,161,163,164]. Genetic studies with conditional null alleles of HDACs revealed that at least three HDAC genes are required for normal skeletal development and bone formation. The genes for HDAC1, HDAC3, HDAC4, and HDAC7 have been conditionally deleted in skeletal tissues and gene ablation results in skeletal abnormalities, including morphological changes in craniofacial bones, shortened limb bones and reduced bone mass [149,150,165].

5.5. Pharmacological inhibition of HDACs

HDAC inhibition (HDACi) by pharmacological agents (e.g., suberoylanilide hydroxamic acid, SAHA) activates osteoblast-related genes and stimulates osteoblast differentiation by increasing the level of H4 acetylation across the genome in MC3T3 cells [62]. This stimulatory effect in committed osteoblasts is associated with modulations in the expression or phosphorylation of components of the insulin signaling pathway such as SLC9A3R1/NHERF1, as well as insulin receptor β, AKT, and FOXO1 to activate the insulin/AKT/FOXO1 signaling axis [62]. The HDACi-mediated stimulation of SLC9A3R1/NHERF1 scaffold results in the activation of protein phosphatase-1α (PP1α; Protein Phosphatase 1 Catalytic Subunit Alpha/PPP1CA) which binds to NHERF1. PPP1CA activation enhances the nuclear localization of transcriptional co-factor TAZ [166]. Because TAZ is linked to the HIPPO/YAP1 pathway [167] and a known regulator of osteogenesis [168], its activation by HDACi provides one plausible mechanism by which increased histone acetylation stimulates osteoblastogenesis. However, HDACi also has undesired effects. In uncommitted mesenchymal stromal cells, HDAC inhibition (using SAHA) modulates SLC9A3R1/NHERF1 and the AKT/FOXO1 pathway, but these events occur concomitant with changes in lineage specific transcription factors that compromise multi-lineage differentiation of mesenchymal stromal cells (MSCs) [19]. The stimulatory effects in osteoblasts and destabilizing effects in mesenchymal stromal cells (MSCs) of HDAC inhibitors render these compounds less than ideal for systemic applications to promote bone formation.

5.6. Nicotinamide adenine dinucleotide (NAD+) dependent protein deacetylases

The NAD(+) class III histone deacetylases (sirtuins, SIRTs) support bone formation in vivo and pharmacological agonists of SIRT1 may provide a new class of drugs to treat low bone mineral density [169]. For example, osteoblast-stimulatory mechanistic effects of SIRT1 are evident from the in vivo bone anabolic effects of pharmacological activation of SIRT1 in two mouse models with low bone mineral density (i.e., ovariectomized female mice and aged male mice) [170]. The SIRT1 agonist SRT1720 enhances bone mass in both models. Furthermore, SIRT1 null mice, and mice in which SIRT is conditionally deleted in osteoblasts show a low bone mass phenotype, consistent with the positive role of SIRT1 in osteogenesis [170].

Mechanistically, SIRT1 may exert its bone stimulatory effects in part by stimulating WNT/β-catenin signaling in SP7/Osterix positive osteoblast precursor cells by blocking the inhibitory interactions of FOXO transcription factors with β-catenin [171]. Notably, SIRT1 activity declines with aging suggesting that strategies involving SIRT1 activation may reverse age-related decreases in bone mass [171]. Corroborating these findings, mice treated with the polyphenol resveratrol results in increased osteoblast activity and stimulated bone growth, presumably via effects on BMP2 and its downstream targets (e.g., alkaline phosphatase, ALPL) [172]. Consistent with the concept that SIRT1 is a key bone stimulatory enzyme, SIRT1 exerts positive effects on osteogenesis because it is also regulates the transactivation potential of the osteoblast-related transcription factor RUNX2. Consequently, loss of SIRT activity (by genetic deletion) reduces expression of RUNX2 target

genes, while increasing SIRT activity (using resveratrol) enhances expression [170]. Activation of SIRT1 and consequently RUNX2 by resveratrol also reverses suppression of osteogenic differentiation of bone marrow derived MSCs by the TNF/NFKB1 signaling pathway [173]. Collectively, these studies suggest that SIRT1 controls at least three major regulatory axes of osteogenesis: WNT/β-catenin, BMP2/RUNX2 and TNF/NFKB1-signaling.

Osteoblast stimulatory effects of SIRT1 may also emerge from SIRT1 mediated deacetylation of the VDR, which forms a heterodimeric transcription factor with retinoid-X-receptor (RXR) and is activated by its bioactive ligand VitD(3) [174]. Deacetylation of VDR in the presence of VitD3 or the SIRT1 activator resveratrol increases the expression of classical VDR target genes (e.g., CYP3A4 and CYP24A1) in HEK293 kidney and TE85 bone cells. These findings suggest that VDR acetylation is part of negative feedback inhibition and that deacetylation of VDR by SIRT1 amplifies VDR signaling [174]. However, other studies indicate that SIRT1 suppresses downstream effects of parathyroid hormone (PTH) signaling in osteoblasts [175]. The PTH target MMP13 exhibits dependent increased expression in SIRT1 null mice [175]. In addition, the activated expression of MMP13 in response to PTH/cAMP/AP1 (FOS/JUN) signaling is enhanced by the SIRT1 inhibitor EX27 and blocked by the SIRT1 activator resveratrol in UMR106–01 osteosarcoma cells [175]. SIRT1 achieves these effects by mediating the deacetylation of JUN protein, which deactivates the protein and reduces expression of osteoblast related targets of PTH [175]. Beyond direct and indirect transcriptional effects of SIRT1 on osteoblast-specific gene expression, SIRT1 and melatonin may also work together to preserve the intracellular antioxidant properties of MSCs [176]. Natural pharmaceutically active compounds (e.g., nutraceuticals) that target SIRT1 provide viable opportunities for improving or preserving bone mass [177]. While many studies focus on the grape-derived polyphenolic compound resveratrol, SIRT1 activity can also be stimulated with other natural compounds (e.g., ferulic acid, N1-methylnicotinamide, melatonin, nicotinamide riboside, glucosamine, and thymoquinone) [177]. Taken together, SIRT1 is an important epigenetic target that controls osteogenesis.

Although SIRT1 function appears to be bone stimulatory, the mitochondrial sirtuin SIRT3 is dispensable for bone formation [178]. Instead, SIRT3 contributes to bone loss via osteoclast mediated bone resorption in aging or ovariectomized (estrogen deficient) mice and the SIRT3 inhibitor LC-0296 impairs multinucleation of differentiating osteoclasts [178]. Thus, different SIRT isoforms appear to have physiologically distinct functions in bone homeostasis. Subsequent studies corroborated these findings, because SIRT3 also is dispensable for new bone formation during murine digit regeneration [179]. Although several studies clearly demonstrate that SIRT3 does not contribute to bone formation, one recent study showed osteocyte related requirements for SIRT3 in controlling bone mass and mechanical responses, based on mice with a SIRT3-specific deletion in osteocytes or mice treated with the SIRT3 activator honokiol [180]. SIRT3 may have a distinct role in osteocyte function by mediating the generation of osteocytic cell extensions (i.e., dendritic processes) and/or osteocyte responses to mechanical loading of bone during exercise in aged mice [180]. Altogether, different SIRT proteins may perform different SIRT-related functions at different stages of the mesenchymal differentiation process from skeletal stem cells to osteoblasts and osteocytes.

5.7. Histone lysine acylation as a new frontier in osteogenesis

Modifications of lysines in histones with short-chain fatty acids, which are linked to acyl-coenzyme levels, represent a less explored mechanism by which epigenetic modifications in chromatin regulate competency for gene expression [101,109–111,116]. These enzymatic modifications have not yet been explored within the context of bone. Acyl-CoA compounds represent metabolic intermediates that are reversibly generated during either β-oxidation or elongation of fatty acids and are key donors of acyl groups for histone modifications. The biochemical similarities between acyl CoA compounds are also reflected by enzymatic conversions between different acyl groups (e.g., butyryl-CoA is reversibly converted into crotonyl-CoA). The types of lysine acylation that have been biochemically characterized to date include propionylation, butyrylation, 2-hydroxyisobutyrylation, succinylation, malonylation, glutarylation, crotonylation, β-hydroxybutyrylation, myristoylation, palmitoylation and methacrylation [101,106,109–111,181,182]. Non-acetyl histone acylation may couple epigenetic control of gene expression with metabolically regulated levels of different acyl-CoA types in a range of biological conditions (e.g., cell stress and tissue injury) [109]. Histone lysine crotonylation (Kcr marks) are of particular interest because Kcr marks are found in active gene regulatory sequences (promoters and enhancers) and include specialized roles in maintaining active status of genes on sex chromosome-associated genes [101].

Multiple enzymes are able to modify histone lysines by acyl modification, including the KATs like EP300 (p300/KAT3B), KAT2A (GCN5) and KAT5 (ESA1/TIP60). KAT2A interacts with succinyl-CoA to mediate succinylation of H3 on lysine 79 (H3K79suc) [183]. Similarly, KAT5 is part of KAT complexes that mediate crotonylation of H3 and H4 to support transcription [184]. Furthermore, acetyl-CoA acyltransferase 2 (ACAA2) selectively mediates acetylation and succinylation of H4 at promoters in which the DNA replication independent histone variant H2A.Z is incorporated into chromatin [185]. Recognition of non-canonical modifications like butyrylated- or crotonylated histone is achieved by a distinct set of bromodomain proteins that recognize fatty acid moieties on lysines, such as BRD9, Cat Eye Syndrome Critical Region Protein 2 (CECR2), and TATA-Box Binding Protein Associated Factor 1 (TAF1).

The removal of histone acylation is mediated by HDACs (HDAC1, HDAC2 and HDAC3) and nicotinamide adenine dinucleotide (NAD)-dependent protein lysine deacetylases (silent mating type information regulation 2 homologs; sirtuins: SIRT1 to SIRT7) [182,186–188]. In mammals, >20 HAT/KATs have the potential to transfer acetyl co-A to lysines in histones and non-histone proteins in either the nucleus or cytoplasm [189], while 18 distinct HDAC/KDACs and SIRTs can potentially reverse protein acetylation or acylation [190]. For example, HDAC1 forms complexes with RCOR1, and LSD1 can deacylate K3K18ac and H3K18cr, which are marks co-localized near transcriptional start sites [187]. SIRT1, SIRT2 and SIRT3 are effective lysine deacetylases compared to SIRT4 to SIRT7. The ability of SIRTs to act as lysine deacylases to remove longer carbon chain moieties such as succinylation, palmitoylation and myristoylation depends on the size of a hydrophobic pocket [182,186]. Structural studies noted that SIRT1, SIRT2, SIRT3 and SIRT6 have active site pockets that can accommodate longer chain acyl groups [182]. Other studies provided analogous structural arguments indicating that SIRT5 represents a lysine demalonylase and desuccinylase [186]. The importance of HDACs and SIRTs in bone formation is evident from other studies cited in this review, but the specific epigenetic effects of histone acylation on chromatin structure of bone related genes remain to be investigated.

Acylated lysines are recognized by bromodomain-containing proteins (BRD1 to BRD9), including “BRD and Extra-Terminal domain” (BET) proteins. These proteins bring RNA polymerase II (RNAP II) complexes to transcriptional start sites and interact with CDK9, which phosphorylates RNAP II to stimulate transcription of osteoblast-related genes. From this molecular perspective, it is likely that histone acylation events are important rate-limiting events for osteogenesis.

The YEATS domain proteins YEATS1 (MLLT1), YEATS2, YEATS3 (/MLLT3/TAF14/AF9) can read acetylated and diacetylated histones, but also interact crotonylated histones [191–200]. YEATS3 (TAF14/MLLT3/AF9) specifically interacts with modified H3K9 and H3K18 residues by methacrylation and crotonylation [181]. Recognition of H3K9 crotonylation by YEATS3 inversely relates fatty acid β-oxidation and the metabolic state of the cell to gene activation via H3K9 acetylation [201]. YEATS3 and YEATS4 (YAF9) recognize histone

crotonylation as part of biological functions related to cellular defense mechanisms for fungal infections [202]. In contrast to bromodomains proteins, YEATS3 prefers crotonyllysine over acetyllysine, and exhibits co-localization with crotonylated H3 to support gene activation [197]. While there are solid biochemical and cellular studies on histone acylation, no current studies link this biochemical modification specifically to bone-related biological processes.

6. Enzymes mediating histone lysine methylation

6.1. Trimethylation of H3 lysines

Three principal lysine methylation marks (H3K4me3, H3K9me3 and H3K27me3) collectively control the extent to which genes are (i) transcriptionally poised or active (marked by H3K4me3), (ii) suppressed in chromatin (e.g., constitutive heterochromatin; marked by H3K9me3), or (iii) rendered conditionally inaccessible (i.e., facultative heterochromatin; marked by H3K27me3) during skeletal development (Figs. 2 and 3). Three types of histone PTMs play critical roles in osteogenesis. Histone methylation is catalyzed by two types of lysine methyltransferases (KMTs) with either SET-domains (e.g., EZH1 & EZH2) or DOT1-like (DOT1L) proteins [189]. KMTs can either stimulate or suppress transcription. Multiple KMTs ($n > 28$) are capable of modifying amino groups with one, two or three methyl groups to either lysine or arginine residues in H3 and H4. KMTs with established functions in bone formation encompass EZH1 (KMT6B), EZH2 (KMT6A), SETD2 (KMT3A) and DOT1L (KMT4). The resulting mono (me), di (me2) and tri (me3) methyl-lysine marks can be removed by multiple lysine demethylases (KDMs; $n > 18$). The KDM class of histone modifiers may also potentially control osteogenesis, and include Jumonji domain proteins (JMJDs; KDM2A-4D, KDM6B), JARID protein (KDM5A-D), UTX (KDM6A) and UTY (KDM6C) proteins [5,189]. The latter enzymes may be highly dependent on the osteogenic effects of VitC and are important for osteogenic differentiation [5]. Hence, the epigenetic mechanisms controlled by these enzymes deserve further analysis.

6.2. H3K27me3 transferase EZH2

Studies focused on the role of H3K27me3 in osteogenesis, have revealed the importance of polycomb repressive complex 2 (PRC2). Mammalian PRC2 contains three subunits: 'Embryonic Ectoderm Development' EED), 'Suppressor of Zeste 12 Protein Homolog' (SUZ12) and 'Enhancer of Zeste Homolog 2' (EZH2), which is the subunit that catalyzes H3K27 trimethylation [203,204]. The EZH2 subunit can be functionally substituted by the related protein EZH1. Biochemically, PRC2 mediated H3K27me3 marks increase chromatin condensation to suppress gene transcription [205]. Genetic studies with EZH2 null mice have shown that EZH2 is required for normal embryo formation and suppressed generation of mesodermal cells [206]. Studies using conditional EZH2 floxed alleles have shown that EZH2 in mesenchymal cells is genetically linked to skeletal patterning (T-Cre, Prrx1-Cre) [59,207], craniofacial development (WNT1-Cre, PRRX1-Cre) [59,208,209], and endochondral bone formation (PRRX1-Cre, COL2A1-Cre, and SP7/Osx-Cre) [59,208,210–212]. The endochondral bone phenotype of EZH2 loss has been further validated by cartilage-specific deletion of the EED subunit that inactivates both PRC2/EZH1 and PRC2/EZH2 complexes [213], as well as double-knockouts of both EZH1 and EZH2 [212]. Furthermore, in vivo pharmacological inhibition of EZH2 in mouse models shows that loss of EZH2 activity stimulates net accrual of bone due to the stimulation of osteoblasts [59] and osteoclasts [214,215]. The bone stimulatory effect of EZH2 inhibition prompted the characterization and development of a larger set of EZH2 inhibitors [138]. Interestingly, dietary differences control the EZH2 dependent ratio of H3K27me3 and H3K27ac marks, and osteoblast differentiation is negatively affected in the calvarial cells of pups from litters with female mice maintained on a high fat diet [216]. Beyond mouse models, mutations in human EZH2 cause Weaver syndrome which includes distinct skeletal abnormalities [217]. Collectively, these in vivo genetic studies establish that EZH2 and the epigenetic mark it generates (H3K27me3) are important for mammalian bone formation [217].

Mechanistically, EZH2 regulates bone formation via effects on lineage-commitment of mesenchymal stem cells, where EZH2 inhibition during differentiation favors osteogenesis over adipogenesis [218–220]. One major function of EZH2 is to maintain stemness of mesenchymal stem cells in conjunction with β-catenin/CTNNB1 [221]. EZH2 activity during osteoblast differentiation appears to be suppressed by upregulation of MIR101, which directly targets the EZH2 mRNA and promotes trabecular bone accrual [222]. Furthermore, EZH2 may function together with the lysine methyl transferase SMYD2 [223] and different arginine methyl transferases (PRMTs) [224]. EZH2 inhibition by itself does not drive differentiation unless there is an osteogenic stimulus (e.g., ascorbate), but in the presence of an osteogenic cocktail (e.g., VitC or exogenous BMP2) it activates endogenous BMP signaling in both mesenchymal stem cells and pre-committed osteoblasts [59]. Co-treatment of EZH2 inhibitors and exogenous BMP2 results in synergistic stimulation of osteogenesis [225]. The latter is consistent with the concept of epigenetic priming whereby the loss of EZH2 results in the disappearance of suppressive H3K27me3 marks promotes the appearance of activating H3K27ac marks, and the concomitant activation of a bone-related gene expression program that supports matrix mineralization.

6.3. Osteogenic effects of EZH2 inhibition

Because pharmacological inhibition of EZH2 stimulates osteoblastogenesis and bone formation, several studies have examined the effects of natural compounds on EZH2 dependent suppression of osteogenic differentiation. Two studies examined the effects of the fungal metabolite Cytochalasin D (CytoD) on human bone marrow or adipose-tissue derived MSCs [220]. CytoD blocks cytoplasmic polymerization of intracellular location of β-actin (ACTB) and enhances nuclear accumulation, polymerization and branching of actin filaments. Importantly, CytoD reduces mRNA and protein expression of EZH2, as well as total genomic H3K27me3 marks, but this positive effect is blocked by inhibitors that prevent actin branching [220]. Meta-analysis of RNA-seq data from different types of MSCs from human and mouse established that CytoD activates differentiation related genes and reduces expression of proliferation related genes, as well as a group of eight uncharacterized genes [226]. Interestingly, CytoD activates VGLL4, a distinct isoform of the Vestigial class of proteins that acts in the Hippo/YAP/TAZ pathway, and inactivation of VGLL4 prevents osteogenic differentiation of human MSCs [226]. Hence, CytoD mediated actin reorganization modulates EZH2 activity and the VGLL4/HIPPP pathway during osteogenesis [226]. In addition, EZH2 activity can be attenuated by microRNAs. Recent studies showed that microRNA-101a suppresses EZH2 activity in osteoblasts, reduces H3K27me3 levels and promotes mineralization of MC3T3-E1 cells [222]. Hence, miRNA-based RNA-therapeutics that target translation or stability of the EZH2 mRNA could be considered for bone regenerative strategies (e.g., in bone tissue engineering using MSCs).

The earliest pathways that are activated upon EZH2 inhibition are also of interest within the context of multi-modality therapeutic reagent screening. For example, EZH2 inhibition increases the expression of the G-protein coupled receptor 5C (GPRC5C) [227]. GPRC5C represents an under-studied member of the G protein coupled receptor family. Its expression is induced by BMP2, VitC or EZH2 inhibitors, but all three compounds together generate the highest level of induction [227]. Loss of function studies in cultured cells revealed that GPRC5C is required for osteoblast differentiation [227]. In vivo studies using mouse cortical and trabecular bone show that GPRC5C is downregulated in trabeculae of ovariectomized mice that exhibit osteopenia and upregulated by estrogen restoration using 17β-estradiol [227]. These studies indicate that

downstream targets of EZH2 are required for bone formation. Because GPRC5C is an orphan receptor, it is an attractive target for the development of agonists that activate GPRC5C signaling to attenuate bone degeneration and promote new bone formation [227].

Other recent studies have shown that EZH2 inhibition by Tazemetostat (EPZ6438) activates the WNT and Hedgehog (Hh)/GLI1 signaling pathways in osteoblasts [60]. Tazemetostat reduces H3K27me3 marks in osteoblast related enhancers in MC3T3 cells near bivalent genes that contain both activating H3K4me3 and repressive H3K27me3 marks [60]. Thus, EZH2 normally suppresses WNT and Hh-GLI1 signaling at bivalent genes to prevent osteogenesis. Collectively, mechanistic findings with GSK126 [227] and Tazemetostat provide new insights into downstream targets that control osteoblast differentiation and bone formation. Agents that modulate the activity of these EZH2 targets could have useful bone anabolic effects that increase bone mass in osteoporotic patients.

6.4. Osteogenic regulation by other lysine methyl transferases

Several studies performed expression surveys using next-generation RNA sequencing (RNA-seq) and semi-automated real-time qPCR platform focusing on mRNAs for epigenetic regulators [58,59,223,224,228]. Evaluating expression of KMTs in osteoblasts revealed that the H4K20 methyl transferase SUV420H2 is expressed more robustly than other methyl transferases (e.g., SUV39H1, SUV39H2, SUV420H1, EZH1, and EZH2). The H3K9 methyl transferases SUV39H1 and SUV39H2 are expressed at higher levels in early stages of osteoblast differentiation (at day 3), while SUV420H1 and SUV420H2 are higher at the subsequent developmental stage (at day 7) prior to formation of a mineralized extracellular matrix. Inactivation of SUV420H2 by RNA interference reduces H4K20 methylation and decreases expression of classical bone-related genes (e.g., ALPL, SP7/Osterix), indicating that SUV420H2 dependent modifications of the H4K20 methylome are required for normal osteoblast differentiation [228].

6.5. Methylation of H3K4, H3K36 and H3K79

Lysine methylation has been extensively studied in the context of histone proteins as a mechanism of epigenetic regulation [229]. Many lysine methyltransferases (KMTs) [230] modify both histone and non-histone proteins at chromosomal, nuclear, and cytoplasmic locations. KMTs are relevant to bone, because transcriptionally active marks (e.g., H3K4me3 and H3K36 methylation) in principle could positively support expression of genes required for osteoblast differentiation once suppressive marks (e.g., H3K27me3 and H3K9me3) have been removed. As discussed below, several important KMTs and components of KMT complexes are known to promote osteoblast differentiation, including WD repeat domain 5 (WDR5), SET and MYND Domain 2 (SMYD2) and Disruptor of telomeric silencing 1-like (DOT1L). The opposing lysine demethylases also have functional roles in osteoblastogenesis, including the lysine-specific histone demethylase 1 (LSD1) and the less characterized histone demethylase NO66/RIOX1.

Early studies focused on the role of the 'WD repeat domain 5' (WDR5), which is part of a KMT complex with either H3K4-specific methyltransferase activity or H4K8-specific acetyltransferase activity [231]. WDR5 was initially characterized as a BMP2 responsive WD-40 repeat protein (BMP2-induced gene 3 kb, BIG-3) [232] that stimulates osteoblastic differentiation both in culture [232] and in vivo upon transgenic over-expression [233]. Binding of WDR5 to osteogenic genes (e.g., IBSP) and concomitant presence of active marks (e.g., H3K4me3, H3K36me3, and H3K acetylation) depends on SP7/Osx [40]. Two KMTs that modify H3K36 have been genetically linked to bone abnormalities (e.g., craniofacial and/or skeletal malformations). Mutations in NSD1 (KMT3B/SOTOS1) are causative for Sotos syndrome (e.g., macrocephaly) [234,235]. NSD2 is associated with Wolf-Hirschhorn syndrome (KMT3G/WHSC1) and is required for chromatin remodeling of osteoblast-related genes [236–238]. These findings highlight the importance of proper regulation and deposition of H3K4 methylation and H3K36 methylation in osteogenesis.

SMYD proteins represent H3K4 methyl transferases that are encoded by five different genes (SMYD1-5) in humans and other mammalian species [239,240]. SMYD proteins have tissue- and cell type-specific functions. For example, SMYD1 is a muscle-specific isoform that contributes to muscle development [241,242] while SMYD2 is more prominently expressed in other mesenchymal tissues and lineages [223]. SMYD proteins contain a catalytic SET domain, which methylates lysines of transcriptional regulatory proteins including histones and growth regulatory proteins (e.g., p53/TP53), as well as a MYND (Myeloid-Nervy-DEAF1) domain that mediates protein-protein interactions [243]. Like most if not all methyl transferases, SMYDs catalyze lysine methylation using S-adenosylmethionine (SAM) as a methyl donor, while releasing S-adenosylhomocysteine (SAH) [244,245]. The methyltransferase activity of SMYD proteins can be targeted by pharmacological inhibitors that target the SET domain [246]. Mono-, di- and trimethylation of lysine residues by SMYD2 in both histone and non-histone proteins contributes to regulation of diverse cellular processes including gene regulation, chromatin remodeling, transcription, signal transduction, cell cycle control, and DNA damage repair [26]. SMYD2 methylation of the growth suppressor P53/TP53 on K370 decreases the ability of P53 to bind DNA, inhibits its ability to activate P53 target genes [247] and prevents P53 mediated apoptosis [248]. Hence, SMYD2 mediated lysine methylation of P53 provides epigenetic regulation of cell growth and survival. Interestingly, RUNX2 also suppresses osteoblast proliferation [249,250], and P53 suppresses RUNX2 activity by stimulating expression of miR-34 [251]. These findings collectively suggest that SMYD2, P53 and RUNX2 form an integrated cell growth regulatory circuit in osteoblasts, consistent with recent loss of function studies [223].

Beyond P53, SMYD2 also methylates EZH2 at lysine 307 (K307) [252] and β-catenin (CTNNB1) [253] to stabilize these proteins in cancer cell types [252]. EZH2 is a principal epigenetic regulator of bone formation and EZH2 inhibitors stimulate bone mineral density in mice and is osteoprotective in ovariectomized estrogen-deficient mice [46,252]. The WNT responsive β-catenin protein regulates mesenchymal stem cell fates and osteogenic lineage commitment in an EZH2 dependent manner [254–256]. Recent studies suggest that SMYD2 works together with EZH2 to control osteoblast growth and differentiation [223]. Depletion of SMYD2 in mouse MC3T3 osteoblasts increases cell proliferation and enhances calcium deposition in the extracellular matrix, while combined loss of SMYD2 and EZH2 display synergistic effects. Consequently, SMYD2 and EZH2 may normally work together to suppress osteoblast differentiation through effects on H3K27me3 [223].

The histone 3 lysine 79 methyltransferase encoded by the Disruptor of telomeric silencing 1-like (DOT1L) is genetically linked to human height [257], because DOT1L is required for normal skeletal development [258,259]. Mesenchymal or cartilage-specific deletion of DOT1L (PRXX1/Cre, COL2A1/Cre & ACAN/Cre) collectively revealed that loss of this enzyme disrupts growth plate development (limb shortening), reduces biosynthesis of extracellular matrix production and mineralization, and reduces trabecular bone formation [258,259]. Natural mutations in DOT1L have been linked to bone sarcomas (i.e., undifferentiated pleomorphic cancers) [260]. The histone methyltransferase DOT1L may also control bone mineral density by attenuating osteoclastogenesis [261].

The association of H3K4 methylation in active genes is counteracted by H3K4 demethylases such as lysine-specific histone demethylase 1 (LSD1). ChIP-Seq analysis revealed that LSD1 is prominently present at gene promoters in osteoblasts. Its binding sites were associated with the di- and tri-methylation of histone 3 at lysine 4 (H3K4me2 and H3K4me3). LSD1 tends to localize at genomic regions enriched for RUNX2 binding sites in osteoblasts, suggesting that the activities of LSD1 and RUNX2 are functionally coupled and that LSD1 is an

important co-factor for proper bone formation [262]. The NO66/RIOX1 enzyme, which is a histone demethylase that doubles as a ribosomal oxygenase, opposes the beneficial function of WDR5 in supporting osteogenesis [231]. RIOX1 may mediate gene suppressive DNA methylation of osteoblast promoters (5mC marks) together with the DNA methyl transferase 1A (DNMT1A), histone deacetylase 1 (HDAC1), and the H3K9me3 binding complex HP1 [40], which contains the chromobox proteins CBX1, CBX3 and CBX5.

6.6. Recognition of histone lysine methylation by chromobox (CBX) proteins

Chromobox proteins (CBX), recognize specific histone PTMs (H3K9me3 and H3K27me3) and play essential roles in maintaining heterochromatin, but their functions in bone development remain to be fully resolved [263]. One group of CBX proteins (CBX1/HP1β, CBX3/HP1γ, and CBX5/HP1α) forms a complex that recognizes H3K9me3, which is an epigenetic mark that is deposited in constitutively inactive heterochromatin. A second group of CBX proteins includes CBX2, CBX4 and CBX6 that recognize H3K27me3, a mark that is generated by the PRC2 complex which contains the H3K27 methyltransferases EZH2 or EZH1 [263]. As discussed below, each of these CBX isoforms has different patterns of expression and distinct epigenetic functions in osteoblasts.

Skeletal hypoplasia and reduced numbers of bone marrow derived multipotent mesenchymal stromal cells are observed in mice lacking CBX2, which recognizes H3K27me3 marks. RNA-sequencing revealed that CBX2 loss alters the expression of genes associated with osteoblastic, adipogenic, and B-cell precursor lineages. Forced expression of CBX2 in CBX2-deficient bone marrow stromal cells rescued fibroblastic colony formation and suppressed adipogenic differentiation. This finding suggests that CBX2 is crucial for maintaining and regulating mesenchymal stromal cells in bone marrow, thereby impacting normal skeletal development [264].

Although studies directly examining CBX4 function in bone are lacking, a few studies have linked CBX4 to molecular pathways in osteosarcoma cells and brain cell mineralization. CBX4 may attenuate calcification in brain cells via a BMP2/miR-181b/CBX4/HDAC3/RUNX2 axis [265]. In this model, upregulation of miR-181b by BMP2 suppresses CBX4 and prevents the recruitment of HDAC3 to the RUNX2 promoter, thus activating RUNX2 expression and mineralization [265]. In osteosarcoma cells (i.e., osteoblastic cancer cells that are defective in osteoblast maturation) CBX4 activity is regulated by the RING finger factor RNF180, which is an E3 Ubiquitin-Protein Ligase that suppresses cell proliferation markers (e.g., MKI67). Here, RNF180 negatively regulates CBX4 to activate RUNX2, suggesting that an RNF180/CBX4/RUNX2 axis may contribute to osteoblastogenesis [266]. Other studies using osteosarcoma cells suggest that CBX4 may also upregulate RUNX2 expression by recruiting the lysine acetyl transferase GCN5/KAT2A [267]. The latter study also showed that CBX4 is normally destabilized levels by casein kinase 1α (CK1α/CSNK2A1) that phosphorylates a degron (at T437) to force ubiquitylation (at K178 or K280) and subsequent degradation by CHIP/STUB1 [267]. While these individuals studies provide some insights into CBX4 dependent molecular mechanisms, additional investigations will be required to gain integrated models for the biological role of CBX4 during normal osteogenic differentiation.

The roles of the HP1 subunits CBX1 (HP1β), CBX3 (HP1γ), and CBX5 (HP1α) proteins have not been studied to a sufficient degree during osteogenesis. Preliminary studies indicate that expression of all three proteins is modulated during osteoblast differentiation and have functionally redundant functions in epigenetic control [263]. Genome-wide association studies strongly indicate that CBX1 may influence bone mineral density and fracture risk [2,3]. Furthermore, one study measured transcript levels of CBX5 and reported that CBX5 expression is reduced during adipogenesis in both porcine adipose tissue derived MSCs and bone marrow derived MSCs. These results suggest that CBX5 is inhibitory for adipogenic differentiation of porcine MSCs [268]. Because the HP1 complex recognizes H3K9me3, it is predicted to have a major role in maintenance of constitutive heterochromatin during osteoblast differentiation resolved [263].

Different CBX proteins have distinct functions involved in maintaining chromatin organization and phenotype identity in various cell types and tissues. These proteins are relatively stable, which has implications for the interpretation of RNA interference (RNAi) experiments targeting epigenetic regulators [263,269]. The most abundant chromatin structural proteins, core histones H2A, H2B, H3, and H4, have long half-lives and do not undergo rapid turnover, so their mRNA molecules are comparatively unstable. This contrast raises the question of whether epigenetic regulatory enzymes (such as EZH2) or proteins that interact with histones through specific modifications (like CBX1 to CBX8 and BRD4) exhibit stability or instability. Blocking mRNA transcription and protein translation in mesenchymal cell types (e.g., mouse MC3T3 osteoblasts, ATDC5 chondrocytic cells and C2C12 myoblasts) indicate that CBX and EZH2 mRNA levels are significantly diminished after ~24 h of inhibition, whereas their corresponding proteins remained intact. These findings indicate that histone code readers and writers are stable chromatin-related proteins required for robust temporal responses to demands for altered gene expression patterns in cells [269].

7. Histone arginine methylation

Arginine methylation by distinct amino-acid selective PRMT proteins results in a range of unique methylation patterns on amino-side chains that either activate or repress transcription [270–272]. The most prominent arginine marks identified to date include H4R3me2a (asymmetric active mark), H4R3me2s (symmetric inactive mark), as well as H3R2me2s (active), H3R17me2a (active mark), H3R26me2a, and H3R42me2a.

PRMT proteins represent enzymes with broad protein substrate specificity for histones and non-histone proteins in either the nucleus or cytoplasm [107,271]. These epigenetic regulators can either activate or repress transcription via asymmetrical arginine methylation (Class I: PRMT1, PRMT2, PMRT3, CARM1/PMRT4, PMRT6 & PRMT8), symmetrical arginine methylation (Class II: PRMT5 & PRMT9) or arginine monomethylation (Class III: PRMT7) with selectivity for different arginine moieties in histones H3 and H4. PRMT1 generates transcriptionally active monomethyl H4R3me1 and asymmetric dimethyl H4R3me2a marks, while CARM1/PRMT4 generates activating asymmetric dimethylarginine (H3R17me2a) marks [273–275]. Thus, PRMT1 and CARM1/PRMT4 represent epigenetic transcriptional co-activators. In comparison, PRMT5 catalyzes the formation of symmetric H3R2me2s (active mark) and H4R3me2s (repressive mark) [270–272] suggesting a bifunctional role in transcriptional control. Only two arginine demethylases have been characterized that can antagonize PRMT activity: JMJD1B/KDM3B and JMJD6. Both enzymes have dual demethylase activities for lysines and arginines [276,277].

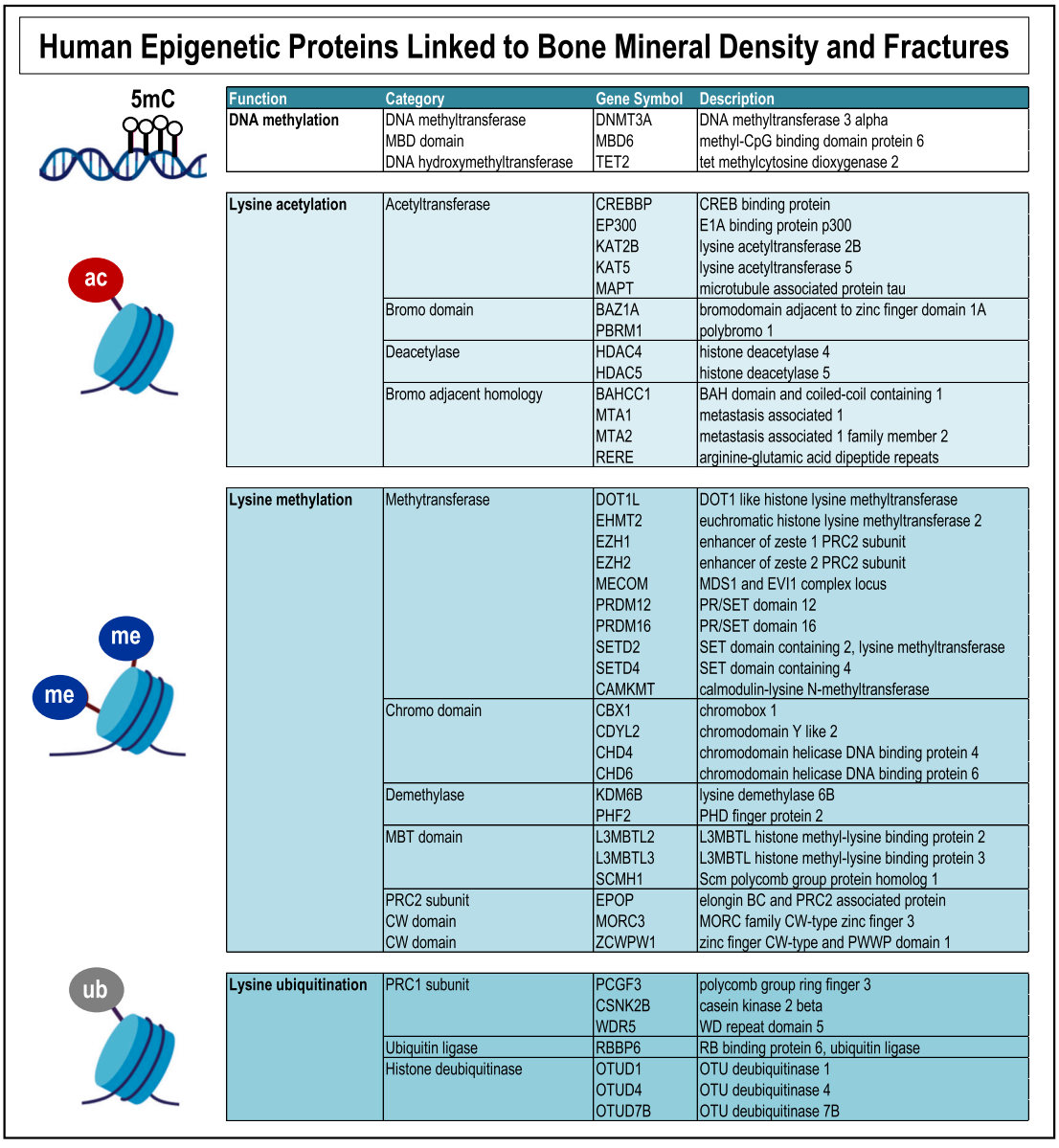
A recent survey of all nine PRMTs suggests that PRMTs influence epigenetic modifications of histones during osteoblast differentiation [224]. PRMT1, PRMT4/CARM1, and PRMT5 are the most prominently expressed PRMT subtypes in musculoskeletal tissues. Loss of PRMT1 enhances calcium deposition in osteoblasts, while loss of PRMT5 inhibits it. Loss of PRMT4/CARM1 has little effect on calcium deposition. These PRMTs modulate the expression of certain osteoblast-related genes but do not significantly affect proliferation. Treatment with the Class I PRMT inhibitor GSK715 enhances osteoblast differentiation by increasing extracellular matrix mineralization [224]. These studies are consistent with other studies that examined the roles of PRMT1, CARM1/PRMT4 and PRMT5 in mesenchymal stem cells during osteogenesis or in osteosarcoma cells [278–282].

Similar to the connection between SMYD2-mediated lysine methylation and EZH2 as a known suppressor of osteogenesis, PRMTs may also

influence osteoblast differentiation through histone arginine methylation and interactions with EZH2 [224]. PRMT5-mediated arginine methylation opposes the activities of the H3K27me3 transferase EZH2 [46,225]. Formation of active H3R2me2s marks by PRMT5 prevents the deposition of suppressive H3K27me3 marks in cancer cells [283]. These findings indicate that loss of PRMT5 may increase osteoblast suppressive H3K27me3 marks, consistent with recent studies in osteoblasts where PRMT5 loss prevents osteoblast differentiation [224]. Furthermore, PRMT1 methylates EZH2 (at R342) which stabilizes the protein [284]. Stabilization of EZH2 by PRMT1 is predicted to suppress osteoblastogenesis. In agreement with this concept, loss of PRMT1 accelerates osteoblast differentiation [224]. Taken together, both PRMT and SMYD proteins work in concert with EZH2 to regulate EZH2 activity and the osteoblast epigenome to support osteoblast maturation.

8. Translational relevance of epigenetic regulators

Within the >500 chromosomal loci that have been statistically linked to bone mineral density and/or bone fractures by GWAS [2,3], there are >80 genes that encode epigenetic proteins (Fig. 4 and Supplementary Table S1). Among these genes, there are a number of epigenetic enzymes that exhibit more robust expression in bone than in non-bone tissues (Fig. 5 and Supplementary Table 2). Furthermore, many of these epigenetic proteins have been genetically ablated in mice and exhibit informative phenotypes, such as (i) DNA methylation related proteins TET2 [5] and DNMT3A [3], (ii) lysine acetylation related proteins like BRD4 [133] and HDAC4 [164], and (iii) lysine methylation related regulators like EZH2 [59], CBX1, DOT1L and SETD4 [3]. Hence, epigenetic regulators are critical for normal bone formation and repair in mouse models and linked to fracture risk in humans. Because many of these proteins can be targeted by pharmacological inhibitors and epigenetic nutrients, there are ample opportunities for



Human Epigenetic Proteins Linked to Bone Mineral Density and Fractures

Function	Category	Gene Symbol	Description
DNA methylation	DNA methyltransferase	DNMT3A	DNA methyltransferase 3 alpha
	MBD domain	MBD6	methyl-CpG binding domain protein 6
	DNA hydroxymethyltransferase	TET2	tet methylcytosine dioxygenase 2
Lysine acetylation	Acetyltransferase	CREBBP	CREB binding protein
		EP300	E1A binding protein p300
		KAT2B	lysine acetyltransferase 2B
		KAT5	lysine acetyltransferase 5
		MAPT	microtubule associated protein tau
	Bromo domain	BAZ1A	bromodomain adjacent to zinc finger domain 1A
		PBRM1	polybromo 1
	Deacetylase	HDAC4	histone deacetylase 4
		HDAC5	histone deacetylase 5
	Bromo adjacent homology	BAHCC1	BAH domain and coiled-coil containing 1
		MTA1	metastasis associated 1
		MTA2	metastasis associated 1 family member 2
		RERE	arginine-glutamic acid dipeptide repeats
Lysine methylation	Methyltransferase	DOT1L	DOT1 like histone lysine methyltransferase
		EHMT2	euchromatic histone lysine methyltransferase 2
		EZH1	enhancer of zeste 1 PRC2 subunit
		EZH2	enhancer of zeste 2 PRC2 subunit
		MECOM	MDS1 and EVI1 complex locus
		PRDM12	PR/SET domain 12
		PRDM16	PR/SET domain 16
		SETD2	SET domain containing 2, lysine methyltransferase
		SETD4	SET domain containing 4
		CAMKMT	calmodulin-lysine N-methyltransferase
	Chromo domain	CBX1	chromobox 1
		CDYL2	chromodomain Y like 2
		CHD4	chromodomain helicase DNA binding protein 4
		CHD6	chromodomain helicase DNA binding protein 6
	Demethylase	KDM6B	lysine demethylase 6B
		PHF2	PHD finger protein 2
	MBT domain	L3MBTL2	L3MBTL histone methyl-lysine binding protein 2
		L3MBTL3	L3MBTL histone methyl-lysine binding protein 3
		SCMH1	Scm polycomb group protein homolog 1
	PRC2 subunit	EPOP	elongin BC and PRC2 associated protein
	CW domain	MORC3	MORC family CW-type zinc finger 3
	CW domain	ZCWPW1	zinc finger CW-type and PWWP domain 1
Lysine ubiquitination	PRC1 subunit	PCGF3	polycomb group ring finger 3
		CSNK2B	casein kinase 2 beta
		WDR5	WD repeat domain 5
	Ubiquitin ligase	RBBP6	RB binding protein 6, ubiquitin ligase
	Histone deubiquitinase	OTUD1	OTU deubiquitinase 1
		OTUD4	OTU deubiquitinase 4
		OTUD7B	OTU deubiquitinase 7B

Fig. 4. Summary of epigenetic regulators linked to the BMD and bone fractures. The tabular figure shows a relevant selection of proteins involved in methylation, acetylation or ubiquitination of chromatin (represented by icons on the left side; genes annotated using ChromoHub) [287]. These proteins are contained within >500 chromosomal loci that exhibit a statistically significant association with bone mineral density and/or bone fractures [2,3]. The full set of epigenetic genes (n = 84), which contains many still under-explored epigenetic proteins is presented in Supplementary Table 1.

pharmacotherapies to improve bone health, including inhibitors for HDACs, EZH2 and BRD proteins.

9. Challenges and limitations of epigenetic studies in bone

Even though major progress has been made on our understanding of epigenetic mechanisms that control bone development, this knowledge remains far behind in both quantity and experimental depth relative to our understanding of cancer epigenetics [64]. In part, these differences in knowledge are driven by the available resources to study bone versus cancer, but also by conceptual and technical considerations.

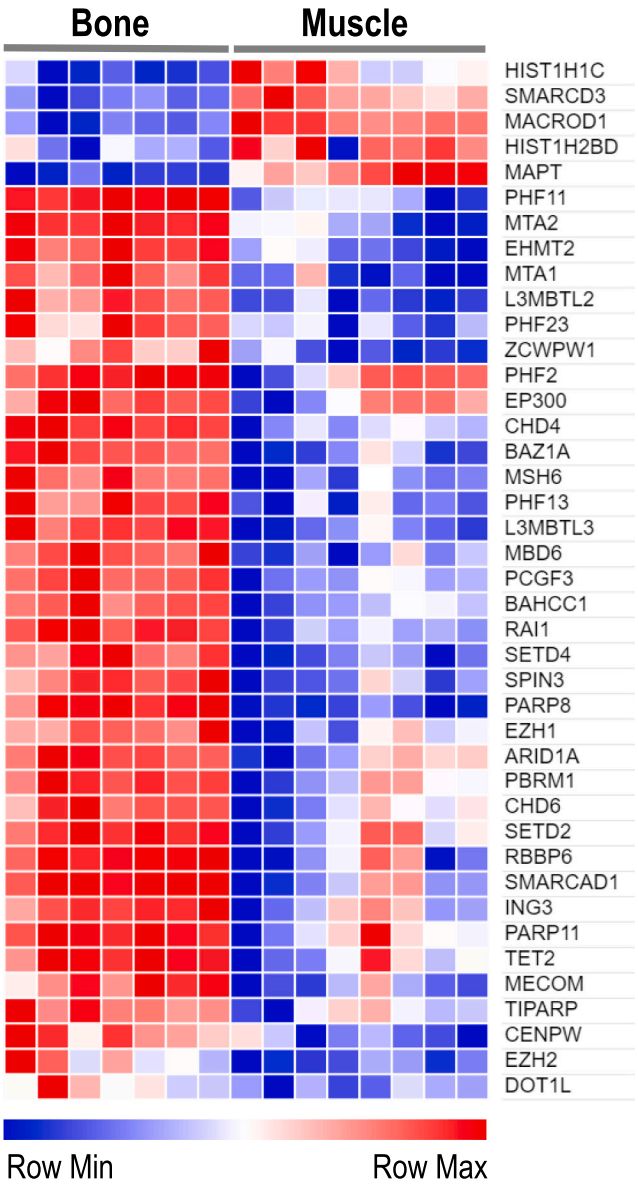


Fig. 5. Bone tissue-specific expression of human epigenetic proteins. The heatmap shows the expression of selected human epigenetic genes ($n = 41$; vertical axis) that are associated with bone mineral density and/or fractures, as well as exhibit lineage-specific expression (human bone, $n = 7$; human muscle, $n = 8$; horizontal axis) (see also Supplementary Table 2). The genes were selected from Supplementary Table 1 and were combined with RNA-seq data selected from a panel of human musculoskeletal tissue samples [288]. Genes were filtered for robust expression (as defined by the frequency of Fragments Per Kilobase of transcript per Million mapped reads: FKPM>1) and a biologically relevant difference ($|\log2FC| > 1$). The heatmap visualization was further refined by t-testing for each gene, while retaining only those genes with acceptable random variation ($p < 0.05$). The heatmap was generated in Morpheus by adjusting FKPM values [Log2(1 + X)] and default settings (One Minus Pearson correlation, column & row clustering; https://software.broadinstitute.org/morpheus).

Almost all enzymes that control lineage commitment are also highly expressed in proliferating cells and required for maintenance of the epigenetic code on replicating chromosomes in cancer and stem cells. It has been challenging to investigate epigenetic mechanisms that specifically promote post-proliferative lineage-specific phenotypes in mesenchymal cells. From a pharmacological perspective, many epigenetic inhibitors have been developed in the cancer field to block cell division and/or cause tumor cell death. One of the principal strategies in the bone field has been to find inhibitors that can reverse epigenetic suppression of bone formation, while avoiding undesired effects on cell proliferation.

Bone remains one of the hardest tissues to work with because molecular studies depend on activities of skeletal stem cells, osteoblasts and osteocytes that form functional cell niches in a mineralized extracellular environment. The mineralized bone matrix complicates many powerful state-of-the-art methodologies, including (i) ChIP-seq to measure histone PTMs, (ii) Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) to measure chromatin accessibility, and (iii) single cell (sc) RNA-seq to measure transcriptomes in heterogenous cell populations. ChIP-seq requires fixatives that cannot be effectively perfused into live osteocytes, ATAC-seq requires genetic introduction of a transposase, while single cell studies are compromised because cell harvest requires time-consuming enzymatic procedures at room temperature or higher that likely will alter the molecular state of the cells that are being isolated. Our group has been able to perform RNA-seq and methyl-DNA-immunoprecipitation sequencing (MeDIP-seq) on mouse bone samples [5], but analysis of many DNA and histone PTMs was by necessity performed at the level of whole tissue analysis using immunoblot based techniques. These limitations severely reduce the power of mouse models for functional analysis of epigenetic regulators in vivo.

Beyond these technical hurdles, studies with most human musculoskeletal tissues and cell types are difficult because it requires a research environment integrated with the orthopedic surgery practice where bone samples can be isolated as surgical waste specimens. Furthermore, bone is one of the last tissues that forms during embryonic development and effects on patterning of primordial precursor tissues and proliferative expansion of these precursor cells must be avoided in genetic studies. Hence, most if not all epigenetic regulators must ideally be studied as bone-specific conditional tamoxifen-inducible mouse knockouts. This approach is technically demanding and time-consuming, and can still be confounded by non-specific bone anabolic effect of tamoxifen and compensation by closely related epigenetic protein isoforms. Therefore, most of our understanding has come from ex vivo cell culture.

Because of the technical necessity of ex vivo studies with cultured cells, epigenetic experimentation has been performed with a large range of cell types including primary human SSCs or MSCs, established transformed or osteogenic tumor derived cell lines, as well as primary mouse SSCs or MSCs and calvarial or established osteoblast and osteocyte cell lines (e.g., MC3T3-E1 preosteoblasts). Economical considerations typically limit the number of analysis types, chromatin parameters, biological conditions and time-points, while different groups use different bioinformatic approaches and default settings. Because of this experimental pluriformity, most of the data that has been obtained to date is rather fragmented and merely provide specific epigenetic snapshots during osteogenic events. Thus, there is a compelling need for comprehensive and standardized approaches, as well as some urgency to find consensus on the use of the best model cell types.

Emerging technologies like CRISPR-based epigenome editing and single-cell ATAC-seq may effectively move the epigenetic frontiers. Yet, single-cell ATAC-seq would at present not be realistic for in vivo analysis but could be applied in SSCs or bone marrow derived MSCs. CRISPR-based editing can remove distal enhancers with specific marks (e.g.,

H3K27ac, H3K4me1) near bone specific genes, or permit the specific positioning of Cas9 variants fused to repressive (KRAB) or activation (VP64) domains. These approaches can be applied in the germline and analyzed during bone formation in vivo. Hence, epigenome editing strategies that selectively alter the epigenetic landscape, modify areas of open chromatin and modulate gene expression, represent an exciting avenue of future research.

10. Conclusions

This review evaluated >200 studies on epigenetic regulators in the context of bone-specific gene expression. These studies collectively indicate that many specific epigenetic proteins control bone formation in mouse models via effects on osteogenic lineage commitment and progression of osteoblast maturation in and/or in vitro. Studies on differentiation into osteogenic versus adipogenic lineages represent fundamental new insights into fetal development that transcend the bone field. In addition, many epigenetic proteins (>80) are present in loci associated with BMD or bone fractures in human subjects, and robustly expressed in bone, reflecting the clinical importance of epigenetic regulators in major bone disorders (e.g., osteoporosis). Essential nutrients (VitC and VitD3) have major roles as epigenetic co-factors, reflecting the interplay between diet and bone health. Because many of epigenetic proteins have structural clefts that permit binding of very specific 'designer drugs', they represent viable targets for local and short-term anabolic pharmacotherapies or tissue-engineering strategies that support accrual or regeneration of bone mass. One of the most exciting frontiers that awaits full exploration is the intricate regulatory interplay between cell metabolism (e.g., TCA cycle, fatty acid metabolism) and epigenetic enzymes that use high energy compounds to modify chromatin (e.g., ATP, Acetyl-Coenzyme A and S-Adenosyl methionine). The current state of the field provides a solid foundation for the next generation of epigenetic studies.

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CRediT authorship contribution statement

Parisa Dashti: Writing – review & editing, Writing – original draft, Conceptualization. **Eric A. Lewallen:** Writing – review & editing, Conceptualization. **Jonathan A.R. Gordon:** Writing – review & editing, Conceptualization. **Martin A. Montecino:** Writing – review & editing, Conceptualization. **James R. Davie:** Writing – review & editing, Conceptualization. **Gary S. Stein:** Writing – review & editing, Conceptualization. **Johannes P.T.M. van Leeuwen:** Writing – review & editing, Conceptualization. **Bram C.J. van der Eerden:** Writing – review & editing, Conceptualization. **Andre J. van Wijnen:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have no conflict of interest to declare.

Data availability

Figure 5 uses a subset of published RNA-seq data cited in the text, and all values used are provided in Supplementary Table 2.

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