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MOLECULAR BIOLOGY OF SKELETAL DEVELOPMENT (T BELLIDO, SECTION EDITOR)

Epigenetic Control of Osteoblast Differentiation by Enhancer of Zeste Homolog 2 (EZH2)

Amel Dudakovic[1] · **Andre J. van Wijnen**[1,2,3]

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Abstract
Purpose of Review Because epigenetic processes are critical during development, there is considerable interest in understanding how epigenetic enzymes control lineage commitment and progression. We review recent studies indicating that methylation of histone 3 at lysine 27 (H3K27), which is a major epigenetic modification that promotes gene silencing by reducing chromatin accessibility, is a principal regulatory mechanism that controls osteogenesis.
Recent Findings Key studies have shown that enhancer of zeste homolog 2 (EZH2/Ezh2), which is the active subunit of the polycomb-repressive complex 2 (PRC2) and catalyzes methylation of H3K27, is critically required for normal skeletal patterning and bone formation. For example, while germline deletion of Ezh2 is embryonically lethal, conditional loss of Ezh2 in the mesenchyme demonstrates that this histone methyltransferase controls normal tissue patterning during fetal development. Furthermore, recent findings show that Ezh2 has an important role in mesenchymal stem cell (MSC) lineage allocation and osteoblast differentiation. Strikingly, suppressing Ezh2 activity in vitro stimulates osteogenic and inhibits adipogenic differentiation. Furthermore, Ezh2 inhibition in vivo has been shown to stimulate bone formation and prevents bone loss associated with estrogen depletion.
Summary These findings collectively suggest that inhibition of the biological activity of EZH2 in human patients may have utility in regenerative therapies that stimulate bone accrual.

Keywords PRC2 · Ezh2 · Ezh1 · Osteoblasts · Mesenchymal cells

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✉ Andre J. van Wijnen
vanwijnen.andre@mayo.edu

[1] Departments of Orthopedic Surgery, Mayo Clinic, 200 First St. SW, Rochester, MN 55905, USA

[2] Biochemistry and Molecular Biology, Mayo Clinic, 200 First St. SW, Rochester, MN 55905, USA

[3] Physiology and Biomedical Engineering, Mayo Clinic, 200 First St. SW, Rochester, MN 55905, USA

Introduction

Modulations in gene expression during development are controlled by major architectural changes in the manner by which DNA is organized in the nucleus. Because chromatin organization controls access of key transcription factors to their cognate sites, epigenetic gene regulation is critical for all biological processes including development, proliferation, differentiation, and disease onset and progression. Pioneering studies by Emil Heitz and colleagues published in 1928 described cytological differences between euchromatin and heterochromatin in plants (i.e., *Plagiochila asplenioides* or liverwort) to provide the foundation for our current understanding of epigenetics [1, 2]. Microscopic alterations in the morphology of chromatin were recognized even earlier [3] and preceded the discovery of DNA as the carrier of genetic traits. The term "epigenetics" traces its root to the concept of the epigenotype that was originally coined by Waddington in 1942 to account for changes in phenotype without changes in the actual genotype (i.e., the DNA sequence) [4]. Since these early discoveries in the twentieth century, the field of epigenetics has considerably evolved and diversified through many scientific innovations, including the advent of next-generation proteomic and genomic technologies. Multiple types of epigenetic processes are recognized at present including DNA methylation,

post-translational modifications of histones (e.g., acetylation and methylation), small non-coding RNAs (microRNAs), long non-coding RNAs (lncRNAs), mitotically transmitted messenger RNAs (mRNAs), and architectural mechanisms by which genes and enhancers are marked during cell division (mitotic bookmarking) [5–9].

The importance of epigenetic mechanisms during skeletal development and specifically during osteoblast differentiation has been evident for some years [8•, 10•, 11, 12, 13, 14•, 15, 16••]. Early studies focused on nuclear hypersensitivity of the bone-specific osteocalcin gene as a paradigm for the accessibility of the transcription factor to their cognate regulatory sequences in promoters [10•, 11, 12], as well as the effects of histone acetylation and steroid hormones on bone-specific gene activation [13, 14•, 15, 16••]. More recently, several high-throughput genomic studies have been performed to describe the overall epigenetic landscape and histone marks in chromatin of differentiating osteoblasts [17••, 18, 19••, 20••]. These studies are motivated with the ultimate goal of designing new strategies based on epigenetic drugs to generate favorable epigenetic conditions that stimulate bone formation in patients with low bone mineral density and increased fracture risk (e.g., osteoporosis), or non-healing fractures.

Many epigenetic regulators are now known to control skeletal development and osteoblast differentiation though chromatin-mediated mechanisms [8•]. This review focuses on enhancer of zeste 2 (EZH2), because studies from a number of laboratories that are discussed below have revealed that it is a very potent inhibitor of osteogenic differentiation and drugs that inhibit its function have shown promise in promoting bone formation in animal models.

Control of Heterochromatin Formation by the Polycomb Repressive Complex 2 and Methylation of H3 Lysine 27

Access of regulatory proteins to promoter and enhancer sequences that control gene expression is restricted by the packaging of DNA into nucleosomes. These fundamental units of chromatin, which are composed of histone octamers composed of two copies each of four core histones (H3, H4, H2A, H2B), can package DNA into densely organized heterochromatin in which gene expression is repressed or more open euchromatin that permits active expression of genes. Histones undergo a large number of covalent post-translational modifications that occur at the N-terminal regions of histones, including acetylation, methylation, and phosphorylation. These modifications also contribute to control of gene expression during skeletal development, because epigenetic enzymes involved in these activities are necessary for normal bone formation [8•].

Generation and maintenance of heterochromatin is a major epigenetic process that controls gene expression. One mechanism by which cells generate heterochromatin is tri-methylation of histone 3 at lysine 27 (H3K27me3), and this epigenetic modification promotes gene silencing by reducing chromatin accessibility [21]. The polycomb-repressive complex 2 (PRC2) catalyzes the mono-, di-, and tri-methylation of lysine 27 of histone H3 (H3K27me1, H3K27me2, and H3K27me3). The PRC2 complex is made up of the catalytic subunit enhancer of zeste homolog 2 (Ezh2) and three structural components, embryonic ectoderm development (Eed), suppressor of zeste 12 (Suz12), and retinoblastoma binding protein 4 (Rbbp4). The catalytic subunit Ezh2 can be replaced by enhancer of zeste homolog 1 (Ezh1) in the PRC2 complex. The activity of the Ezh2-containing PRC2 complex is balanced by the activity of three histone demethylases (Jhdm1d, Kdm6a, and Kdm6b) that remove KH3K27 methyl groups [22]. Studies discussed in further detail below have revealed that the biochemical activity of Ezh2 is genetically required for selective silencing of bone-related genes until the final stages of skeletal development.

Evolutionarily Conserved Role of the PRC2 Complex in Chromatin Condensation

A full appreciation of the critical role of Ezh2 in bone formation requires an understanding of the role of PRC2 proteins as members of the evolutionarily conserved polycomb group (PcG) family of proteins, which mediate formation of transcriptionally repressive chromatin in many eukaryotic species. Because of their function in repressing developmentally regulated genes, PcG proteins have been identified through genetic screens of mutants with developmental abnormalities [23, 24]. PRC2 complex proteins were initially discovered in *Drosophila* as proteins that repress the expression of Hox genes to regulate cell identity and promote proper body plan development [25]. The PRC2 complex is well-conserved and present in various unicellular eukaryotes, but not present in *Schizosaccharomyces pombe* or *Saccharomyces cerevisae* [26]. In general, invertebrates have only one copy of the PRC2 complex genes [27]. However, *Drosophila* contains two copies of the Eed homolog (e.g., Esc and Esc-like), which are expressed at all stages of development and appear to have identical functions [28]. The mammalian and *Drosophila* homologs of Ezh2 have been extensively studied, although less is known about the mammalian-specific homolog Ezh1, which actually was the first homolog cloned in humans [29]. Early studies suggested that the expression of Ezh1 and Ezh2

are inversely correlated [30••]. Recent evidence from our laboratory supports this model both in mesenchymal stem cells (MSCs) and osteoblasts, where we have found that Ezh2 is expressed in proliferating mesenchymal cells, while Ezh1 is upregulated when cells exit the cell cycle and adopt a more committed cellular phenotype [31••, 32].

Accumulating data suggest that there are functional differences in PRC2 complexes that contain Ezh1 instead of Ezh2. For example, Margueron and colleagues elegantly demonstrated that the Ezh1-PRC2 complex exhibits lower methyltransferase activity than the Ezh2-PRC2 complex [33••]. Mechanistically, PRC2-Ezh1 may play a role in maintaining H3K27me3 that were previously established and perhaps promote additional chromatin compaction of genes in post-proliferative cells [21, 33••]. Consistent with biochemical differences between Ezh1 and Ezh2, genetic evidence discussed below indicates that Ezh1 and Ezh2 perform distinct biological functions during pre- and post-natal development.

EZH2 and Cancer

Like other epigenetic regulators, EZH2 is actively expressed in proliferating cells, and consequently, the function of EZH2 has been assessed in significant detail during tumorigenesis in many different cancer types [25, 34]. Findings on EZH2 in cancer cells are relevant to its role during normal development, because tumor-derived cells exhibit abrogated cell growth and differentiation relationships. For example, expression of EZH2 correlates with tumor grade in breast and prostate cancers [35, 36], while somatic mutations in EZH2 enhance its catalytic activity in patients with non-Hodgkin lymphoma [37, 38]. It is now understood that EZH2-PRC2 activity in cancer can be deregulated by several mechanisms including increased gene expression, copy number amplifications, and missense mutations that stimulate EZH2 methyltransferase activity. Aberrant EZH2 activity is associated with increased proliferation, migration, and metastasis, as well as with interference in DNA damage repair [25].

Given the importance of the tumor promoting effects of EZH2, several drugs have been developed to inhibit the activity of this epigenetic regulator. The availability of EZH2-specific drugs provides an opportunity to repurpose these pharmacological agents for applications in regenerative medicine, including possible bone anabolic therapies discussed below. The function of the PRC2 complex in generating H3K27me3 marks can be inhibited with 3-deazaneplanocin A (DZNep), a cyclopentyl analog of 3-deazaadenosine that interferes with *S*-adenosyl-L-homocysteine hydrolase (SAH) [39]. This compound induces apoptosis in cancer cells, but its therapeutic utility is limited by its lack of specificity for EZH2 [40]. Several potent and more specific inhibitors of EZH2 have been developed. One important broadly used inhibitor is GSK126, which inhibits the growth of lymphomas with an activating EZH2 mutation in vitro and in vivo. This drug specifically inhibits both wild-type and mutant forms of EZH2, and is 150-fold more specific for EZH2 compared to EZH1 and 1000-fold more specific than other methyltransferases [41]. The major limitation of GSK126 and some of the earlier EZH2 inhibitors (e.g., EPZ005687) is that they cannot be orally administered and effective therapy would require repeated injections. This limitation does not apply to UNC1999, a compound that selectively blocks both wild-type and mutant EZH2 and inhibits EZH1, but is 10-fold less potent when compared to EZH2 [42].

EPZ-6438 was recently developed and shown to be a potent, selective, and orally bioavailable small-molecule inhibitor of EZH2 [43]. This study showed that treatment of *SMARCB1*-mutan mice with EPZ-6438 reduced regression of malignant rhabdoid tumors by reducing H3K27me3 levels. With pre-clinical success, a combined phase 1 and 2 clinical trial was launched to assess the effects of EPZ-6438 in patients with advanced solid tumors or with B cell lymphomas. Preliminary data from these studies have been encouraging and showed partial or complete responses in patients (http://www.epizyme.com/media-center/publications/). Other clinical trials are currently enrolling patients to test the effects of EZH2 inhibitors in human cancers (https://clinicaltrial.gov). These clinical trials with cancer patients provide critical safety data for EZH2 inhibitors that could be considered for short-term musculoskeletal regenerative therapies.

EZH2 Suppresses Osteogenic Differentiation and Permits Adipogenic Differentiation

Chromatin organization is actively modulated during bone cell differentiation, and many epigenetic regulators and isoforms may contribute to chromatin remodeling in the osteoblast lineage. To define which of these regulatory proteins are functionally expressed in osteoblasts, our laboratory designed a semi-automated RT-qPCR platform that detects expression of a large cohort of human epigenetic regulators. We combined this RT-qPCR platform with next-generation sequencing (RNA-Seq) of mRNA to identify differentially expressed epigenetic regulators during osteogenic commitment of human mesenchymal/stromal cells (MSCs) [31••, 32]. This screen identified numerous epigenetic regulators that are upregulated and others, including EZH2, that are strongly downregulated during osteogenic differentiation of MSCs. Our studies focused on EZH2 for several reasons. First, it is a rapidly downregulated gene during initial commitment of MSCs to the osteogenic lineage. We assumed that accelerating its loss of function could stimulate osteogenic differentiation of progenitor cells. Second, its function as a H3K27

methyltransferase (i.e., suppressor of gene expression) made it an exciting epigenetic target, because its inhibition is predicted to induce expression of osteogenic genes that stimulate osteoblastogenesis. Furthermore, there are already many small molecule drugs available that target this epigenetic enzyme (e.g., GSK126, UNC1999, and EPZ-6438) and have established safety profiles in animal models or patients. This availability of effective inhibitors allows for manipulation of its function in cell culture and animal models, as well as perhaps rapid clinical translation for bone-related pathological conditions.

Because EZH2 as a gene suppressor is downregulated during osteogenic lineage commitment, we hypothesized that inhibition of EZH2 would stimulate bone-related genes and promote the osteoblast cell fate in multipotent MSCs that are not yet committed to a specific mesenchymal lineage. To test this hypothesis, our laboratory performed studies assessing the effects of EZH2 inhibition in mesenchymal stem cells and pre-osteoblasts. Inhibition (GSK126) and/or knockdown (siRNA) of EZH2 significantly stimulate osteogenic commitment of human MSCs, while suppressing adipogenic differentiation [31••]. Gene expression profiling by RNA-Seq analysis suggests that EZH2 inhibition enhances the expression of cell cycle inhibitory proteins and bone-related extracellular matrix (ECM) proteins in MSCs. Together, the suppression of cell growth and concomitant deposition of ECM proteins supports development of an osteogenic MSC phenotype. Similar observations were also made by Hemming and colleagues [44••] who showed that knockdown or inhibition of EZH2 promotes osteogenic commitment, while its overexpression promotes adipogenic differentiation of MSCs. Interestingly, promoting demethylation of H3K27 by overexpression of the cognate demethylase (KDM6A) produces the exact opposite effects and enhances ectopic bone formation of MSCs in vivo. The anti-osteogenic effects of EZH2 in human MSCs are also highlighted in studies by Chen and colleagues [45, 46] who suggest that EZH2 and the histone deacetylase HDAC9 control lineage commitment of MSCs. These studies also provided evidence for the pro-adipogenic and anti-osteogenic roles of EZH2 in MSCs. Taken together, studies from several laboratories indicate that EZH2 plays a key role in mesenchymal lineage allocation and development of osteogenic versus adipogenic cell fates.

Recent studies from our group also indicate that EZH2 suppresses maturation of mesenchymal cells committed to the osteoblast lineage. We assessed the effects of Ezh2 on differentiation of MC3T3 pre-osteoblasts, a well-established tissue culture model of osteogenesis [47••]. Similar to uncommitted human MSCs, inhibition or knockdown of Ezh2 in these committed osteoblasts greatly enhances differentiation of MC3T3 cells. RNA-Seq profiling revealed that Ezh2 inhibition enhances expression of bone-related gene regulators and ECM proteins. Mechanistically, the upregulation of these genes as a result of Ezh2 inhibition is linked to decreased H3K27me3 near transcriptional start sites as demonstrated by genome-wide sequencing of chromatin immunoprecipitation (ChIP-Seq) assays. Specifically, Ezh2 inhibition increases BMP-dependent phosphorylation of Smad1/5 and enhances expression of bone stimulatory proteins like Wnt10b and Pth1r. In addition, the combination of BMP2 and EZH2 inhibition further stimulates osteogenic commitment of MC3T3 pre-osteoblasts. Taken together, our studies suggest that EZH2 inhibition may accelerate osteoblast differentiation by promoting bone-stimulatory paracrine signaling events.

While EZH2 normally suppresses osteoblast differentiation, it appears to be necessary for differentiation of lineage-committed pre-adipocytes. EZH2 may facilitate adipogenesis by blocking bone-stimulatory WNT signals, based on previous studies indicating that the EZH2-containing PRC2 complex occupies the genomic regions of Wnt genes [48–50]. Wang and colleagues provided evidence that EZH2 regulates Wnt genes in the context of adipogenic differentiation of primary pre-adipocytes [51•]. This study demonstrated that EZH2 suppresses the expression of several Wnt genes (e.g., Wnt1, Wnt6, Wnt10a, and Wnt10b) to facilitate differentiation of cells committed to the fat lineage. This observation is consistent with our findings that Wnt10b is also suppressed in osteoblasts unless EZH2 is inactivated [47••]. Collectively, these studies are consistent with a model in which EZH2 actively sustains H3K27me3 marks on Wnt genes in pre-adipocytes and pre-osteoblasts (e.g., WNT10B), while the selective loss of EZH2 during osteoblast differentiation decreases H3K27me3, activates the Wnt/β-catenin axis through paracrine signaling, and stimulates osteogenesis while inhibiting adipogenesis.

Regulation of EZH2 Activity During Proliferative and Post-Proliferative Stages of Osteoblast Differentiation

Studies by Wei and colleagues demonstrated that the mitosis-related cyclin-dependent kinase 1 (CDK1) phosphorylates EZH2 at Thr-487 to control its enzymatic activity in proliferating cells [52]. Using immuno-precipitation assays, these authors showed that phosphorylation at Thr-487 of EZH2 disrupts its binding to the PRC2 components SUZ12 and EED, resulting in impaired methyltransferase activity. Importantly, phosphorylation (Thr-487) of EZH2 correlates with enhanced differentiation of MSCs into the osteogenic lineage, consistent with a model in which CDK-mediated phosphorylation controls the suppressive function of EZH2 during osteoblast differentiation.

Post-transcriptional regulation of EZH2 activity is also achieved by interactions with non-coding RNAs. For example, initial studies showed that genomic loss of miR-101

enhances expression of EZH2 in prostate cancer cells [53]. Consistent with our findings that EZH2 is downregulated during osteogenic differentiation [47••] and that EZH2 is a miR-101 target in prostate cancer [53], Wang and co-workers demonstrated that EZH2 is a biologically relevant functional target gene of miR-101 in MSCs [54]. These authors also showed that expression of miR-101 increases during progression of osteogenic differentiation in human MSCs, and that the loss of miR-101 inhibits while upregulation of miR-101 enhances osteogenic differentiation of MSCs. Importantly, over-expression of miR-101 in human MSCs enhances bone formation in a mouse calvarial defect model [54]. The key findings of these studies have recently been corroborated by studies in our laboratory using mouse MC3T3 pre-osteoblasts (unpublished data) and by Huang and colleagues [55]. The molecular model that emerges from these studies is that miR-101 and EZH2 form a reciprocal negative feedback loop in which expression of miR-101 is initially suppressed by EZH2 and that reduced activity of EZH2 and/or increased levels of miR-101 initiate an escalating regulatory cascade in which EZH2 is suppressed and miR-101 is elevated in mature osteoblasts.

Beyond suppression by miR-101 as a small non-coding RNA, EZH2 also appears to be targeted by long-non coding RNAs (lncRNAs) during osteogenic differentiation. One lncRNA from the Angelman syndrome chromosome region (ANCR) appears to control EZH2 activity during osteoblast differentiation. Consistent with its inhibitory function in osteoblastogenesis, silencing of ANCR by RNA interference enhances while over-expression inhibits differentiation of immortalized human fetal osteoblastic cells (hFOB1.19) [56]. These authors also show that ANCR is suppressed during human differentiation of hFOB1.19 cells, and advance a model in which interactions between ANCR and EZH2 suppress RUNX2 gene expression. While other studies support the general idea that EZH2 is a principal epigenetic suppressor of RUNX2 [57], this model may need adjustments and further testing will be required to define the exact role of ANCR. For example, recent studies in breast cancer cells [58] suggest that ANCR facilitates the inhibitory phosphorylation of EZH2 by CDK1 on Thr-487 (see above) [52]. The latter is predicted to destabilize EZH2 and would qualify ANCR as an indirect molecular antagonist of EZH2.

Apart from ANCR, the lncRNA HoxA-AS3 has been shown to interact with EZH2 in MSCs. Zhu and colleagues demonstrated that HoxA-AS3 is upregulated during adipogenic commitment, while its expression remained relatively constant during osteogenic differentiation of human MSCs [56]. A physiological role in adipogenic versus osteogenic lineage commitment is further suggested by the observation that suppression of HoxA-AS3 decreases adipogenic but enhances osteogenic commitment of human MSCs. Mechanistically, the authors demonstrate that the loss of HoxA-AS3 dissociates EZH2 from the RUNX2 promoter to enhance the expression of this osteogenic transcription factor. Interestingly, a recent study by Wu and colleagues suggested that RUNX2 interactions with the EZH2 locus are enhanced during MC3T3 osteoblast differentiation and thus may play a key inhibitory role in controlling the expression of EZH2 [59•], a model which is also supported by recent findings from our laboratory (unpublished data).

In summary, EZH2 activity is controlled at multiple levels including transcriptional suppression at the EZH2 locus, suppression of EZH2 translation by miR-101, CDK1 phosphorylation that controls EZH2 activity, and two lncRNAs (ANCR and HoxA-AS3) that modulate the suppressive activity of its bone-specific target gene promoters (e.g., RUNX2 gene). Feedback regulation in which RUNX2 controls EZH2 and EZH2 controls miR-101 results in several intersecting molecular regulatory loops that may maintain a molecular balance during bone formation.

Genetic Roles of PRC2 Proteins During Fetal Development and Skeletal Patterning

Heterochromatin formation is quite important during normal development. Therefore, it is not surprising that mice lacking expression of PRC2 components like Eed [60], Ezh2 [61], or Suz12 [62] are not viable and die during early stages of fetal development. Remarkably, a global knockout (KO) of Ezh1 results in viable mice that exhibit a normal phenotype [63]. Thus, the function of the Ezh2-PRC2 complex is required for normal development, while the Ezh1-PRC2 complex appears to be dispensable.

Several studies have utilized conditional knockout mouse models to assess the role of the PRC2 complex during early stages of fetal development when skeletal patterning occurs and at later post-natal stages of development (Table 1). The first study demonstrating phenotypic changes in the osteochondroprogenitor lineage due to PRC2 complex disruption was described by Wyngaarden and colleagues in 2011 [64••]. The floxed Ezh2 allele ($Ezh2^{f/f}$) was used in this and other studies (below) to assess the functional role of Ezh2 in osteochondroprogenitor cells [71]. The loxP within the Ezh2 gene sites flank the SET domain, the catalytic activity domain of Ezh2, to disrupt the methyltransferase activity of Ezh2 protein. In addition to the inhibition of its function, the deletion of the SET domain of Ezh2 has been reported by others [71] and observed in our laboratory (unpublished data) to destabilize the Ezh2 protein and lead to its degradation. The authors deleted Ezh2 using two different drivers: the T-Cre [72] driver, in which Cre is expressed from the Brachyury/T promoter to inactivate Ezh2 in the early stages, and Prrx1-Cre [73], which controls Cre from the paired-related homeodomain protein

Table 1 Mouse genetic models assessing the role of the PRC2 complex on skeletal development and function

Publication	Targeted gene	Targeting method	Mouse phenotype
Wyngaarden et al. [64••] (2011)	Ezh2	T-Cre (mesoderm, embryonic stage)	-Abnormal expression of Hox genes -Altered antero-posterior axis pre-patterning and proximo-distal segment elaboration -Most embryos die by E13.5 -Abnormal no. of digits -Cell death
		Prrx1-Cre (mesenchyme)	-Shortened segment lengths -Mice survive post embryonic stages -Shortened proximo-distal limb segments -Abnormal expression of Hox genes
Schwarz et al. [65••] (2014)	Ezh2	Wnt1-Cre (neural-crest cells)	-Embryonic lethal -Missing craniofacial structures -De-repression of Hox genes
Dudakovic et al. [31••] (2015)	Ezh2	Prrx1-Cre (mesenchyme, post-embryonic)	-Multiple skeletal abnormalities (e.g., craniosynostosis, clinodactyly, shortened limbs, reduced cartilage and bone formation) -Enhanced expression of Hox genes, transcription factors, osteogenic genes, and cyclin-dependent kinase inhibitors
Hemming et al. [66•] (2016)	Ezh2	Prrx1-Cre (mesenchyme, post embryonic)	-Skeletal abnormalities in cKO animals -Expansion of the skeletal size, weight, and growth plate in HET animals -Trabecula expansion in cKO animals -Enhanced cortical and trabecular bone formation, but decreased mechanical strength in WT and HET animals -Enhanced tri-lineage potential of MSCs derived from HET animals
Mirzamohammadi [67] (2016)	Eed	Col2a1-Cre (chondrocytes)	-Severe form of kyphosis, decreased chondrocyte proliferation, growth defects, cell death (altered Hif1a levels), accelerated hypertrophic differentiation, and induction of multiple signaling pathways -Inhibition of TGFβ pathway rescues the proliferation and growth defects in cKOs -Inhibition of Wnt pathway improves spinal deformity in cKO animals
Lui [68•] (2016)	Ezh2 and Ezh1	Ezh1 (whole body) and Col2a1-Cre deletion of Ezh2 (chondrocytes)	-Loss of Ezh2 alone insufficient to induce a chondrocyte phenotype -Combined loss of Ezh1 and Ezh2 severely impair skeletal development (abnormalities in growth plate development, chondrocyte proliferation, and chondrocyte hypertrophy) -Loss of Ezh1 and Ezh2 reduces induces Cdkn2a and Cdkn2a to halt proliferation -Loss of Ezh1 and Ezh2 suppresses IGF signaling to stimulate inappropriate chondrocyte hypertrophy

Prrx1, to inactivate Ezh2 in the later stages of the mesoderm lineage.

The deletion of functional Ezh2 using T-Cre results in abnormal expression of Hox genes as a result of altered Hand2 and Gli3 expression. In turn, deregulation of Hox gene expression alters pre-patterning of the antero-posterior axis, as well as proximo-distal segment elaboration in conditional KO (cKO) animals. The authors note that few embryos lacking expression of Ezh2 under the control T-Cre survive past E13.5. Their data reveal a shortening of primary proximo-distal limb segments, antero-posterior patterning anomalies, abnormal number of digits in limbs, and cell death in the mutant limbs. The deletion of Ezh2 using Prrx1-Cre resulted in shortened segment lengths, which affected the antero-posterior axis, but to a lesser degree when compared to T-Cre mutants. Similar to our studies that focus on bone and cartilage formation of mice that lack expression of Ezh2 in the mesenchyme [31••] (discussed below), the deletion of Ezh2 in Prrx1-Cre-expressing cells resulted in mice that survive past the embryonic stages, have shortened proximo-distal limb segments, and exhibit abnormal expression of Hox genes.

In 2014, Schwarz and colleagues utilized the Wnt1-Cre transgene system to conditionally delete functional Ezh2 in

neural crest-derived cells (NCCs) [65••]. The authors did not observe any births of Ezh2 conditional knockout (cKO) animals, but embryos did survive until late developmental stages allowing for in utero analysis. The Ezh2 cKO embryos exhibited a severe craniofacial bone and cartilage phenotype, which is characterized by a complete loss of craniofacial structures. The conditional inactivation of Ezh2 using Wnt1-Cre does not alter the migration of NCCs, neural development, cell survival, or cell cycle progression. However, the inactivation of Ezh2 in NCCs leads to a significant upregulation of Hox genes (e.g., Hoxa10, Hoxa9, and Hoxa8) when compared to wild-type (WT) embryos. The authors conclude that the derepression of Hox genes, and potentially some other genes, causes the craniofacial defects observed in Ezh2 cKO embryos.

From the studies above, it is apparent that Ezh2 is critical for normal body and tissue patterning during fetal development. These early developmental phenotypes may be due to the important function of Ezh2 in controlling H3K27me3 levels during active cell proliferation that occurs when progenitor cell populations expand to enlarge primordial tissues.

Genetic Roles of the PRC2 Complex in During Bone Formation and Post-Natal Bone Homeostasis

The cell culture studies discussed above clearly indicate that H3K27me3 formation by EZH2 is a major mechanism that controls phenotype commitment of MSCs into osteogenic versus adipogenic lineages, with reduced H3K27me3 levels favoring osteoblastic versus adipogenic cell fates. Furthermore, EZH2 has also been shown to control myogenesis [74, 75] and neuronal differentiation [57, 76, 77] of mesenchymal stem cells. For these reasons, there is considerable interest in understanding the role of EZH2 in mediating mesenchymal stem cell fate during post-natal stages of skeletal development and bone formation (Table 1).

Our laboratory published a comprehensive study detailing the biological and mechanistic effects of mesenchyme-specific loss (Prrx1-Cre) of Ezh2 in post-embryonic mice [31••]. Conditional deletion of Ezh2 results in viable offspring that exhibit multiple defects including shortened forelimbs, shorter stature, reduced cartilage and bone formation, craniosynostosis (premature cranial suture fusion), and clinodactyly (abnormal digit bending). Similar to the observations made by earlier studies [64••, 65••], RNA-Seq analysis of mRNAs from calvarial bone demonstrates a de-repression of Hox genes (e.g., Hoxb2, Hoxb3, and Hoxc4) in Ezh2 cKO animals. Loss of Ezh2 in the calvarial tissue enhances the expression of other transcription factors, osteogenic markers (e.g., Ibsp, Sparc, Bglap), and cyclin-dependent kinase inhibitors (e.g., Cdkn2a, Cdkn2b, and Cdkn1c). This study suggests that the observed phenotype in mesenchyme-specific depletion of Ezh2 is attributable to cell cycle arrest and altered expression of Hox genes and other developmentally regulatory genes that together force precocious commitment of MSCs into the osteogenic lineage as demonstrated by premature fusion of calvarial bones due to de-repression of osteogenic genes.

Hemming and colleagues also assessed the phenotype of Ezh2 conditional loss in the mesenchymal lineage [66•]. In addition to assessing WT and cKO mice, the authors of this publication also included analysis of Ezh2 heterozygous ($Ezh2^{+/flox}$, HET) mice. Similar to the observations made by previous studies [31••, 64••], Ezh2 cKO animals exhibited skeletal deformities, smaller overall body size, and reduced weight and growth plate size. Interestingly, the HET animals exhibited expansion of skeletal size, weight, and growth plate when compared to WT animals. While trabecular patterning is similar between WT and HET animals, the trabeculae expand throughout the entire hind limbs of cKO animals. The authors also reported enhanced cortical and trabecular bone formation in HET animals, but these animals nonetheless exhibit decreased mechanical strength when compared to WT animals. Further analysis demonstrated that cKO and HET animals exhibit increases in osteoclast number and activity, which provides a reasonable explanation for the observed differences between enhanced bone formation rates and reduced bone quality in cKO and HET mice. The authors also show that MSCs isolated from HET animals exhibit enhanced trilineage (osteogenic, adipogenic, and chondrogenic) differentiation potential when compared to WT animals. Similar to studies from our laboratory [31••, 47••], the gene expression studies presented by Hemming and Gronthos suggest that Ezh2 loss upregulates expression of Ezh2 target genes implicated in Wnt and BMP signaling to promote osteogenic differentiation at the expense of adipogenic commitment of MSCs. Thus, the findings from multiple groups begin to converge on the same concept that endogenous WNT and BMP signaling is stimulated during osteogenic phenotype commitment upon physiological reduction of Ezh2 expression or Ezh2 inhibition.

Mirzamohammadi and colleagues published a paper describing mice that have a cartilage-specific loss of Eed, which is one of the structural components of the PRC2 complex [67•]. Cartilage-specific cKO animals were generated by crossing $Eed^{f/f}$ mice [78] with Col2a1-Cre-expressing mice [79]. Animals that lack Eed expression in chondrocytes exhibit a severe form of kyphosis, decreased chondrocyte proliferation, growth defects, cell death related to reduced Hif1a levels, and accelerated hypertrophic differentiation. Western blotting and qPCR analysis demonstrated that chondrocyte-specific deletion of Eed induces multiple signaling pathways, including Wnt and TGFβ signaling. Administration of the TGFβ pathway inhibitor, Y364947, into pregnant or nursing mothers rescues the proliferation and growth defect observed in cKO animals.

However, treatment of cKO animals with the Wnt inhibitor C59 improves spinal deformity, subdues premature hypertrophic differentiation, and prevents premature growth plate closure between the vertebral body and transverse processes in cKO animals.

In a recent study by Lui and co-workers, the authors generated a first compound mice in which both Ezh1 and Ezh2 function is perturbed in chondrocytes [68•]. The $Ezh2^{f/f}$ allele was used in Col2a1-Cre animals (described above) to delete functional Ezh2 in chondrocytes, while a whole body knockout of Ezh1 was accomplished by using mice described by Ezhkova and others [63]. Key findings include the observation that loss of Ezh2 alone in chondrocytes is not enough to induce an appreciable phenotype in mice, and these data are independently confirmed by observations in our research group (unpublished data). However, combined loss of Ezh1 and Ezh2 in chondrocytes interferes with proper growth plate development, chondrocyte proliferation, and chondrocyte hypertrophy and together these events severely impair skeletal development. At a molecular level, the Ezh1 and Ezh2 dual loss reduces chondrocyte proliferation by enhancing the expression of Cdkn2a and Cdkn2b, while inappropriate chondrocyte hypertrophy is the product of suppressed IGF signaling.

Interestingly, the dual loss of Ezh1 and Ezh2 in chondrocytes resembles the phenotype observed with the loss of Eed in the same tissue [67•, 68•]. Both of these chondrocyte-specific losses of PRC2 complex function are, in general, very similar to the phenotype observed with the loss of Ezh2 in the mesenchyme utilizing the Prrx1-Cre system [31••, 64••, 66•]. Because of the severe phenotypes of Ezh1/Ezh2 double knockout mice and the lack of obvious phenotypes in mice in which either Ezh1 or Ezh2 is ablated in chondrocytes, it appears that Ezh1 and Ezh2 can functionally compensate in the PRC2 complex. However, because the double knockout compromises the PRC2 complex, it is clear that formation of this complex is required for proper cartilage formation during skeletal development and endochondral bone formation. We note that Ezh1 and Ezh2 cannot fully compensate for each other at the molecular level as is indicated by work from Margueron and colleagues, who showed data suggesting different mechanisms by which Ezh1 and Ezh2 repress gene expression [33••]. Additional studies will be necessary to resolve the specific roles of Ezh1 and Ezh2 in the mesenchymal lineage and assess their PRC2-dependent and independent roles in bone formation and bone homeostasis.

Bone Anabolic and Osteoprotective Effects of Ezh2 Inhibition

Three recent studies have demonstrated bone anabolic and osteoprotective effects of Ezh2 inhibition in vivo [47••, 69, 70] (Table 2). Jing and colleagues [69] observed that the expression of Ezh2 is upregulated in MSCs derived from ovariectomized (OVX) mice. The enhanced expression of Ezh2 leads to enhanced H3K27me3 of Wnt1, Wnt6, and Wnt10a promoters to inhibit β-catenin signaling shifting MSC commitment into adipocytes. The suppression of Ezh2 by knockdown or inhibition (DZNep) experiments rescued the osteogenic phenotype of MSCs by de-repressing Wnt signaling. To assess whether DZNep could prevent bone loss associated with estrogen depletion (OVX surgery), the authors intraperitoneally (IP) injected 0.1 mg/kg DZNep every other day (starting 1 week after surgery, for 6 weeks) into 8-week-old animals that underwent SHAM or OVX surgeries. The authors demonstrate that DZNep prevents trabecular bone loss as measured by microCT analysis (e.g., BV/TV and Tb.N), inhibits bone marrow fat formation, and enhances osteogenic differentiation of MSCs. One limitation of this study is that the effects of DZNep on SHAM-treated animals were not reported. Also, DZNep is not a specific inhibitor of Ezh2, but it is widely considered as a global methylation inhibitor [34, 40, 80]. Thus, the possibility exists that the beneficial effects with DZNep may not be directly related to Ezh2 inhibition.

Encouraged by the in vitro effects of Ezh2 inhibition and knockdown in human MSCs [31••], our laboratory assessed the effects of Ezh2 inhibition and knockdown in mouse preosteoblasts, as well as in vivo during bone homeostasis in mice [47••]. We demonstrate that inhibition of Ezh2 significantly enhances osteogenic differentiation of MC3T3 preosteoblasts and promotes the expression of bone-related genes and ECM proteins by reducing H3K27me3 near transcriptional start sites. Mechanistically, in vitro Ezh2 inhibition enhances osteogenic differentiation by increasing the expression of Wnt10b and Pth1r and enhancing the phosphorylation of Smad1/5, a BMP2 target. In our first in vivo study, we examined the effect of Ezh2 inhibitor GSK126 in 6-week-old C57/B6 wild-type mice. The animals were IP injected daily with 15 and 50 mg/kg GSK126 for 5 weeks. We did not observe gross adverse reactions as demonstrated by similarities in body and spleen weights between vehicle and GSK126 groups. μCT analysis demonstrates a significant increase in cortical bone volume and thickness (femoral diaphysis) and a trend toward increased cancellous bone thickness (distal femoral metaphysis) with low and high doses of GSK126. In support of μCT results, histomorphological analysis of the distal femoral metaphysis shows a significant increase in bone formation rate, number of osteoblasts, and mineral apposition rate in the 50 mg/kg GSK126 group. We did not observe any significant changes in osteoclast numbers. Because of the bone anabolic effects of GSK126, we performed a second study to assess for potential osteo-protective effects of this Ezh2 inhibitor. Twelve-week-old SHAM and OVX animals received a daily dose of vehicle or 50 mg/kg GSK126 for 6 weeks. Similar to our first study with skeletally immature

Table 2 In vivo effects of Ezh2 inhibition in wild-type and estrogen-depleted mice

Publication	Mouse line	Drug	Outcome
Jing et al. [69] (2015)	8-week-old C57/B6	0.1 mg/kg DZNep IP administration (every other day, 6 weeks, starting 1 week after OVX)	-Prevents trabecular bone loss -Inhibits bone marrow fat formation -Enhances osteogenic differentiation of MSCs
Dudakovic et al. [47] (2016)	6-week-old C57/B6 12-week-old C57/B6	15 and 50 mg/kg GSK126 IP administration (every day, 5 weeks) 50 mg/kg GSK126 IP administration (every day, 6 weeks, starting day after OVX)	-No gross adverse reactions -Increase in cortical bone volume and thickness -Increased cancellous bone thickness (not significant) -Increase in bone formation rate, OB no., and mineral apposition rate -No changes in OC no. -No gross adverse reactions -Increase in cortical thickness of femoral diaphysis -Increase in trabecular thickness of femoral metaphysis -Partial restoration of L5 vertebral bone volume, trabecular number, and trabecular thickness
Fang et al. [70]	11-week-old C3H	150 mg/kg of GSK126 IP administration (5× per week, starting 3 weeks after OVX)	-Restores several trabecular bone parameters (BV/TV, Tb.Th, Tb.Sp, Tb.N) in tibiae—no positive effects observed in femurs

mice at 6 weeks, GSK126 treatment of skeletally mature mice at 12 weeks revealed that inhibition of Ezh2 stimulates net bone accrual. In the OVX group, GSK126-treated animals exhibit an increase in cortical thickness of femoral diaphysis and trabecular thickness of femoral metaphysis. Along these lines, L5 vertebral bone volume, trabecular number, and trabecular thickness are partially restored when OVX animals are treated with GSK126. These studies indicate that EZH2 inhibition appears to have selective effects on bone mineral accrual in different skeletal elements.

Fang and colleagues assessed the role of Ezh2 activity during osteoclast differentiation [70]. The authors demonstrate that knockdown or inhibition of Ezh2 inhibits the differentiation of human osteoclast precursors into osteoclasts in vitro. Mechanistically, they show that Ezh2 inhibits the expression of Irf8, an inhibitor of osteoclast differentiation, by altering H3K27me3 marks on its promoter. Thus, the inhibition of Ezh2 activates Irf8 expression, which then acts as an inhibitor of osteoclastogenesis. To test the effects of Ezh2 inhibition in vivo, SHAM and OVX were performed on 11-week-old C3H mice which were treated with vehicle or 150 mg/kg of GSK126 for 5 weeks by IP injections (5 times per week) starting at 3 weeks post surgery. The results indicate that GSK126 partially restores several trabecular bone parameters (e.g., BV/TV, Tb.Th, Tb.Sp, Tb.N) from tibiae. While the authors do not mention vertebral bone quality, they state that positive effects seen in the tibiae were not observed in the femurs of the animals, consistent with stimulatory effects of GSK126 in some but not all bone types.

In summary, all three reports [47••, 69, 70] demonstrate an osteo-protective effect of Ezh2 inhibition in vivo. One of our studies [47••] also suggests that Ezh2 has bone anabolic effects in young animals. Two of the studies utilized a specific Ezh2 inhibitor GSK126, but at different doses (50 versus 150 mg/kg), in different strains of mice (C57/B6 versus C3H), and in different treatment regimens. For example, in the Dudakovic study, treatment started immediately after surgery every day of the week [47••], while mice were treated after a 3-week hiatus and then treated only five times per week in the Fang study [70]. The third study by Jing [69] utilized DZNep, a non-specific methylation inhibitor, in C57 mice. Hence, a complete and direct comparison between the three studies is rather difficult.

Conclusions and Future Directions

The current literature on the role of EZH2 suggests that this epigenetic enzyme plays a critical role in suppressing osteogenic, myogenic, and neuronal differentiation of MSCs. Interestingly, this epigenetic regulator appears to favor adipogenesis as a default lineage of MSCs. For this reason, targeting EZH2 in the MSC compartment may have beneficial effects in promoting differentiation into preferred tissues (e.g., bone, muscle, neurons) at the expense of the undesirable fat tissue.

The current findings demonstrating the involvement of Ezh2 in osteogenic differentiation is summarized in Fig. 1. Ezh2 inhibition enhances in vitro and in vivo bone formation and protects animals from estrogen depletion-induced osteoporosis. Mechanistically, Ezh2 inhibits the expression of key pro-osteogenic genes to suppress osteogenic commitment of MSCs. In turn, Ezh2 is regulated by CDK1-induced phosphorylation, Runx2, microRNAs, and long non-coding RNAs to suppress the activity of this epigenetic enzyme. The conditional deletion animal models of Ezh2 paint a more

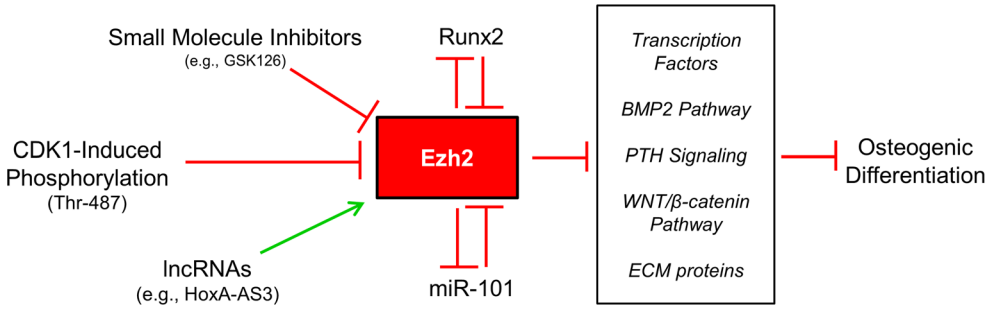

Fig. 1 Ezh2 is an epigenetic inhibitor of osteogenic differentiation. Ezh2 suppresses the activity of several pro-osteogenic genes and pathways to suppress osteogenic differentiation of MSCs. Several cellular mechanisms have been shown to modulate the activity of Ezh2. miR-101, Runx2, and CDK1 have been show to inhibit Ezh2 function, while lncRNAs (e.g., HoxaA-AS3 and ANCR) have been shown to promote Ezh2 activity. Small molecule inhibition of Ezh2 (e.g., GSK126) has shown promise in promoting osteogenic differentiation in vitro and vivo

complex picture of the role of Ezh2 in mesenchymal development. These recent studies reveal that inactivation of Ezh2 appears to stimulate the osteogenic phenotype, but these changes are also accompanied by obvious physical abnormalities that can be attributed to inhibition of proliferation. Thus, it appears that Ezh2 inhibition is detrimental to the developing skeleton, but it may be beneficial to the adult skeleton.

Several questions still remain unanswered in regards to Ezh2 and its role in osteogenic differentiation. One question that should still be addressed is the methyltransferase-dependent and methyltransferase-independent functions of Ezh2 in osteoblast differentiation. Recent studies have suggested that methyltransferase-independent functions of Ezh2 contribute to cellular phenotypes [57, 81]. Although the widely studied Ezh2 mouse model utilizes loxP sites that flank the SET domain, the excision of this portion of the gene makes the protein unstable and undetectable in Cre-expressing tissues. Thus, a mouse model that utilizes an inactivating mutation in Ezh2, but retains protein expression, would help understand methyltransferase-dependent and methyltransferase-independent function of this epigenetic regulator. Furthermore, future studies should also account for the epigenomic functions of Ezh2 on chromatin and its emerging role as a general lysine trimethyl transferase of critical regulatory proteins (e.g., β-catenin) [82].

The status of the PRC2 complex in the absence of Ezh2 has not been sufficiently considered. A PRC2 complex that contains Ezh1 does exist, but the extent to which Ezh1 can compensate for Ezh2 is still not fully understood. It is well known that Ezh2 is expressed in undifferentiated proliferating cells, while Ezh1 expression is most commonly observed in post-proliferative/differentiated cells. The possibility exists that Ezh1 cannot compensate for Ezh2 because (i) their expression patterns are different, (ii) the PRC2-Ezh2 and PRC2-Ezh1 complexes target different genes, or (iii) methyltransferase activity of Ezh1 is not sufficient enough to methylate Ezh2 target genes. Furthermore, additional animal models should be considered to assess bone anabolic and osteo-protective effects of Ezh2 inhibition.

The abovementioned studies were able to demonstrate beneficial effects, but Ezh2 inhibitors were only administered for several weeks. Longer administration in mice is warranted, which should be followed up by bigger animal models (e.g., rat, rabbit, and sheep). In addition, because of the anti-proliferative potential of Ezh2 inhibitors (chronic administration of Ezh2 inhibitors for osteoporosis treatment may not be feasible), the effects of Ezh2 inhibition should also be assessed in models of bone disease that only require short-term treatment (e.g., non-union fractures, critical size defect models, osseo-integration, spinal fusion). These studies should also consider combing Ezh2 inhibitors with currently approved treatments for these particular disease states (e.g., BMP2). Finally, it would be beneficial to monitor bone health of cancer patients currently treated with Ezh2 inhibitors. The possibility exists that the patients may experience a decreased tumor burden and enhanced bone formation as a result of Ezh2 inhibition. This would be a welcome "side effect" in patients undergoing an anti-cancer treatment regimen. Notably, Ezh2 inhibition can alleviate bone-specific metastatic cancer burden (e.g., breast, prostate, melanoma) while also improving bone qualities as suggested by in vitro studies [83]. Therefore, bone-targeting tumor models should be considered that focus on assessing tumor burden as well as local and global bone health in experimental animals. In addition, local and/or short systemic delivery of Ezh2 inhibitors alone or in combination with other treatments (e.g., BMP2, PTH, WNT agonist, SOST antibody) may aid in clinical settings in which bone repair is required (e.g., spinal fusion, osseo-integration, non-healing unions, radiation-induced lesions).

In summary, epigenetic mechanisms play a critical role in osteoblast differentiation. Ezh2 is a key epigenetic enzyme that controls lineage allocation and osteogenic differentiation of MSCs. Ezh2 inhibition has shown pro-osteogenic effects in vitro and in vivo, and future studies could be considered to assess the clinical utility of Ezh2 inhibition for bone-related diseases.

Compliance with Ethical Standards

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Conflict of Interest Amel Dudakovic and Andre J. van Wijnen each declare no potential conflicts of interest.

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