

# Signaling Networks that Control the Lineage Commitment and Differentiation of Bone Cells

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**ABSTRACT:** Osteoblasts and osteoclasts are the two major bone cells involved in the bone remodeling process. Osteoblasts are responsible for bone formation while osteoclasts are the bone-resorbing cells. The major event that triggers osteogenesis and bone remodeling is the transition of mesenchymal stem cells into differentiating osteoblast cells and monocyte/macrophage precursors into differentiating osteoclasts. Imbalance in differentiation and function of these two cell types will result in skeletal diseases such as osteoporosis, Paget's disease, rheumatoid arthritis, osteopetrosis, periodontal disease, and bone cancer metastases. Osteoblast and osteoclast commitment and differentiation are controlled by complex activities involving signal transduction and transcriptional regulation of gene expression. Recent advances in molecular and genetic studies using gene targeting in mice enable a better understanding of the multiple factors and signaling networks that control the differentiation process at a molecular level. This review summarizes recent advances in studies of signaling transduction pathways and transcriptional regulation of osteoblast and osteoclast cell lineage commitment and differentiation. Understanding the signaling networks that control the commitment and differentiation of bone cells will not only expand our basic understanding of the molecular mechanisms of skeletal development but will also aid our ability to develop therapeutic means of intervention in skeletal diseases.

**KEY WORDS:** osteoblasts, osteoclasts, signaling pathways, transcriptional regulation, skeletal disease, bone genes

## I. INTRODUCTION

Bone is an essential mineralized tissue with critical mechanical and metabolic functions. It has the capacity to adapt to its functional environment in such a way that its morphology is "optimized" for the mechanical demand.<sup>1</sup> Physiological bone turnover can be divided into two temporal phases: modeling, which occurs during development, and remodeling, a lifelong process involving tissue renewal. Bone integrity and function are maintained by an exquisite balance between the osteoblast and the osteoclast, the two major bone cells involved in the remodeling process. Remodeling starts with removal by osteoclasts of matrix, a mixture of insoluble proteins in which type I collagen is predominant (>90%) and a poorly crystalline, chemically modified hydroxyapatite. Follow-

ing resorption, osteoblasts are recruited to the site, where they secrete and mineralize new matrix. Bone is continuously remodeled throughout life and an imbalance in this process can result in bone disease. The increased activity of osteoclasts caused by estrogen withdrawal causes bone loss and osteoporosis, a frequent low-bone mass disorder in postmenopausal women leading to structural instability and a high fracture risk. A recent study has shown that estrogen actually induces osteoclast apoptosis.<sup>2</sup> Estrogen deficiency is known to play a critical role in the development of osteoporosis, while calcium and vitamin D deficiencies and secondary hyperparathyroidism also contribute.<sup>3</sup> Osteoporosis is a factor in more than 1.5 million fractures each year in the United States alone. Costs have been estimated at more than \$17 billion a year, particularly from hip fractures. More

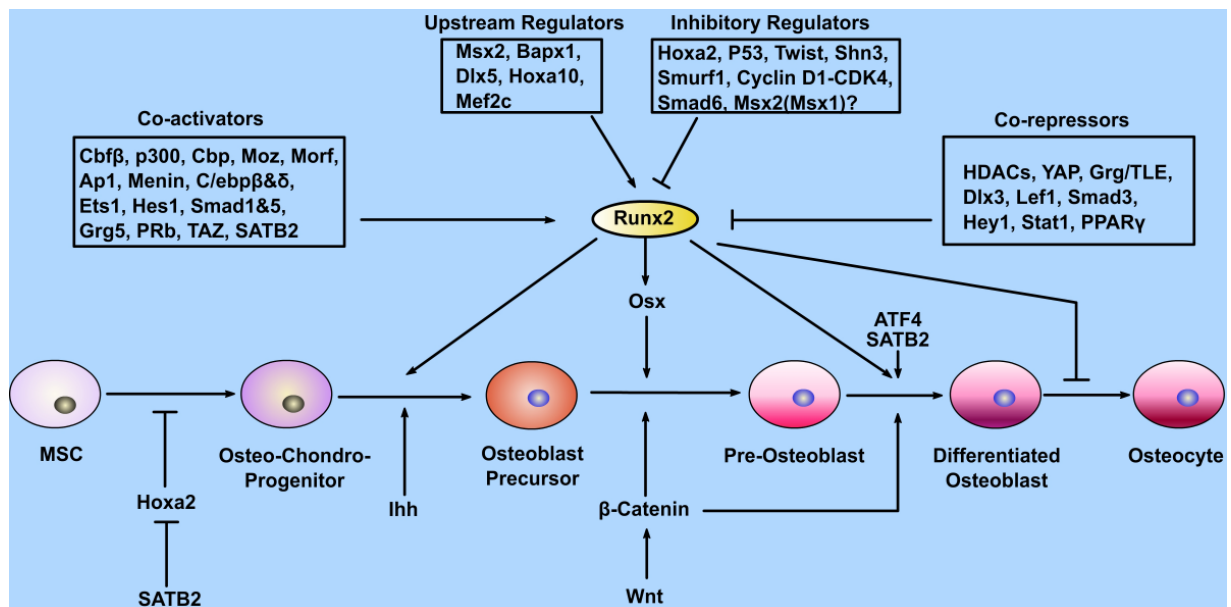
than 75% of which occur in women. A better understanding of bone quality, coming from biochemical markers and refined imaging techniques, will help predict who is most at risk of debilitating fractures. One of the main approaches to gleaning details about the quality of bones is to measure the activity of osteoclasts and osteoblasts, the cells that remodel bone and thus influence its structural properties.<sup>4</sup> The recent discoveries of signal transduction pathways and transcription factors critical for the differentiation and function of osteoblasts and osteoclasts have opened up new approaches to understanding the pathogenesis of osteoporosis. We review what is known about the transcription factors and cytokines that regulate the stages of differentiation in osteoclasts and osteoblasts.

## II. BONE CELL ORIGIN AND CELL LINEAGE

### A. Osteoblast Lineage

Osteoblasts, which play central roles in bone formation, are derived from undifferentiated mesen-

chymal cells (Fig. 1), which also have the capacity to differentiate into chondrocytes, adipocytes, and myoblasts.<sup>5</sup> There are three major stages of osteoblastogenesis: proliferation, matrix maturation, and mineralization. Osteoblast progenitors can first be identified within the inner perichondrium adjacent to, and coincident with, the first appearance of hypertrophic chondrocytes. This tight linkage reflects a crucial role for Indian hedgehog (Ihh) signaling (Fig. 1), discussed below. Markers of this program in the endochondral-derived skeleton are dependent on an initial Ihh input. During normal development, Ihh signaling appears to act as a switch within a specific population of inner perichondrial mesenchyme to initiate a program of bone formation.<sup>6</sup> Failure to activate this switch results in cells adopting an alternative chondrocyte pathway of development. Ihh acts in differentiation of osteoblast progenitors into runt-related transcription factor 2 (Runx2)<sup>+</sup> osteoblast precursors. Wnt/ $\beta$ -catenin signaling acts later in the differentiation pathway to osterix<sup>+</sup> osteoblast precursors and then to bone-secreting osteoblasts (Fig. 1).<sup>6</sup> In addition, alkaline phosphatase (ALP), bone sialoprotein (BSP), and collagen type 1 alpha 1



**FIGURE 1.** Transcription factors and signaling involved in the osteoblast differentiation pathway. Osteoblasts and chondrocytes are derived from common mesenchymal stem cell precursors. Runx2 stimulates terminal differentiation. A number of transcription factors are involved in Runx2 regulation and function, either upstream of Runx2 or as coactivators or corepressors. Runx2 functions upstream of Osx, which is required after Runx2 for osteoblast differentiation. Ihh and Wnt/ $\beta$ -catenin are key signaling molecules in osteoblastogenesis.

(Col1a1) are early markers of osteoblast differentiation, while parathyroid hormone/PTH-related peptide (PTH/PTHrP) receptor (PPR) and osteocalcin (OCN) appear late, concomitantly with mineralization. Osteopontin (OPN) peaks twice, during proliferation and then again in the later stages of differentiation.

Following initial lineage commitment, a phase of lineage expansion ensues that culminates normally in permanent cell cycle withdrawal. The initial cell division is asymmetric, giving rise to another stem cell (self-renewal) and a committed osteoprogenitor. Following commitment, the stem cell gives rise to the transit-amplifying compartment.<sup>7</sup> This phase is associated with intensive proliferative activity. The preosteoblast is an intermediate stage, which expresses STRO-1, ALP, PPR, and type I collagen, and is committed to the osteoblast lineage with extensive replicative capacity, but no self-renewal capacity.<sup>8</sup> In vitro, the use of agents such as retinoic acid can induce further differentiation in the preosteoblast. The mature osteoblast expresses ALP, OPN, BSP, and OCN, and lies adjacent to newly synthesized osteoid. This stage, which is responsible for the laying down of bone, has limited replicative potential.<sup>9</sup> The cumulative effect of the recruitment of stem cells and their expansion, and the functional capacity of mature osteoblasts, is measured by rates of bone formation in vivo. The second key step initiates terminal differentiation and permanent cell cycle withdrawal. The terminal stage of the bone lineage is the postmitotic osteocyte, often found isolated within bone, presumably embedded within advancing osteoids. As an alternate fate, a proportion of cells in the transient amplifying compartment may also terminate in apoptosis.

It was recently found that calcineurin plays a role in osteoblast differentiation. Calcineurin  $\alpha$  null mice display severe osteoporosis and reduced Runx2, BSP, and OCN expression.<sup>10</sup> A range of cytokines modulate osteoblast differentiation, including bone matrix-derived transforming growth factor beta (TGF- $\beta$ ), bone morphogenic protein 2 (BMP-2), BMP-4, and BMP-7, and their inhibitors noggin, chordin, gremlin, and sclerostin, the last identified by positional cloning of families with increased bone mass. Similarly, numerous hormones impact osteoblast function positively including insulinlike growth factor-1 (IGF-1),

PTH, PTHrP, 1,25(OH)2D3, leptin, glucocorticoids, the Notch pathway, and members of the leukemia inhibitory factor/interleukin-6 (IL-6) family. Deletion of the neuropeptide Y2 receptor was shown to increase bone mineralization by increasing the number of mesenchymal progenitor cells and osteoblast activity.<sup>11</sup> Y1 null mice also showed elevated bone mass, showing the inhibitory effects of neuropeptide Y on bone cells via the Y receptors.<sup>12</sup>

## B. Osteoclast Lineage

Osteoclast (OC) lineage, phenotype, and gene expression are associated with OC differentiation. Transplantation studies have demonstrated that the OC precursor is a mononuclear cell that is hematopoietic in origin.<sup>13</sup> Earlier studies performed in vivo<sup>14</sup> and in vitro<sup>15-17</sup> suggested that OCs are derived from cells of the mononuclear phagocyte system. However, more recent evidence indicates that although macrophages and OCs share a common precursor, these lineages diverge on further differentiation.<sup>18</sup> OCs possess a distinct phenotype and functional capabilities compared to cells of the macrophage series, particularly the ability to resorb bone. OC hematopoietic precursors migrate via vascular pathways to the skeleton. Osteoblasts, chondrocytes, and their mineralized matrices, together with stromal and endothelial cells, provide the microenvironment for homing of these precursor cells. Stromal cells produce cytokines, including macrophage-colony stimulating factor (M-CSF) and IL-6, which induce and modulate growth and differentiation of the precursors to mature OCs.<sup>19,20</sup> Annexin II is an OC stimulatory factor that stimulates OC formation by activating T cells to secrete granulocyte macrophage colony-stimulating factor (GM-CSF), which expands the OC precursor pool.<sup>21</sup> In addition, the vitamin D metabolite calcitriol and the parathyroid hormone support OC development.<sup>22,23</sup> Usui et al. found that hypertrophic chondrocytes regulate osteoclastogenesis through BMP-2-induced receptor activator of NF- $\kappa$ B ligand (RANKL) expression.<sup>24</sup> BMP-2 regulation of RANKL activity was abolished by Runx2 mutation. It was recently discovered that follicle-stimulating hormone (FSH) effects osteoclast

formation.<sup>25</sup> FSH was found to activate several signaling mechanisms in osteoclasts and their precursors, such as Erk kinase/mitogen-activated protein kinase (MEK/Erk), nuclear factor kappa-B (NF- $\kappa$ B), thymoma viral proto-oncogene (Akt), and inhibitory kappa-B alpha ( $\text{I}\kappa\text{B}\alpha$ ).

The OC precursor cells have multiple Golgi complexes and abundant mitochondria,<sup>14</sup> and are positive for nonspecific esterase<sup>26</sup> and possibly type IV collagenase. In a suitable microenvironment, OC precursors differentiate into preosteoclasts (preOCs) or mononuclear osteoclasts (Fig. 2). The term "preOC" has been used to describe a direct mononuclear precursor showing morphological and cytochemical features that are similar to those of the multinucleated OC.<sup>27-30</sup> They are tartrate-resistant acid phosphatase (TRAP)-positive cells that express mRNAs for all OC-associated phenotypes [e.g., TRAP, calcitonin receptor (CTR), and cathepsin K], and are poised to fuse into multinucleated OCs on further differentiation.<sup>29</sup> OCs possess a highly specialized proton-generating mechanism for the rapid dissolution of mineral, and secrete collagenases,<sup>31</sup> cathepsin K,<sup>32</sup> and other hydrolases active in the degradation of bone matrix proteins. OC formation *in vitro* can be studied either in organ culture of murine embryonic metatarsals,<sup>19</sup> in a coculture system in which hematopoietic precursor cells (spleen or marrow cells) are cultured with osteoblasts,<sup>33</sup> or in a cloned SV40-transformed OC precursor cell line, that is, MOCP-5.<sup>34</sup> Soluble RANKL and M-CSF allow spleen or marrow cells to differentiate into OCs without coculture with osteoblasts.

### III. BONE CELL SIGNALING

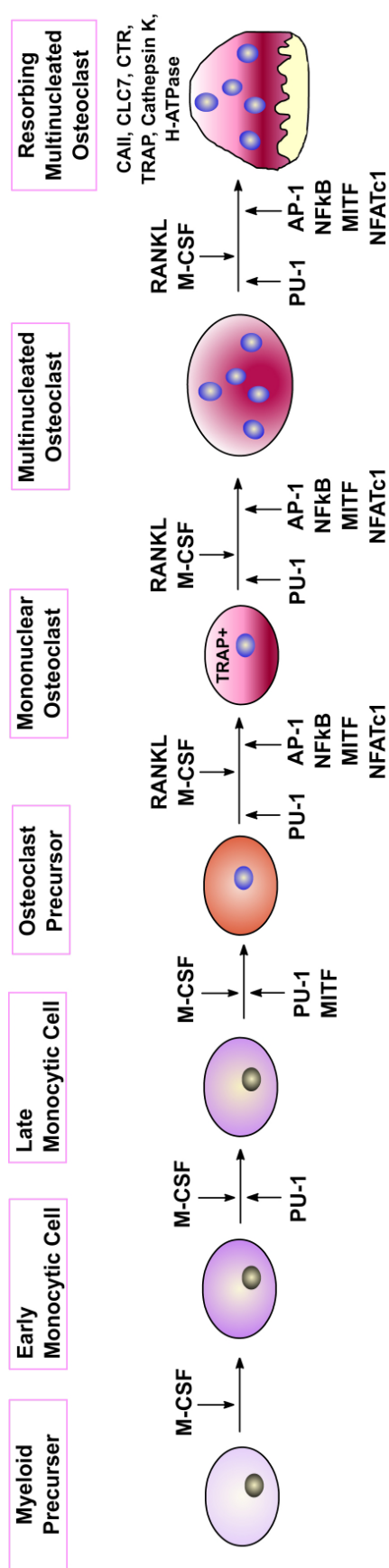
#### A. Osteoblast Signaling

##### 1. Wnt Signaling Pathway

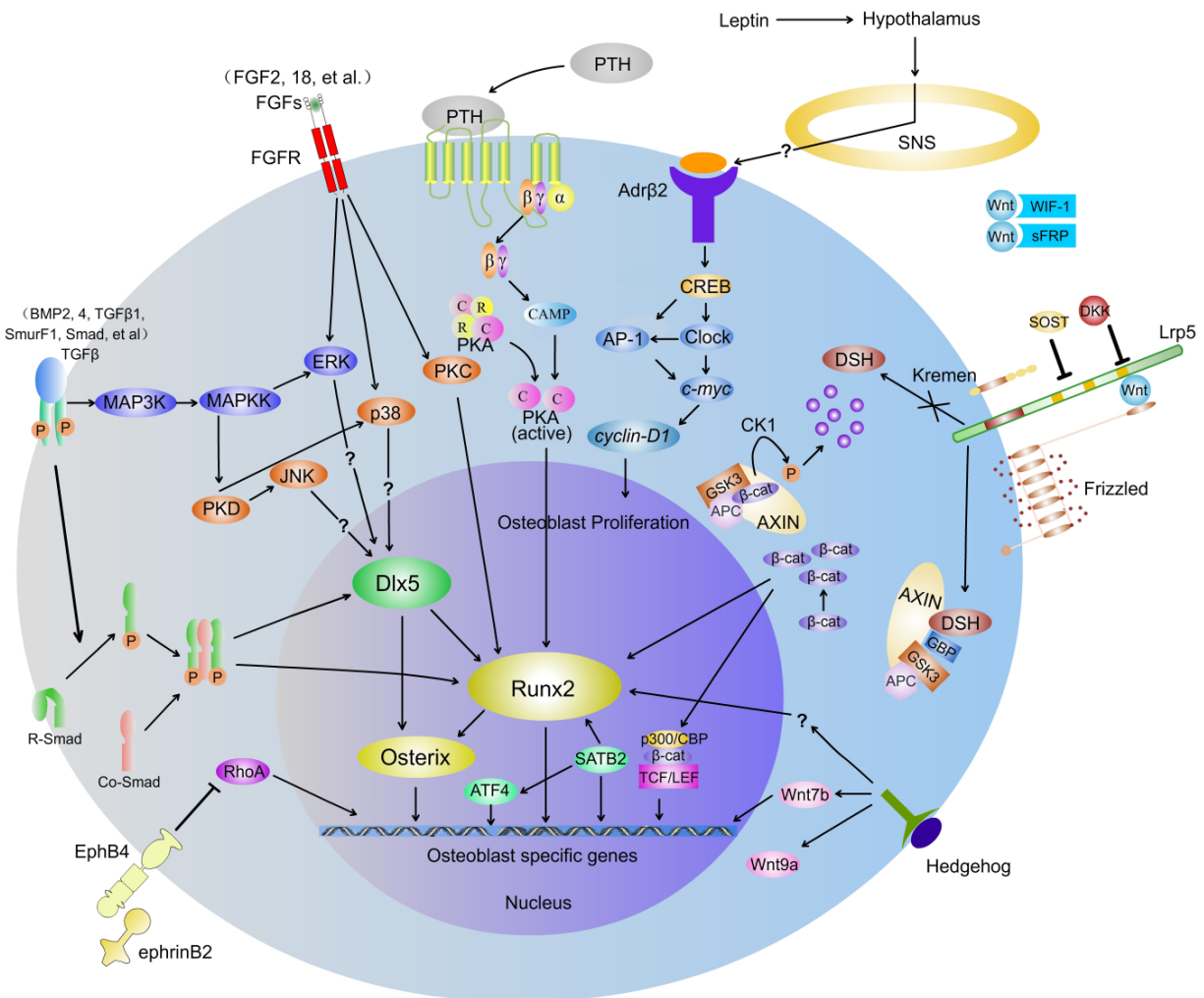
Wnt proteins signal through several pathways to regulate cell growth, differentiation, function, and death. The Wnt/ $\beta$ -catenin, or canonical, pathway appears to be particularly important for bone cell signaling.<sup>35-38</sup> Here we outline the current model of Wnt signal transduction, shown in Figure 3. Wnt proteins released from or presented on the surface of signaling cells act on

target cells by binding to the frizzled homolog (*Drosophila*)/low-density lipoprotein receptor-related protein 5 and 6 (FZD/LRP5/6) complex at the cell surface. Signals are generated through the proteins Disheveled, Axin, and Frat-1, which disrupt the protein complex and inhibit the activity of glycogen synthase kinase 3 (GSK3), thus causing hypophosphorylation of its substrate,  $\beta$ -catenin (Fig. 3).<sup>39</sup> The on state involves increasing the post-translational stability of  $\beta$ -catenin, through Wnt-dependent inhibition of GSK3 (Fig. 3). Stabilized  $\beta$ -catenin accumulates in the cytosol and translocates to the nucleus to activate target gene transcription. The lymphoid enhancer-binding factor/T-cell factor (Lef/Tcf) transcription factor family members are well-studied nuclear partners of  $\beta$ -catenin.<sup>40,41</sup>  $\beta$ -catenin displaces corepressors of Lef/Tcfs [e.g., Groucho, silencing mediator of retinoid and thyroid receptors and nuclear receptor corepressor (SMRT/NCoR)], and forms heterodimers with the Lef/Tcf proteins to bind DNA and initiate the transcription of target genes.<sup>42</sup>

Extracellular Wnt ligands can interact with a host of secreted antagonists, including the secreted FZD-related protein (sFRP) family and Wnt inhibitory factor 1 (WIF-1), preventing activation of the pathway. Knockout of sFRP1 in mice leads to a high bone mass phenotype, early induction of collagen type 10a1, and activation of the Runx2 transcription factor leading to increased chondrocyte differentiation.<sup>43</sup> Loss of sFRP1 in mice results in reduced osteoblast apoptosis while enhancing proliferation and differentiation of osteoblasts, leading to heightened trabecular bone mass.<sup>44</sup> LRP5/6 coreceptor activity is inhibited by members of the SOST (Sclerosteosis gene product)<sup>45</sup> and Dickkopf (Dkk) families,<sup>46</sup> all of which bind LRP5/6.<sup>47</sup> Both Dkk1 and Dkk2 antagonize canonical Wnt signaling by simultaneously binding to LRP5/6<sup>47</sup> and a single-transmembrane protein called kremen.<sup>46</sup> Diarra et al. were able to reverse the bone-destructive pattern of a mouse model of rheumatoid arthritis to the bone-forming pattern of osteoarthritis by inhibiting Dkk1.<sup>48</sup> Tumor necrosis factor (TNF)- $\alpha$  was identified as a key inducer of Dkk1 in human rheumatoid arthritis, suggesting that Wnt signaling is a key regulator of joint remodeling. Dkk2 has been shown to be involved in terminal osteoblast



**FIGURE 2.** Stages of osteoclast differentiation from hematopoietic lineage cells. M-CSF and RANKL are essential external stimuli for osteoclastogenesis. PU.1, Mitf, NF- $\kappa$ B, AP-1, and NFATc1 are required for differentiation of mature osteoclasts.



**FIGURE 3.** Seven important signaling networks of osteoblast differentiation. Binding of Wnt to the FZD receptor induces  $\beta$ -catenin accumulation, which translocates to the nucleus to activate target gene transcription. Several transcription factors have been found crucial for osteoblast differentiation downstream of this signaling pathway, such as Runx2, Osterix, and ATF4. They are essential for differentiation of mesenchymal stem cells into differentiated osteoblasts and also function in the transcription of osteoblast-specific genes.

differentiation, although the precise mechanisms need further investigation.<sup>49</sup>

In the absence of Wnt expression, degradation of  $\beta$ -catenin is facilitated via interactions with a protein complex consisting of adenomatous polyposis coli (APC), axin, and GSK3. APC and axin act as scaffold proteins allowing GSK3 to bind and phosphorylate  $\beta$ -catenin, identifying it for degradation by the beta-transducin repeat-containing protein ( $\beta$ -TrCP)-mediated ubiquitin/proteasome pathway. In the nucleus, prospective target genes of the pathway are kept in a repressed state by interacting with TCF and LEF transcription factors,

with associated corepressors (Fig. 3). In this off state, cells maintain low cytoplasmic and nuclear levels of  $\beta$ -catenin, although  $\beta$ -catenin is associated with cadherins at the plasma membrane, which spares it from the degradative pathway.<sup>41</sup> Despite the prominent role of canonical Wnt signaling in osteoblast biology, the role of the TCF/LEF family of transcription factors is unclear.

Indisputably, Wnts are involved in embryonic skeletal patterning, fetal skeletal development, and adult skeletal remodeling.<sup>50-52</sup> Unraveling the function(s) of Wnt proteins in the regulation of skeletogenesis has been a complex problem,

however, confounded by questions of functional redundancy, multiple times and sites of action, and the presence of other molecules that compete with Wnt function. The first indication that Wnt signaling plays a critical role in bone formation came from human studies where mutations in the Wnt coreceptor LRP5 are causally linked to alterations in human bone mass.<sup>53–59</sup> Eight LRP5 missense mutations (D111Y, G171R, A214T, A214V, A242T, T253I, G171V, M282V) have been found to cause high bone mass,<sup>53,54,56,58,59</sup> while several homozygous or heterozygous nonsense, frameshift, and missense mutations have been identified in osteoporosis-pseudoglioma patients leading to low bone density.<sup>55,57</sup> LRP5<sup>-/-</sup> mice also have low bone mass.<sup>60</sup> One of the mechanisms whereby Wnt signaling increases bone formation is via stimulation of the development of osteoblasts, and there is considerable *in vitro* evidence supporting a role for canonical Wnt/ $\beta$ -catenin signaling in this process.<sup>61–63</sup> Higher levels of  $\beta$ -catenin enhance bone formation with associated increases in expression of osteoblast-specific genes,<sup>61,64</sup> whereas conditional knockdown of the  $\beta$ -catenin gene at an early developmental stage causes ectopic chondrogenesis and abnormal osteoblast differentiation.<sup>64–66</sup> Most recently, Hill et al.,<sup>64</sup> Day et al.,<sup>65</sup> Glass et al.,<sup>67</sup> and Hu et al.<sup>68</sup> provide compelling evidence that Wnt signaling represents both a cell-autonomous mechanism for inducing osteoblastic and suppressing chondrocytic differentiation in early osteochondroprogenitors, and a mechanism in fully differentiated osteoblasts for stimulating the production of osteoprotegerin (OPG), an inhibitor of osteoclast formation.<sup>64,65,67,68</sup>

Recent experiments examining the conditional inactivation of  $\beta$ -catenin in skeletal progenitors and using different Cre lines revealed that  $\beta$ -catenin activity is essential for the differentiation of mature osteoblasts and, consequently, for bone formation in endochondral bones (the long bones of the limbs) and membranous bones (in the skull).<sup>64,65,68</sup> These variable results likely arise because Wnt/ $\beta$ -catenin signaling regulates bone development and accrual through different mechanisms at different stages of life.<sup>69</sup> This concept is supported by the results of studies using mouse models in which targeted deletion of  $\beta$ -catenin occurs early or late in osteoblasto-

genesis. It is likely that  $\beta$ -catenin activity is required in a bipotential precursor of the osteoblast lineage, the so-called osteochondroprogenitor, and indeed its absence steers the fate of mesenchymal precursors toward chondrogenesis.<sup>64,65</sup> Because Runx2, but not osterix, is expressed in  $\beta$ -catenin<sup>-/-</sup> mesenchymal cells,<sup>65,68</sup>  $\beta$ -catenin seems to be required for osteoblast differentiation at the preosteoblast stage. Furthermore,  $\beta$ -catenin/TCF1 enhances Runx2 expression and Runx2 promoter activity.<sup>70</sup> By contrast, for differentiation into the chondrocyte lineage,  $\beta$ -catenin levels must be low.<sup>64,65,68</sup> A recent study of the mechanism of Wnt/ $\beta$ -catenin-induced osteoblastogenesis revealed that Wnt/ $\beta$ -catenin signaling allows activation of transcription factors important in osteoblastogenesis by suppressing CCAAT/enhancer binding protein alpha (C/EBP $\alpha$ ) and peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ).<sup>71</sup> Knockdown of C/EBP $\alpha$  or PPAR $\gamma$  expression in ST2 cells and mouse embryonic fibroblasts reduced adipogenic potential and caused spontaneous formation of osteoblasts.

Recently, inactivation of  $\beta$ -catenin function in more mature osteoblasts using a Col1a1- and an OCN-Cre line revealed a novel role for canonical Wnt signaling in postnatal bone homeostasis.<sup>66,67</sup> Mice deficient in  $\beta$ -catenin develop osteopenia, while activation of  $\beta$ -catenin function in osteoblasts resulted in increased bone mass and an osteopetrotic phenotype.<sup>66,67</sup> However, no change in osteoblast activity or histomorphometric evidence of bone formation was observed. The altered bone resorption was caused by deregulation of OPG, a major inhibitor of osteoclast differentiation.<sup>67</sup> OPG is a direct target gene of the  $\beta$ -catenin-TCF complex in osteoblasts and Tcf1 is probably the relevant transcription factor required for OPG regulation; nevertheless, a possible role for Tcf4 cannot be excluded.<sup>62,67,72</sup> These mice demonstrate that  $\beta$ -catenin regulates osteoclastogenesis through effects on expression of osteoprotegerin and RANKL.<sup>66</sup>

On the other hand, recent evidence has revealed that noncanonical Wnt signaling also plays a role in osteoblastogenesis. Takada et al. found that the noncanonical Wnt pathway through CaMKII-TAK1-TAB2-NLK promotes osteoblastogenesis by transcriptionally repressing PPAR $\gamma$  transactivation and inducing Runx2

expression.<sup>73</sup> Tu et al. have shown that Wnt3a and Wnt7b each function in osteoblastogenesis through a protein kinase C delta (PKC $\delta$ ) pathway.<sup>74</sup> Wnt3a signals through G protein G $\alpha_{q/11}$  subunits to activate PKC $\gamma$ , and PKC $\gamma$ -deficient mice exhibit a deficit in embryonic bone formation. Ablation of Wnt7b in skeletal progenitors also leads to reduced bone formation in mouse embryos.

## 2. TGF- $\beta$ Superfamily Signaling

Once activated, TGF- $\beta$  can interact with its receptor to induce signaling. All members of the TGF- $\beta$  superfamily signal through a dual receptor system of type I and type II transmembrane serine/threonine kinases. The mothers against decapentaplegic homolog (Smad) signaling turned out to play a central role in the transmission of signals from all receptors activated by the TGF- $\beta$  superfamily members to target genes in the nucleus. Several members of the TGF- $\beta$  superfamily, such as BMPs, have potent osteogenic effects. BMPs are a group of phylogenetically conserved signaling molecules, and were initially identified by their capacity to induce endochondral bone formation.<sup>75-77</sup> BMP-1 through BMP-7 are expressed in skeletal tissue, and BMP-2, -4, and -6 are the most readily detectable BMPs in osteoblast cultures.<sup>75,78</sup> BMPs are unique because they are implicated in the specification of both chondrocytes and osteoblasts,<sup>79</sup> as well as in the subsequent modification of the osteogenic program, where some BMPs promote bone formation, such as BMP-2, BMP-7, BMP-6, and BMP-9,<sup>80</sup> although BMP-3 acts as a negative regulator of bone formation.<sup>81</sup>

On receptor activation, BMPs transmit signals through Smad-dependent and Smad-independent pathways, including Jun N-terminal kinase (JNK), and p38 mitogen activated protein (MAP) kinase (MAPK) pathways.<sup>82</sup> Smads are the major signal transducers for the serine/threonine kinase receptors.<sup>83</sup> There are three classes of Smads: (1) receptor-regulated Smads (R-Smads) that can be BMP activated, such as Smad 1, 5, and 8 (referred to as BR-Smads in this article), or TGF- $\beta$  activated, such as Smad 2 and 3 (TR-Smads); (2) common partner BMP and TGF- $\beta$  mediator Smads (Co-Smads), such as Smad 4; and

(3) inhibitory Smads (I-Smads), such as Smad 6 and 7. On ligand stimulation and activation by type II receptors, type I receptors phosphorylate R-Smads, which in turn form complexes with Co-Smads (Fig. 3).<sup>84</sup> The R-Smad/Co-Smad complexes then translocate into the nucleus and regulate transcription of target genes by interacting with various transcription factors and transcriptional coactivators or corepressors. The third class of Smads, I-Smads, negatively regulates signaling by the R-Smads and Co-Smads. Runx2 and BR-Smads physically interact with each other on activation of BMP signaling, and cooperatively regulate the transcription of target genes, leading to osteoblast differentiation of mesenchymal progenitor cells.<sup>85-87</sup> BMP induces Runx2 expression in mesenchymal progenitor cells through the action of BR-Smads,<sup>88</sup> and BR-Smads in turn interact with Runx2 and further induce osteoblastic differentiation. BMP does not directly induce the expression of Runx2 in mesenchymal cells,<sup>89</sup> but it facilitates expression of distal-less homeobox 5 (Dlx5) in osteoblasts,<sup>90,91</sup> and Dlx5 then induces expression of Runx2 in osteoprogenitor cells. Osteoprogenitor cells, for example, C2C12 cells, have been widely used for the identification of BMP target genes during osteoblastic differentiation. Hairly/enhancer of split related with YRPW motif 1 (Hey1; also termed HesR1 and Herp2) and Tcf7 are transcription factors specifically expressed in osteoblast cells by BMP-2 treatment, and are involved in Notch and Wnt signaling, respectively.<sup>92</sup> Using constitutively active BMP type I receptors, Korchynskiy et al. identified several genes as targets of BMP receptors in C2C12 cells, including transcription factors Hey1, ITF-2 (Tcf4), and interferon regulatory factor 8 (ICSBP).<sup>93</sup>

Although the Smads are critical mediators in the TGF- $\beta$  signaling pathway, a substantial body of evidence illustrates the existence of additional, Smad-independent pathways. BMP-2 can activate ERK, JNK, and p38 in osteoblastic cells and provide evidence that these MAP kinases have distinct roles in regulating alkaline phosphatase and osteocalcin expression (Fig. 3).<sup>94,95</sup> Recent reports suggest that during osteoblast differentiation, BMP-2 activates JNK and p38 via protein kinase D (PKD), independent of PKC activity.<sup>96</sup> It has been demonstrated that following TGF- $\beta$



and BMP induction, both the Smad and p38 MAPK pathways converge at the Runx2 gene to control mesenchymal precursor cell differentiation.<sup>97</sup> Runx2 plays a central role in the BMP-2–induced transdifferentiation of C2C12 cells at an early restriction point by diverting them from the myogenic pathway to the osteogenic pathway.<sup>89,98</sup> It has been found that the homeobox gene *Dlx5* is an upstream target of BMP-2 signaling and that it plays a pivotal role in stimulating the downstream osteogenic master transcription factor Runx2.<sup>90</sup> In turn, Runx2 acts simultaneously or sequentially to induce the expression of bone-specific genes that represent BMP-2–induced osteogenic transdifferentiation. However, inhibition of BMP signaling was shown to disrupt the ability of RUNX2 to stimulate osteoblast differentiation and transactivate an osteocalcin gene promoter–luciferase reporter in C3H10T1/2 cells.<sup>99</sup> In conclusion, we can state that the JNK, ERK, and p38 MAPK pathways contribute considerably to all TGF- $\beta$ –induced responses, but further characterization is needed to assess their importance in relation to the Smad-dependent and other TGF- $\beta$ –induced signaling pathways.

BMP-2 has been reported to induce *Osterix* (*Osx*) expression in mouse progenitor cells and chondrocytes.<sup>100,101</sup> Moreover, BMP-2–induced *Osx* expression is mediated by *Dlx5* but is independent of Runx2.<sup>102</sup> In the bone microenvironment, BMPs act in conjunction with other growth factors. Celil et al. identified the involvement of BMP-2 and IGF-I in mediating *Osx* expression in human mesenchymal stem cells (hMSCs).<sup>103</sup> The BMP-2–induced effect on *Osx* expression was mediated via p38 but not via Erk. Under osteogenic culture conditions, both Erk and p38 were involved in mediating *Osx* expression.<sup>103</sup>

In the past several years, ubiquitin-mediated proteasomal degradation has been implicated in the regulation of BMP-2 and TGF- $\beta$  signaling pathways in various cell types.<sup>104,105</sup> Recently, several studies highlighted the importance of this mechanism in regulating the *in vivo* effects of TGF- $\beta$  (Fig. 3).<sup>106,107</sup> Dupont et al. redefined the role of a previously identified Smad 1 ubiquitin ligase, Smurf-1. The absence of Smurf-1 causes the accumulation of MEK kinase 2 (MEKK2), resulting in activation of JNK, an event that is both necessary and sufficient for BMP sensitiza-

tion in osteoblasts.<sup>106</sup> Sapkota et al. found that MAPK-dependent linker phosphorylation of Smad 1 allows recognition by Smurf-1 and restricts Smad 1 activity, controlling BMP signaling during mouse osteoblast differentiation.<sup>108</sup> BMP also triggers linker phosphorylation, creating feedback control. Yamashita et al. identified and characterized a novel Smad 4 ubiquitin ligase, Ectoderm (Ecto), and provided convincing evidence that Ecto represents the elusive determinant of ectoderm formation, acting as a critical inhibitor of all Smad-dependent TGF- $\beta$  signaling during vertebrate development.<sup>107</sup>

Recently, Tsuji et al. found that BMP-2 is a necessary component of the signaling cascade that governs fracture repair.<sup>109</sup> Mice lacking the ability to produce BMP-2 in their limb bones have spontaneous fractures that do not resolve with time and the earliest steps of fracture healing seem to be blocked. The presence of other osteogenic stimuli cannot compensate for the absence of BMP-2, identifying BMP-2 as an endogenous mediator necessary for fracture repair.

### 3. Hedgehog Signaling

Osteoblast progenitors can first be identified within the inner perichondrium adjacent to, and coincident with, the first appearance of hypertrophic chondrocytes. This tight linkage reflects a crucial role for *Ihh* signaling (Fig. 1).<sup>110</sup> *Ihh* is produced by prehypertrophic chondrocytes and appears to act directly on perichondrially located osteoblast progenitors to specify the osteoblast precursors.<sup>111,112</sup> The failure of activation of Runx2, a crucial early determinant of the osteoblast lineage, indicates that hedgehog (Hh) signaling acts to initiate an osteogenic program.<sup>113</sup> Furthermore, Hh activates osteoblast development in a variety of mesenchymal and skeletogenic cell types *in vitro*.<sup>111,114,115</sup> Genetic manipulation of smoothed (Smo), which encodes an obligatory component of the Hh signaling pathway, has revealed that cells devoid of Smo, and hence Hh signaling, fail to undergo osteoblast differentiation.<sup>111</sup> Although *Ihh* signaling plays the crucial role in regulating the temporal and spatial program of early osteoblast commitment, *Ihh* does not play an ongoing role beyond this stage.<sup>6</sup> When Smo activity is

removed in *Osx1*<sup>+</sup> osteoblast precursors, normal bone-secreting *Oc*<sup>High</sup> osteoblasts are generated, and the endochondral skeleton at birth is indistinguishable from wild type.<sup>6</sup> Whether this is also true in the adult is currently under investigation.

The interaction between Hh and Wnt signaling is probably complex. It has been demonstrated that nuclear localization of  $\beta$ -catenin as well as expression of target genes for the Wnt canonical pathway were abolished in the perichondrium in *Ihh*<sup>-/-</sup> embryos.<sup>68</sup> This could, among other possibilities, be due to the downregulation of Wnt expression in the absence of Hh signaling. Indeed, expression of *Wnt9a* and *Wnt7b* was either reduced or abolished in the perichondrium in *Ihh*<sup>-/-</sup> embryos. In addition, both genes were induced by Hh signaling in C3H10T1/2 cells (Fig. 3). Alternatively, the Hh and Wnt signaling pathways could intersect intracellularly via common regulators such as Suppressor of fused [*Su(fu)*]<sup>116</sup> and GSK3.<sup>117,118</sup> Other pathways in addition to canonical Wnt signaling also contribute to Hh-induced osteogenesis. Of note, some groups reported that Hh-induced osteogenesis in C3H10T1/2 cells required BMP signaling.<sup>119,120</sup>

#### 4. FGF Signaling

The fibroblast growth factors (FGFs) are a family of secreted polypeptides that act through four related tyrosine kinase receptors (*Fgfr1*–*Fgfr4*) to regulate a plethora of developmental processes, and they are critical for the control of endochondral and intramembranous ossification.<sup>121</sup> Human diseases that manifest the precocious osseous obliteration of sutures, known as craniosynostosis, often result from gain-of-function mutations in FGF receptors 1–3 (Fig 3).<sup>122,123</sup> *Fgfrs* 1–3 are expressed in the developing and mature skeleton in patterns suggestive of both unique and redundant function.<sup>121</sup> In the developing growth plate, both *Fgfr1* and *Fgfr2* are expressed in condensing mesenchyme that will give rise to cartilage. *Fgfr2* remains expressed in reserve chondrocytes and appears to be downregulated in proliferating chondrocytes, whereas *Fgfr1* is expressed in hypertrophic chondrocytes. Later in development, *Fgfr1* and *Fgfr2* are both expressed in the perichondrium and periosteum, tissues that give rise to osteoblasts

and cortical bone. In contrast to *Fgfr1* and *Fgfr2*, *Fgfr3* is prominently expressed in proliferating chondrocytes where it regulates cell growth and differentiation<sup>124</sup> and in differentiated osteoblasts where it regulates bone density and cortical thickness.<sup>125,126</sup> Mutations in *Fgfrs* account for many of the craniosynostosis and chondrodysplasia syndromes in humans.<sup>121,127,128</sup>

Embryos lacking *Fgfr1* (*Fgfr1*<sup>-/-</sup>) die shortly after gastrulation,<sup>129</sup> necessitating a conditional knockout approach to address function later in development. Hypomorphic alleles of *Fgfr1* or conditional inactivation of *Fgfr1* prior to limb bud initiation affects digital patterning and the formation of some skeletal elements.<sup>130–132</sup> *Fgfr1* signaling in the osteoprogenitor cell normally acts to stimulate differentiation, whereas it functions to suppress differentiation in differentiated osteoblasts. Thus, *Fgfr1* signaling has stage-specific effects on osteoblast maturation.<sup>133</sup>

The FGF ligands that signal to *Fgfr1* in osteoblasts are not known; however, three FGFs (FGFs 2, 9, and 18) are likely candidates for this role. FGF9 and FGF18 are expressed in the perichondrium/periosteum, and mice lacking these FGFs show delayed ossification during midgestation skeletogenesis.<sup>134–136</sup> FGF2 is expressed in periosteal cells and osteoblasts,<sup>137,138</sup> and adult *FGF2*<sup>-/-</sup> mice showed a loss of trabecular bone volume; however, no skeletal dysmorphology was reported in neonatal *FGF2*<sup>-/-</sup> mice.<sup>139</sup> These observations suggest that FGFs 2, 9, and 18 may act alone or redundantly to regulate osteoblast activity and physiology, and that FGFs 9 and 18 may constitute the predominant signals during embryonic development, whereas FGF2 may be more important during postnatal stages. Consistent with a role for FGF2 in more differentiated osteoblasts, bone marrow stromal cultures from *FGF2*<sup>-/-</sup> mice showed a significantly decreased ability to mineralize in vitro.<sup>139</sup> It has been demonstrated that *Runx2* is phosphorylated and activated by FGF2 via the MAPK pathway and suggests that FGF2 plays an important role in regulation of *Runx2* function and bone formation.<sup>140</sup>

*Fgfr2*<sup>-/-</sup> mice die at embryonic day 10.5 (E10.5), prior to skeletal development.<sup>141</sup> The contribution of *Fgfr2* signaling to skeletal development has been clarified to some extent by using splice form–

specific knockouts and conditional gene deletion approaches in mice. These studies demonstrated that *Fgfr2* positively regulates bone growth and the anabolic function of osteoblasts. The resulting phenotype of mice lacking mesenchymal *Fgfr2* included skeletal dwarfism, decreased bone density, incomplete formation of the dorsal vertebrae, and tarsal joint fusion.<sup>142,143</sup> Alternative splicing of *Fgfr2* is tissue specific, resulting in epithelial variants (b splice forms) and mesenchymal variants (c splice forms).<sup>144–146</sup> Ligand-binding studies demonstrate that mesenchymally expressed ligands such as FGF7 and 10 activate *Fgfr2b*, whereas FGF2, 4, 6, 8, and 9 activate *Fgfr2c*.<sup>147,148</sup>

As adults, *Fgfr3*<sup>-/-</sup> mice were osteopenic, suggesting a role for *Fgfr3* signaling in differentiated, *Fgfr3*-expressing osteoblasts.<sup>125</sup> Mice lacking either FGF18 or *Fgfr3* exhibited expanded zones of proliferating and hypertrophic chondrocytes and increased chondrocyte proliferation, differentiation, and Indian hedgehog signaling. These data suggest that FGF18 acts as a physiological ligand for *Fgfr3*.<sup>135</sup> In addition, FGF18<sup>-/-</sup> mice had decreased endochondral and intramembranous bone formation, suggesting that FGF18 positively regulates osteogenesis and/or osteoblast function independent of *Fgfr3*.<sup>135</sup>

Two lines of evidence indicate that engrailed 1 (*En1*) regulates signaling mediated by *Fgfrs*. First, the activation ERK, normally restricted to the mature endosteal osteoblasts of wild-type calvarial bone, is severely impeded in *En1* mutants. Second, *En1* ablation results in loss of the FGF target gene sprouty homolog 2 (*Drosophila*) (*Spry2*) in ectoperiosteal osteoblasts. Furthermore, *En1* may regulate alternative FGF-signaling effectors known to affect osteoblast differentiation, such as p38 MAPK or PKC.<sup>149–151</sup> A precise temporal and spatial delineation of these intracellular pathways will enable a better understanding of how osteoblastic differentiation is coordinated by *En1* and FGFs.

To study the effects of growth factors on hMSC, Kratchmarova et al. tested the effects of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), FGF, and nerve growth factor (NGF) on cellular responses and observed that EGF and PDGF elicited the strongest responses.<sup>152</sup> They also found that the differentiation of human mesenchymal stem cells into

bone-forming cells is stimulated by EGF but not PDGF. Mass spectrometry-based proteomics analysis demonstrated that more than 90% of these signaling proteins were used by both ligands, whereas the phosphatidylinositol 3-kinase (PI3K) pathway was exclusively activated by PDGF, implicating it as a possible control point. Indeed, chemical inhibition of PI3K in PDGF-stimulated cells removed the differential effect of the two growth factors, bestowing full differentiation effects onto PDGF.

## 5. Ephrin Signaling

Ephrins have the capacity for bidirectional signaling. That is, when a cell expressing an ephrin receptor contacts a cell expressing an ephrin ligand, signals are transduced into both the ephrin receptor-expressing cell (forward signaling) and the ephrin ligand-expressing cell (reverse signaling). There are two classes of ephrins, the B class (ephrin B1 to B3) are ligands for EphB tyrosine kinase receptors (B1 to B6), whereas class A ephrins (A1 to A5) are ligands for glycosylphosphatidylinositol (GPI)-anchored EphA receptors (A1 to A10).<sup>153</sup> In bone biology, ephrinB and EphB receptors control patterning of the developing skeleton,<sup>154</sup> and disruption of ephrin signaling is implicated in a syndrome called craniofrontonasal syndrome [CFNS (MIM 304110)].<sup>155</sup> Zhao et al. now suggest that ephrin signaling is critical to the two-way communication between osteoclasts and osteoblasts.<sup>156</sup> This bidirectional signaling is mediated by the transmembrane ephrinB2 ligand in osteoclasts and EphB4, a tyrosine kinase receptor, in osteoblasts (Fig. 3). Using osteoblast-osteoclast coculture assays, as well as loss- and gain-of-function studies in vitro, the authors demonstrated that reverse signaling from EphB4 in osteoblasts to ephrinB2 in osteoclast progenitors leads to the inhibition of osteoclast differentiation. On the other hand, EphB4 expression is constitutive and the forward signaling through EphB4 induces osteogenic regulatory factors, such as *Dlx5*, *Osx*, and *Runx2*, in calvarial osteoblasts, suggesting that EphB4 is at the top of the regulatory cascade during osteoblast differentiation. Zhao et al. demonstrated that forward signaling between the extracellular domains of ephrinB2

and EphB4 in osteoblasts stimulates their differentiation, a process that may be dependent on ras homolog gene family, member A (RhoA), inactivation in osteoblasts. Expressing active RhoA in osteoblasts may block the ability of ephrinB to promote osteoblast differentiation. By contrast, McBeath et al., using human mesenchymal stem cells, suggest that active RhoA enhances osteoblast differentiation (Fig. 3).<sup>157</sup> Therefore, the involvement of RhoA in EphB4 forward signaling will need to be confirmed by future pharmacological or genetic studies. This study establishes the concept that ephrin-Eph signaling contributes to bone homeostasis.

## 6. PTH Signaling

PTH can be used pharmacologically to build bone. PTH has been used as an effective treatment for osteoporosis due to the fact that PTH exerts either a catabolic or anabolic effect, depending on the method of administration. New insight into the structure of PTH, PTHrP, and the PTH/PTHrP receptor has stimulated the field of calcium and bone biology and posed new questions about the role of PTH and PTHrP.<sup>158</sup> Currently, PTH(1–34) is the only FDA-approved anabolic agent for the treatment of osteoporosis. Pettway et al. aimed to gain an understanding of the mechanism allowing PTH(1–34) to work as a treatment.<sup>159</sup> They found that three weeks of PTH treatment on bone marrow stromal cells implanted in immunocompromised mice resulted in an anabolic response. PTH-treated ossicles showed increased donor cell proliferation. When zoledronic acid was combined with PTH, PTH-induced proliferation was reduced without reducing bone volume, indicating that combining PTH and bisphosphonate therapy warrants further investigation.

The PTH signaling pathway involves  $G\alpha$  proteins that bind to the PTH receptor.<sup>160</sup>  $G\alpha$  proteins activate cAMP, which binds the regulatory subunits of protein kinase A (PKA) to release the catalytic subunits of the enzyme. PKA then phosphorylates proteins to cause changes in the structure and function of Runx2. Other  $G\alpha$  proteins on the PTH receptor interact with phospholipase C (PLC) to activate PKC.

It was recently found that there is a link between PTH and noncanonical Wnt4 signaling.<sup>161</sup> Wnt4 signaling in response to PTH implicates cross talk of multiple signaling pathways, showing PTH's anabolic effect in bone. PTH stimulation of Wnt4 is primarily through the PKA pathway. Swarthout et al. found that PTH regulates genes and proteins through the PKA pathway,<sup>162</sup> which was supported in vivo by microarray data from peptides PTH(1–34), PTH(1–31), and PTH(3–34).<sup>163</sup> Guo et al. generated mice with mutant PTH/PTHrP receptor that does not activate PLC.<sup>164</sup> The mice exhibited abnormalities in embryonic endochondral bone development including delayed ossification and increased chondrocyte proliferation, indicating that PLC signaling through the PTH/PTHrP receptor slows proliferation and promotes differentiation of chondrocytes.

## 7. Sympathetic Signaling

Neural control of bone metabolism, both trophic and atrophic, has been suggested by numerous experimental and clinical observations. Osteoblasts have been reported to express receptors for several neuropeptides, suggesting that they could indeed integrate multiple neuronal signals.<sup>165</sup> Immunolabeling studies have revealed a close association between glutamate-, catecholamine-, or peptide-containing nerve fibers and osteoblasts or osteoclasts in the endosteum.<sup>166</sup> Blockade of glutamate receptors was reported to reduce the DNA-binding activity and expression of Runx2 in cultured osteoblasts.<sup>167</sup> The effect of the sympathetic nervous system (SNS) on bone formation has only recently been elucidated using genetic models.<sup>168</sup> These studies revealed that leptin induced bone loss through SNS-derived signals originating in the ventromedial hypothalamic nuclei.<sup>166</sup> Sato et al. found that leptin- or SNS-mediated inhibition of bone formation was abolished in neuromedin U null mice, which show high bone mass due to an increase in bone formation.<sup>169</sup>

Fu et al. indicated that an important, new regulator of bone remodeling is the circadian cycle.<sup>170</sup> The model that emerges from the results of Fu et al. suggests that signaling by  $\beta$ 2-adrenergic receptors first activates the transcription factor

cAMP responsive element binding protein (CREB) (Fig. 3). CREB in turn stimulates expression of clock genes, which mediate the antiproliferative function by inhibiting G1 cyclin expression, and activator protein 1 (AP1) genes, which stimulate proliferation of osteoblasts. Fu et al.'s work provided evidence that the inhibition of osteoblast proliferation by clock proteins is the dominant effect. Pharmacological or genetic ablation of adrenergic neurotransmission indicates that norepinephrine (NE) signaling controls granulocyte-colony stimulating factor (G-CSF)-induced osteoblast suppression.<sup>171</sup> Based on studies describing leptin-mediated neuronal control of osteoblast function<sup>166,172</sup> and the fact that leptin and G-CSF receptors display a high degree of homology,<sup>173</sup> it has been proposed that G-CSF signals directly in the hypothalamus through the leptin receptor.<sup>171</sup>

Sequence-specific DNA-binding proteins are frequently encoded by gene families. Such proteins display highly conserved DNA-binding properties, yet are assumed to retain promoter selectivity. Yamamoto et al. addressed the factor-specificity issue with his update on the varied roles of the glucocorticoid receptor (GR).<sup>174</sup> They presented evidence that two ligands, the small nuclear hormone and the larger DNA molecule, act to direct the activities of GR. Most fascinating were the data showing that different hormone ligands could affect promoter selectivity.<sup>174</sup>

## 8. Osteocyte Signaling

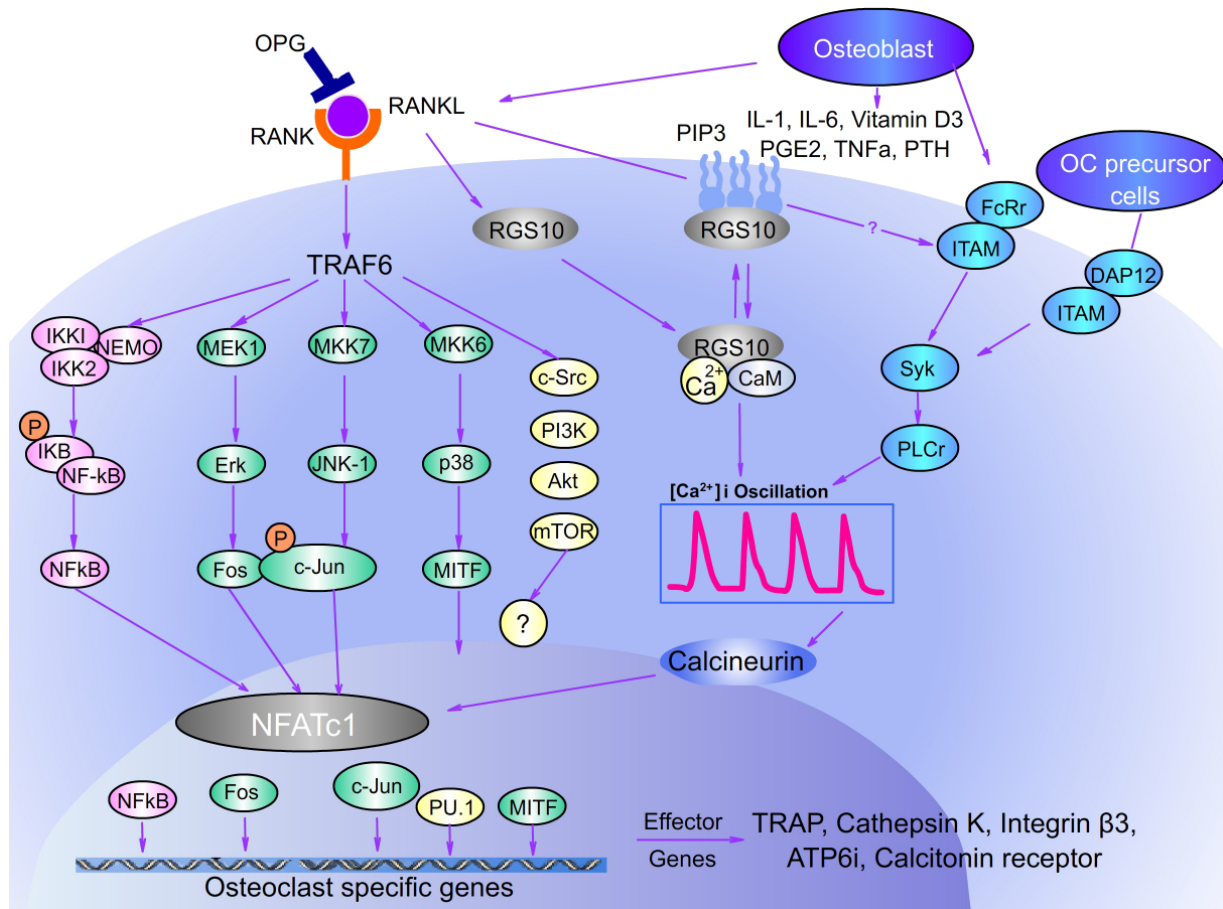
A recent study has shed light on the role of osteocytes in bone remodeling: osteocytes deep inside the bone serve an important function in regulating the activities of osteoclasts and osteoblasts at the bone surface. Tatsumi et al. ablated osteocytes *in vivo* using diphtheria toxin and found that mice exhibited osteoporosis characterized by fragile bone with intracortical porosity and microfractures, osteoblastic dysfunction, and trabecular bone loss.<sup>175</sup> They also found that osteocytes play a role in bone homeostasis by sending signals to osteoclasts to maintain bone mass or initiate bone loss. Osteocytes may therefore comprise an important target for the development of diagnostics and therapeutics for bone disease, as exemplified by osteoporosis.

## B. Osteoclast Signaling

Osteoblasts, chondrocytes, and their mineralized matrices, together with stromal and endothelial cells, provide the microenvironment for homing of osteoclast precursor cells. Osteoblasts/stromal cells produce cytokines including M-CSF and RANKL that induce and modulate growth and differentiation of the precursors to mature osteoclasts. M-CSF binds to its receptor, colony stimulating factor 1 receptor (c-Fms), present on osteoclast precursors, providing the signals for macrophage survival and proliferation. Intracellular RANK signaling by its interaction with RANKL induces recruitment and activation of cytoplasmic tumor necrosis factor receptor-associated factors (TRAFs), leading to the activation of multiple signaling cascades such as MAP, NF- $\kappa$ B, Rous sarcoma oncogene (Src), and Akt (Fig. 4).<sup>176</sup> Of the several TRAF proteins that have been described in conjunction with RANKL, including TRAF-1, -2, -5, and -6, TRAF-6 is indeed an essential adaptor required for RANK-associated signaling. It is necessary for RANK-induced NF- $\kappa$ B activation and *in vitro* osteoclastogenesis and the deletion of TRAF-6 leads to osteopetrosis. By contrast, the contributions of TRAF-2 and TRAF-5 to osteoclastogenesis are minor.<sup>177,178</sup> Osteoclast signaling molecules also play a role in bone disease. Macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) has been found to be an important osteoclast stimulatory factor in multiple myeloma.<sup>179,180</sup> MIP-1 $\alpha$  enhances osteoclast formation induced by IL-6, PTHrP, and RANKL, all of which are implicated in myeloma bone disease.

### 1. M-CSF

After commitment to the OC lineage, the entrance of mononuclear cells into the early preOC pathway is a response to M-CSF (Fig. 2). M-CSF is required for the proliferation, differentiation, and survival of hematopoietic cells in the monocytic lineage.<sup>20,181,182</sup> Treatment with M-CSF alone results in the formation of macrophagelike cells. It also contributes to their differentiation and regulates the cytoskeletal changes that accompany bone resorption. The absence of a functional M-CSF in the *op/op* mouse causes not only a



**FIGURE 4.** RANK signaling network of osteoclast differentiation. Binding of RANKL to its receptor RANK induces various intracellular signaling cascades through TRAF-6, such as MAP (ERK, p38, JNK), NF- $\kappa$ B, Src, and NFATc1. Several transcription factors have been found crucial for osteoclast differentiation downstream of RANKL/RANK signaling such as NF- $\kappa$ B, NFATc1, c-Fos, c-Jun, and Mitf. RGS10 functions downstream of RANKL to regulate calcium oscillations and NFATc1 expression. These transcription factors are essential for differentiation of mononuclear precursors into multinucleated osteoclasts and are essential for the transcription of osteoclast-specific genes.

macrophage deficiency, but also a lack of OCs, resulting in an osteopetrotic phenotype.<sup>183,184</sup> These deficiencies can be restored with injections of M-CSF.<sup>183,185</sup> Op/op marrow stromal cells can support the differentiation and proliferation of OC progenitors from inoculated stem cells, as shown by experimental evidence that M-CSF is not essential for the early stages of OC development.<sup>186</sup> Furthermore, M-CSF, although necessary for entry of precursors into the early preOC pathway, was found to inhibit osteoclastogenesis at high doses.

Binding of M-CSF to its receptor c-Fms recruits adapter proteins and cytosolic kinases, thereby activating a variety of intracellular signals.

Tyrosines 559 and 807 in the cytoplasmic tail of c-Fms play distinct roles in OC differentiation and function. Changes in M-CSF-receptor expression appear to modulate the final lineage selection of the pluripotent monoblastic progenitor.<sup>187</sup> M-CSF-induced genes are necessary for a direct response to RANKL and interleukins. Cappellen et al. reported that M-CSF induced the receptor for RANKL (RANK) and other RANK/NF- $\kappa$ B pathway components [TRAF2A, PI3-kinase, MEKK3, and receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIPK1)], providing a molecular explanation for the synergy of M-CSF and RANKL. Interleukins, interferons, and their receptors [IL-1 $\alpha$ , IL-18, interferon (IFN)- $\beta$ ,

IL-11R $\alpha$ 2, IL-6/11R gp130, IFN $\gamma$ R] were also induced by M-CSF.<sup>188</sup> Furthermore, M-CSF acts as a survival factor for the OC precursors through Bcl-X(L)-induced inhibition of caspase-9 activation, which inhibits apoptosis of OC precursors.<sup>189</sup> Akiyama et al. found that ubiquitylation of the B-cell leukemia/lymphoma 2 (Bcl-2) family member BCL2-like 11 (apoptosis facilitator) (Bim) regulates apoptosis in osteoclasts and bone marrow cells from Bim-null mice show prolonged survival in the absence of M-CSF, but the bone-resorbing activity of osteoclasts was reduced.<sup>190</sup>

## 2. RANKL

RANKL, also called TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), or OC differentiation factor (ODF), is a member of the TNF family. RANK<sup>-/-</sup> mice demonstrated profound osteopetrosis resulting from an apparent block in OC differentiation, revealing that RANK provides critical signals necessary for OC differentiation.<sup>191</sup> RANK connects to its extracellular signal factor RANKL and induces recruitment and activation of its adaptor TRAF6, leading to multiple downstream cascades. There are six known main signal pathways: MAP (ERK, p38, and JNK), NF- $\kappa$ B, Src, and Akt, shown by recent research results. OPG, encoded by Tnfrsf11b, as a decoy receptor of RANKL, is also expressed by OCs and preOCs. It is secreted and competes with RANK by binding to RANKL. Overexpression of OPG causes osteoclast-deficient osteopetrosis, while deletion of OPG leads to osteoporosis due to increased OC number and activity. Autosomal recessive osteopetrosis in humans involves mutations in the RANKL gene, which result in a lack of osteoclasts.<sup>192</sup>

The role of RANKL in rheumatoid arthritis has recently been elucidated at sites of pannus invasion into bone. Pettit et al. found that RANKL protein expression and RANK-expressing osteoclast precursor cells were confined to sites of osteoclast-mediated erosion at the pannus-bone interface and subchondral bone erosion.<sup>193</sup> OPG protein expression, on the other hand, was limited at sites of bone erosion. These results implicate RANKL in the pathogenesis of rheumatoid arthritis and show that it contributes to the generation

of a local environment that favors osteoclast differentiation and activity.

## 3. Src

Src is a member of a family of nine nonreceptor tyrosine kinases (NRTKs) that associate with the cytoplasmic surface of cellular membranes.<sup>194</sup> Deletion of the gene encoding c-Src produces an osteopetrotic skeletal phenotype that is the consequence of the inability of the mature OC to efficiently resorb bone. Src<sup>-/-</sup> OCs exhibit reduced motility and abnormal organization of the apical secretory domain (the ruffled border) and attachment-related cytoskeletal elements that are necessary for bone resorption. A key function of Src in OCs is to promote the rapid assembly and disassembly of the podosomes, the specialized integrin-based attachment structures of OCs and other highly motile cells.<sup>195</sup> Dynamin, a GTPase, colocalizes with Casitas B-lineage lymphoma (Cbl), an adaptor protein, in these actin-rich podosomes and forms a complex in osteoclasts, which is decreased by Src tyrosine kinase activity.<sup>196</sup> Phosphorylation of Cbl and the subsequent recruitment and activation of PI3K may be critical signaling events downstream of Src in osteoclasts.<sup>197,198</sup> In addition to Cbl, Src forms a complex with protein tyrosine kinase 2 (Pyk2). Src osteoclastic bone resorption requires both c-Src kinase activity and the targeting of Src kinase by Pyk2.<sup>197</sup> Src<sup>-/-</sup> osteoclasts were examined to determine the specific functions of Src in the organization and dynamics of podosomes.<sup>199</sup> Src was found to regulate the formation, structure, life span, and rate of actin polymerization in podosomes and in the actin cloud. Kinase activity and the SH2 or SH3 binding domain are required for Src to restore normal podosome organization and dynamics. The absence of Src affects the bone-resorbing activity of mature OCs, but does not affect OC formation.<sup>200,201</sup> In fact, the number of OCs in bones of Src<sup>-/-</sup> mice is more than twice that in normal mice, suggesting that in vivo OC differentiation and/or survival is enhanced in the absence of Src. Recently, it was reported that c-Src kinase activity, not only on the plasma membrane but also within mitochondria where it phosphorylates cytochrome c oxidase (Cox), is essential for the regulation of osteoclastic bone resorption.<sup>202</sup>

Of the several signaling mechanisms that are activated downstream of RANK, only the sequential activation of PI3K and Akt is known to involve Src and the Cbl proteins.

Besides these pathways, c-Src interaction with TRAF-6 was found to enhance the kinase activity of c-Src, activating the Akt/PI3K pathway, skeletal rearrangement, and cell motility. The molecular adapter growth factor receptor-bound protein 1 (Grb-1)-associated binder-2 (Gab2) was also found recently to associate with RANK and mediate RANK-induced activation of NF- $\kappa$ B, Akt, and Jnk (Fig. 4).<sup>203</sup> Src homology 2-containing inositol-5-phosphatase (SHIP) was shown in negatively regulated osteoclast formation and function. SHIP blunts PI3K signaling by dephosphorylating its major substrate, phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>). SHIP<sup>-/-</sup> mice show a twofold increase in osteoclast number, due to the prolonged life span of these cells and to hypersensitivity of precursors to M-CSF and RANKL.<sup>204</sup> Similar to pagetic osteoclasts, SHIP<sup>-/-</sup> osteoclasts are enlarged and exhibit enhanced resorptive activity, and serum levels of interleukin-6 are markedly increased.

#### 4. NF- $\kappa$ B

NF- dimers are normally rendered inactive in the cytosol. Activation of this transcription factor is a consequence of phosphorylation of its inhibitory protein, I $\kappa$ B proteins (I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , I $\kappa$ B $\gamma$ , Bcl-3), by an upstream kinase complex termed I $\kappa$ B kinase (IKK), resulting in the release and translocation of NF- $\kappa$ B to the nuclear compartment. The NF- $\kappa$ B pathway is relevant for RANKL-RANK-regulated osteoclast development and osteoclast function not only in mice, but also in humans as can be seen from patients with X-linked osteopetrosis, lymphedema, anhidrotic ectodermal dysplasia, and immunodeficiency (OLEDA-ID syndrome), which carry a X420W point mutation in IKK $\gamma$  and have osteopetrosis.<sup>205</sup> Besides, NF- $\kappa$ B, TRAF proteins also activate the upstream MAP kinase (MAPK) MEKK1, which is followed by activation of a large number of MAP kinase mediators, eventually leading to induction of three distinct MAP kinase families, namely, JNK/c-Jun, ERK1/2,

and p38 kinases. Dominant-negative forms of various MAP kinases and selective inhibitors of the MAP kinase pathways inhibited osteoclastogenesis or reduced osteoclast survival.<sup>206-208</sup> Activated p38-MAPKs downstream of RANK can directly phosphorylate signal transducer and activator of transcription 1 (STAT1) and hence control expression of a variety of target genes.<sup>209</sup> JNKs and their direct upstream kinase MKK7 have been shown to be involved in osteoclastogenesis through in vitro cell culture.<sup>210,211</sup> JNK activation facilitates the phosphorylation of c-Jun and increases its transcriptional activity, leading to AP-1 activation. ERK, which activates the other component of AP-1, c-Fos, is also activated on RANK stimulation. Inhibition of MEKs (ERK kinases) by PD98059 or U0126 does not, however, attenuate osteoclast differentiation, but rather increases osteoclastogenesis.

Expression of IL-1 is regulated by NF- $\kappa$ B and IL-1 mediates bone resorption in a variety of diseases affecting bone. TNF induces IL-1 expression and activates c-Fos in osteoclast formation. Yao et al. found that IL-1 is capable of inducing osteoclast formation directly from osteoclast precursors overexpressing c-Fos without addition of cytokines.<sup>212</sup> IL-1 was secreted from osteoclast precursors on bone, stimulated by bone matrix proteins dentin sialoprotein and osteopontin, to induce c-Fos-mediated differentiation, which was more prevalent in eroding inflamed joints. These results indicate that osteoclast progenitors expressing c-Fos may interact with bone matrix to produce IL-1 by an autocrine mechanism and induce their differentiation into osteoclasts. McHugh et al. have proposed the idea that certain osteoclast genes and transcriptional pathways are induced by interaction of osteoclast precursors with specific components of the bone matrix.<sup>213</sup> Xing et al. found that NF- $\kappa$ B p50 or p52 is required in osteoclasts and their precursors for IL-1-mediated bone resorption.<sup>214</sup>

#### 5. ITAM-Dependent Costimulatory Signals

Most recently, Koga et al. reported that mice lacking immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptors, Fc receptor common  $\gamma$  subunit (FcR $\gamma$ ) and DNAX-activating



protein (DAP) 12, exhibit severe osteopetrosis owing to impaired osteoclast differentiation. In osteoclast precursor cells, FcR $\gamma$  and DAP12 associate with multiple immunoreceptors and activate calcium signaling through PLC $\gamma$ . Thus, ITAM-dependent costimulatory signals activated by multiple immunoreceptors are essential for the maintenance of bone homeostasis (Fig. 4).<sup>215</sup> However, in ITAM-mediated costimulatory signaling, the ligands for the Ig-like receptors are not identified. The molecules that connect RANK with ITAM signaling are also unknown. DAP12<sup>-/-</sup> FcR $\gamma$ <sup>-/-</sup> bone marrow cells show impaired phosphorylation of spleen tyrosine kinase (Syk), in addition to failure to differentiate into multinucleated osteoclasts or resorb bone.<sup>216</sup> Syk<sup>-/-</sup> progenitors are also defective in osteoclast development and bone resorption, indicating that recruitment of Syk to phosphorylated ITAMs is critical for osteoclastogenesis. SH3-domain binding protein 2 (Sh3bp2) mutation plays a role in “cherubism” mice through the Syk pathway.<sup>217</sup> Sh3bp2 mutant myeloid cells show increased responses to M-CSF and RANKL stimulation through increased Syk phosphorylation/activation, forming unusually large, hyperactive osteoclasts, reflecting Sh3bp2 gain of function and leading to trabecular bone loss, TNF- $\alpha$ -dependent inflammation, and cortical bone erosion.

## 6. RGS10 and RGS12

Despite the importance of the calcium-nuclear factor of activated T-cells (NFAT) pathway, it remained unclear how RANKL activates calcium signals leading to the induction of NFAT, cytoplasmic, calcineurin-dependent 1 (NFATc1). Recent work in our lab reveals more details of the RANKL-induced signaling pathway controlling calcium oscillations and NFATc1 activation. The regulator of G-protein signaling (RGS) proteins are a family of 21 proteins, all containing the RGS domain. We found that RGS10A is important in osteoclast signaling.<sup>218</sup> This isoform of RGS10 is specifically expressed in human osteoclasts and RNAi silencing of RGS10A blocked calcium oscillations, NFATc1 expression, and osteoclast terminal differentiation. Target components of RGS10A are different from those of RGS12;

RGS10A interacts with calmodulin while RGS12 interacts with the calcium-sensing receptor. We also found that RGS10A acts upstream of calcineurin in the RANKL-calcium oscillation-calcineurin-NFATc1 pathway. The specificity of RGS10A as a key component in the osteoclast differentiation signaling pathway makes it an excellent target for potential therapies.

To further define the role of RGS10 in osteoclast signaling, we went on to generate RGS10-deficient mice, which exhibited severe osteopetrosis.<sup>219</sup> We found that RGS10 competitively interacts with the calcium/calmodulin complex and PIP<sub>3</sub> in a calcium-dependent manner to mediate PLC $\gamma$  activation and calcium oscillations. Our results provide *in vivo* evidence that RGS10 specifically regulates the RANKL-evoked RGS10/calmodulin-calcium oscillation-calcineurin-NFATc1 signaling pathway in osteoclast differentiation. Based on our data from this study, we proposed an RGS10 working model (Fig. 4): RANKL mediates DAP12 and FcR $\gamma$ , the membrane adaptor molecules that contain an ITAM motif and that activate PLC $\gamma$ . PLC $\gamma$  hydrolyzes PIP<sub>2</sub> to generate inositol 3-phosphate (IP<sub>3</sub>). IP<sub>3</sub> then triggers a transient initial release of calcium from intracellular stores. Intracellular calcium release allows an increase in intracellular calcium to reach peak concentration and leads to formation of the calcium/calmodulin complex. The calcium/calmodulin complex competes for the PIP<sub>3</sub>-binding site on RGS10 and frees the bound PIP<sub>3</sub>. Once the calcium concentration reaches its peak formation, intracellular calcium begins to reload into the endoplasmic reticulum (ER) in the absence of further PLC $\gamma$  activation, and the combination of calcium reloading in the ER and binding to calmodulin causes the calcium concentration to decrease. The calcium/calmodulin complex dissociates from RGS10 at the low calcium concentration. Free PIP<sub>3</sub> activates PLC $\gamma$  and then binds RGS10 again without calcium/calmodulin complex competition. PLC $\gamma$  activation triggers a release of calcium from intracellular stores by generating IP<sub>3</sub> to cause a second peak. This process continues to cycle, causing calcium oscillations. In this way, RGS10 mediates PLC $\gamma$  activation and calcium oscillations through its calcium-dependent dual interaction with calcium/calmodulin and PIP<sub>3</sub>. The RGS10-

mediated intracellular calcium oscillations activate calcineurin and NFATc1 expression for osteoclast terminal differentiation (Fig. 4).

In addition to our work on RGS10, we identified RGS12 as a new signaling protein in osteoclasts.<sup>220</sup> RNA interference (RNAi) silencing of RGS12 expression impaired phosphorylation of PLC $\gamma$  and blocked calcium oscillations, NFATc1 expression, and osteoclast differentiation. RGS12 was further found to directly interact with N-type calcium channels. Our results revealed that RGS12 is essential for RANKL-induced terminal differentiation of osteoclasts.

### **7. Cathepsin K Regulates Osteoclast Apoptosis and Senescence**

Cathepsin K was cloned from human osteoclastomas in our lab<sup>32</sup> and from rabbit and human osteoclasts in other labs.<sup>221–224</sup> The important role of cathepsin K in osteoclast function was first suggested by the finding of clinical research that mutations in this gene caused pycnodysostosis, a rare, autosomal, recessive, skeletal disorder caused by mutations in the cathepsin K gene at 1q21, which codes for cathepsin K protein, a lysosomal cysteine protease. Mutation in this gene affects the metabolism of the skeletal system, causing defects in bone resorption and bone remodeling.<sup>225,226</sup> Recently, we discovered a novel pycnodysostosis mouse model in the 129/Sv background that exhibited many characteristics of the human pycnodysostosis phenotype.<sup>227</sup> This model revealed that cathepsin K<sup>-/-</sup> osteoclasts lacked normal apoptosis and senescence and have reduced expression levels of p19, p53, and p21, leading to unusually high osteoclast numbers. Cathepsin K<sup>-/-</sup> osteoclasts did not begin to shrink or exhibit condensed nuclei, had little to no chromosome degradation, and survived through 36 hours, while no wild-type cells survived that period. Cathepsin K was associated with senescence in wild-type cells, which indicates that impaired senescence might be the major cause of the extraordinarily high number of osteoclasts in cathepsin K<sup>-/-</sup> mice. This is the first evidence of the role of cathepsin K in osteoclast apoptosis and senescence. The mechanism by which cathepsin K regulates osteoclast apoptosis and senescence is under characterization.

### **8. Vav3**

The regulation of osteoclast activation has remained unclear; however, significant progress has recently been made by Faccio et al.<sup>228</sup> They reported that Rho family guanine nucleotide exchange factor Vav3 is essential for stimulated osteoclast activation and bone density. Osteoclasts from Vav3-deficient mice show defective actin cytoskeleton organization, polarization, spreading, and resorptive activity resulting from impaired signaling downstream of the M-CSF receptor and  $\alpha_v\beta_3$  integrin. These mice also have increased bone mass and are protected from bone loss induced by systemic bone resorption stimuli such as parathyroid hormone or RANKL. In addition, Faccio et al. discovered a role for Syk tyrosine kinase as a crucial upstream regulator of Vav3 in osteoclasts.<sup>228</sup>

### **IV. OSTEOBLAST GENE TRANSCRIPTION: TRANSCRIPTION FACTORS THAT REGULATE OSTEOBLAST DIFFERENTIATION**

Transcription factors that regulate osteoblasts include a range of homeodomain proteins: the AP family members Jun, Fos, and Fra, Smads, C/EBP $\beta$  and C/EBP $\delta$ , lymphoid-enhancing factor (a Wnt effector), twist, activating transcription factor 4, Runx2, and osterix, the last three of which are considered master genes for osteoblast differentiation.

Commitment of mesenchymal stem cells (MSCs) to tissue-specific cell types is orchestrated by transcriptional regulators that serve as “master switches.” A central regulator of bone formation is the Runx2 transcription factor, which fulfills its role as a master regulatory switch through unique properties for mediating the temporal activation and/or repression of cell growth and phenotypic genes as osteoblasts progress through stages of differentiation.<sup>229</sup> Although Runx2 is essential for osteoblast differentiation, this differentiation program also requires other genes, such as osterix, which encode a transcription factor genetically “downstream” of Runx2.<sup>230</sup> Thus, multiple genes regulate Runx2 activity and the effectiveness of Runx2 in stimulating osteo-

blast formation. It is perhaps not surprising then that Runx2 expression in osteoblast precursors predates by several days the first evidence for osteoblast activity.

## A. Runx2

Runx2 is a member of the Runx family of transcription factors [previously known as acute myeloid leukemia (AML) factor, polyomavirus enhancer binding protein 2 (PEBP2), and core binding factor (CBF)]. The family members, Runx1 (also called PEBP2aB, CBFA2, and AML1), Runx2 (also called PEBP2aA, CBFA1, and AML3), and Runx3 (also called PEBP2aC, CBFA3, and AML2), are encoded by distinct unlinked genes but share a common DNA recognition motif (TGTGGT) and heterodimerize with the ubiquitous subunit CBF $\beta$  for stable DNA binding.<sup>231</sup> Their highly conserved DNA binding domain is homologous to that from the *Drosophila* segmentation gene runt.<sup>232</sup> In addition to the Runx DNA binding domain, Runx2 contains an active transactivation domain, rich in glutamine and alanine residues, and activates the Osteocalcin and Col1a1 genes.<sup>233–236</sup> Thus, Runx2 is an initial marker of the osteogenic cell lineage.

Runx2 is expressed in the thymus and testes (in T-lymphocytes tendon), is abundantly expressed in calcified cartilage and bone tissues, and is absent from the brain, heart, lung, gut, and liver.<sup>237–239</sup> The function of Runx2 in bone formation (Fig. 1) has been demonstrated by analyzing its role in regulating the expression of the principal osteoblast-specific genes and by studying Runx2 null mice.<sup>233,238,240</sup> Targeted disruption of Runx2 results in the complete lack of bone formation by osteoblasts, revealing that Runx2 is essential for both endochondral and membranous bone formation.<sup>238</sup> The haploinsufficiency of the Runx2 gene, which leads to cleidocranial dysplasia, a genetic disease in humans that is characterized by hypoplastic clavicles, large open spaces between the frontal and parietal bones of the skull, and other skeletal dysplasias, is caused by heterozygous mutations in the Runx2 gene.<sup>241</sup> Moreover, Runx2 is sufficient to induce osteoblast differentiation. This is true in cell culture, where forced expression of Runx2 in skin

fibroblasts leads to osteoblast-specific gene expression,<sup>233</sup> and in vivo, since ectopic expression of Runx2 leads to endochondral ossification in parts of the skeleton that would normally never ossify.<sup>242,243</sup> Importantly, Runx2 may function as an inhibitor of proliferation of progenitors, thus providing a mechanism for regulating the transition from growth to a postproliferative stage as a component of cellular commitment to the osteogenic lineage.<sup>244</sup> Thus, Runx2 may be expressed in early osteoprogenitors to induce a program of gene expression required for lineage determination and differentiation of mesenchymal cells. Finally, Runx2 is also required for osteoblast function beyond differentiation.<sup>245,246</sup> Overexpression of Runx2 inhibits osteocyte formation from osteoblasts, showing that Runx2 maintains the supply of osteoblasts (Fig. 1).<sup>247</sup> These functions, along with its role during hypertrophic chondrocyte differentiation and vascular invasion, identify Runx2 as the most pleiotropic regulator of skeletogenesis.<sup>230</sup> The literature now embraces the concept that Runx2 functions as a scaffold for the interaction with coregulatory proteins at subnuclear foci to provide an architectural basis for accommodating the requirements of biological control.

## B. Runx2 Upstream Proteins

### 1. *Msx2* and *Bapx1*

Msh homeobox 2 (*Msx2*), which encodes a homeobox-containing transcription factor, is expressed in osteoblasts during development. The role of *Msx2* during skull formation was first uncovered by human genetic studies. Indeed, one syndrome characterized by increased bone formation around the cranial suture, Boston-type craniosynostosis, is caused by an activating mutation in *MSX2*.<sup>248</sup> *Msx2* inactivation in mice causes a marked delay of ossification in the bones of the skull and an overall decrease in bone volume. This phenotype is accompanied by a downregulation of Runx2 expression, indicating that *Msx2* directly or indirectly regulates Runx2 expression (Fig. 1).<sup>249</sup> Recently, Cheng et al. and Ichida et al. reported that a homeobox gene, the *Msx2* gene, stimulates the commitment of mesenchymal cells into an

osteoblast lineage in association with inhibition of adipogenesis.<sup>250,251</sup> NK3 homeobox 2 (Bapx1), another homeobox protein encoding gene, is required for axial skeleton formation. In Bapx1-deficient mice Runx2 expression is downregulated in the axial skeleton,<sup>252</sup> suggesting that Bapx1 is another activator of Runx2 expression.

## 2. *Dlx5*

*Dlx5* expression in osteoblasts is facilitated by BMP, and *Dlx5* goes on to induce expression of Runx2 in osteoprogenitor cells.<sup>90,91</sup> *Dlx5* has been found to enhance Runx2 P1 promoter activity.<sup>90,253</sup> Furthermore, there is increasing evidence that *Dlx5* promotes activation of Osteocalcin by forming heterodimers with *Msx2*, which antagonizes the *Msx2*-mediated repression of Osteocalcin.<sup>254,255</sup>

## 3. *Twist*

Twist-1 (previously called Twist) and Twist-2 (previously called Dermo-1) encode vertebrate basic helix-loop-helix transcription factors homologous to *Drosophila* Twist, a mediator of dorsal-ventral patterning and mesoderm formation. Knockout of Twist-1 in mice leads to lethality at E10.5 due to failure of neural tube closure.<sup>256</sup> Twist-1 heterozygotes (both in mice and in humans) exhibit craniosynostosis, a disease caused by premature osteoblast differentiation in the skull. Bialek et al. show that Runx2-induced osteoblast gene expression only occurs when expression of Twist genes disappears in osteoblast precursors.<sup>257</sup> Twist-1 heterozygosity reverses skull abnormalities in Runx2<sup>+/-</sup> mice, and Twist-2<sup>-/-</sup> reverses clavicular abnormalities in Runx2<sup>+/-</sup> mice and accelerates osteoblast differentiation in bones formed through endochondral bone formation. Twist proteins' antiosteogenic function is mediated by a novel domain, the Twist box, which interacts with the Runx2 DNA binding domain to inhibit its function. They conclude that Twist-1 and Twist-2 regulate the developmental action of Runx2 in bone formation through the direct interaction of these proteins. Bialek et al., by bringing together the actions of Twist and Runx proteins, have clarified

an important stage in bone formation and set a research agenda for the future.<sup>258</sup>

## 4. *p53*

It has long been recognized that the p53 tumor suppressor plays a pivotal role in preventing cancer. Two independent studies have addressed the role of p53 in bone differentiation in mouse models.<sup>259,260</sup> In one case, Wang et al. examined skeletal structure and bone metabolism in p53 knockout mice.<sup>260</sup> Conversely, Lengner et al. analyzed the effects of hyperactive p53 on bone formation caused by the conditional deletion of transformed mouse 3T3 cell double minute 2 (*Mdm2*) in osteoblasts.<sup>259</sup> Surprisingly, and in contrast to the *in vitro* studies,<sup>261</sup> both groups came to the same conclusion that p53 suppresses differentiation. Specifically, p53<sup>-/-</sup> osteoblasts displayed a marked propensity to differentiate, which was manifested by a modest but significant increase in bone formation and bone density in adult p53 knockout mice. Consistent with these results, the conditional deletion of *Mdm2* in osteoblasts interfered with terminal differentiation, leading to late-stage embryonic lethality, where the embryos displayed more porous and shorter bones. These findings suggest that the interplay between p53 and *Mdm2* could either positively or negatively impact bone development. The studies of both Lengner et al. and Wang et al. provide compelling evidence that p53 suppresses osteoblast differentiation by repressing the expression of either Runx2 or Osterix.<sup>259,260</sup> The subtle discrepancy that exists between the two studies (whether Runx2 or Osterix is the target of p53 action) may be related to how p53 activity is targeted and whether this mechanism alters the stage of cell differentiation. In either case, the concept that the absence of a tumor suppressor gene can enhance cell proliferation while favoring the differentiation of mesenchymal stem cells is intriguing but counterintuitive. It is likely that p53-deficient osteoprogenitors can still exit the cell cycle on terminal differentiation, which may be enhanced as a result of the elevated expression of Runx2 and Osterix. These findings clearly establish p53 as a negative regulator of osteoblast differentiation both *in vitro* and *in vivo*.

## 5. Schnurri-3

Schnurri-3 (Shn3), a large zinc finger protein, was originally identified as a DNA binding protein of the heptameric recombination signal sequence required for V(D)J recombination of immunoglobulin genes<sup>262</sup>; however, it also functions as an adapter protein in the immune system.<sup>263</sup> Jones et al. found that Shn3 is an essential regulator of adult bone formation.<sup>264</sup> Mice lacking Shn3 display adult-onset osteosclerosis with increased bone mass due to augmented osteoblast activity. Shn3 was found to control protein levels of Runx2 by promoting its degradation through recruitment of the E3 ubiquitin ligase WWP1 to Runx2. By this means, Runx2-mediated extracellular matrix mineralization was antagonized, revealing an essential role for Shn3 as a central regulator of postnatal bone mass.<sup>264</sup>

## 6. Cyclin-D1-Cdk4

Cyclin D1-cyclin-dependent kinase 4 (Cdk4) also induces Runx2 degradation by ubiquitination. Shen et al. studied the functional significance of post-translational modification of Runx2 to find that mutation of Runx2 in a consensus Cdk site increases the half-life of Runx2 and causes loss of sensitivity to cyclin D1-induced Runx2 degradation.<sup>265</sup> This presents a novel mechanism through which Runx2 activity is regulated together with the cell cycle in bone cells.

## 7. Hoxa10 and Hoxa2

Homeobox A10 (Hoxa10) has recently been shown to activate Runx2 and directly regulate osteoblast phenotype genes.<sup>266</sup> Hoxa10 is induced by BMP-2 and, in addition to Runx2, activates alkaline phosphatase, osteocalcin, and bone sialoprotein. Hoxa10 associates with the promoters of these genes through chromatin remodeling prior to Runx2 recruitment. Hassan et al. propose that Hoxa10 activates Runx2 in mesenchymal cells and contributes to the onset of osteogenesis.<sup>266</sup> On the other hand, besides defects in branchial arch patterning, Hoxa2<sup>-/-</sup> mice show an upregulation of the cartilage- and bone-specifying genes

SRY (sex determining region Y)-box containing gene 9 (Sox9) and Runx2.<sup>267</sup> This area of osteoblast biology is still in its infancy, and the transcription factors that act upstream of Runx2 to control its expression remain to be identified.

## C. Runx2 Coactivators and Corepressors

### 1. Cbfb

Runx2 protein is shown to interact with a number of transcriptional coactivators. The most important coregulatory protein, essential for enhancement of Runx DNA binding, is Cbfb (also known as PEBP2 $\beta$ ), the non-DNA-binding partner of all three Runx proteins. Inactivation of Cbfb causes embryo lethality in mice between E11.5 and E13.5. This results from hemorrhaging in several tissues and the absence of liver hematopoiesis because Cbfb is a heterodimerizing partner of Runx1 and Runx3, which are essential for haematopoiesis. The timing of embryonic lethality precludes examining the role of the Cbfb subunit in osteoblast differentiation. Transgenic rescue of embryonic lethal Cbfb-null mice and “knock-in” of Cbfb fused in-frame to a cDNA encoding green fluorescent protein resulted in mice that exhibited delayed ossification, indicating a role for Cbfb in bone.<sup>268</sup> However, unlike Runx2-null mice that completely lack bone and osteoblasts, ossification is initiated in these mice, suggesting that Runx2 can act in the absence of Cbfb. Similar observations were made simultaneously by two other groups.<sup>269,270</sup>

### 2. p300, CBP, MOZ, MORF

Runx2 also interacts physically and/or functionally with other well-characterized coactivators, including p300, CREB binding protein (CBP), MYST histone acetyltransferase monocytic leukemia 3 (MOZ), and MYST histone acetyltransferase monocytic leukemia 4 (MORF). p300 and CBP physically interact with various R-Smads on ligand stimulation and enhance Smad-dependent transcription of target genes.<sup>271,272</sup> Neither p300 nor CBP has been coprecipitated with Runx2. Two members of the MYST family

of HATs, MOZ and MORF, however, do interact with the activation domain of Runx2 and enhance activation of the osteocalcin promoter.<sup>273</sup> This interaction may be functionally relevant to intramembranous bone formation, since mice with a mutation in the MYST gene have craniofacial defects.<sup>274</sup> Amino-terminal enhancer of split (Grg5), retinoblastoma 1 (pRb), and tafazzin (TAZ) are other proteins that enhance Runx2-mediated transactivation.<sup>275–278</sup> Although most Grg/transducin-like enhancer (TLE) proteins are corepressors, Grg5 appears to be a dominant negative form of longer Grg/TLE proteins, and thereby enhances Runx2 activity *in vivo*.<sup>278</sup> The 14-3-3-binding protein, TAZ (transcriptional coactivator with PDZ-binding motif), coactivates Runx2-dependent gene transcription while repressing PPAR $\gamma$ -dependent gene transcription, indicating that TAZ functions as a molecular rheostat to fine-tune the balance between osteoblast and adipocyte development.<sup>276</sup>

### 3. Histone Deacetylases

Histone deacetylases (HDACs) remove acetyl groups from lysine residues on many proteins, including histones. The elimination of the acetyl group alters chromatin structure by removing a mark needed to recruit coactivating proteins and by facilitating chromatin condensation to promote transcriptional repression.<sup>279</sup> General HDAC inhibitors, such as trichostatin A, increase Runx2-mediated activation, and Runx2 associates with several HDACs, including HDAC3, HDAC4, and HDAC6.<sup>280–282</sup> HDAC3 interacts with the N terminus of Runx2. Suppression of HDAC3 expression in differentiating MC3T3-E1 cells accelerates matrix mineralization and the expression of bone marker genes such as osteopontin, bone sialoprotein, and osteocalcin.<sup>280</sup> Vega et al. claimed that HDAC4 inhibits Runx2 activity by blocking Runx2 DNA binding.<sup>281</sup> HDAC4-null mice display premature ossification due to early onset chondrocyte hypertrophy, and overexpression of HDAC4 inhibits chondrocyte hypertrophy, suggesting that Runx2 activity is controlled by HDAC4 in prehypertrophic chondrocytes.<sup>281</sup> On the other hand, muscle and cardiovascular transcription factor myocyte enhancer factor 2C (Mef2c) has been

found to control bone development by impairing hypertrophy, cartilage angiogenesis, ossification, and longitudinal bone growth in mice.<sup>283</sup> Bone deficiency of Mef2c mutant mice can be rescued by an Hdac4 mutation, showing that endochondral bone formation is sensitive to the balance between Mef2c and HDAC4. Runx2 expression was also dramatically diminished in the endochondral cartilage of Mef2c mutant mice, suggesting that Mef2c is required, either directly or indirectly, for Runx2 expression. HDAC6 was identified as a Runx2 binding protein in co-immunoprecipitation experiments designed to identify corepressors that bind to the potent C terminus repression domain of Runx2.<sup>282</sup> Moreover, several other groups have reported that HDAC inhibitors increased Runx2-dependent activation of the osteocalcin promoter<sup>280</sup> and osteoblast maturation and differentiation.<sup>284,285</sup> However, results from a new study support a requirement for HDAC4 and -5 deacetylase activity to regulate acetylation, abundance, and activity of Runx2.<sup>286</sup> As the phenotypes of other HDAC mutations are probed in detail, it will be exciting to learn whether these proteins have a general role controlling osteoblast differentiation. Recently, Runx2 was found to form complexes with RNA polymerase I transcription factors upstream binding transcription factor, RNA polymerase I (UBF1), and TATA box binding protein (TBP)-associated factor, RNA polymerase I (SL1) in osteoblasts, co-occupy the rRNA gene promoter, and affect chromatin histone modifications at rDNA regulatory regions, whereby Runx2 directly represses rDNA promoter activity and so rRNA synthesis.<sup>287</sup> This shows another mechanism of lineage commitment and cell proliferation control by Runx2.

### 4. Grg/TLE and YAP

Grg/TLE proteins are coexpressed with Runx2 in skeletal cells.<sup>288</sup> The induction of the OCN gene correlates with downregulation of the level of Grg/TLE in mice skeletal tissues between E14 and birth, and Grg/TLEs were shown to inhibit Runx2-dependent activation of OCN gene transcription.<sup>289</sup> However, Grg5, a dominant negative form of long Grg/TLE proteins, can enhance Runx2 transcriptional activity *in vitro*.<sup>278</sup> Depletion

of *Grg5* alone, with normal activity of *Runx2*, causes postnatal growth retardation in about 50% of the mice, and in *Grg5* null *Runx2*<sup>+/-</sup> mice, the lack of *Grg5* function combined with the heterozygous loss of *Runx2* activity resulted in a growth deficiency that was more pronounced than would have been expected. This finding suggests that *Grg5* and *Runx2* interact with each other in vivo and that their combined activity is necessary for the activation of another factor important for bone and cartilage development. It is highly probable that the factor regulated by *Grg5*-*Runx2* interaction is *Ihh*.<sup>278</sup> *Runx2* also interacts with Yamaguchi sarcoma viral (v-yes) oncogene homolog 1 (yes)-associated protein (YAP), a mediator of Src/Yes signaling, in the cytoplasm and translocates it to the nuclear matrix where YAP represses *Runx2*-mediated activation of the osteocalcin promoter.<sup>290</sup> It is not yet known whether *Runx2* associates with multiple corepressor complexes, or whether all of the corepressors mentioned above are components of the same complex.

## 5. *Smurf1*

One of the mechanisms by which transcription factors are regulated is by modulation of degradation. The proteasome degradation pathway decreases *Runx2* protein levels to slow osteoblast differentiation.<sup>291</sup> Smad-specific E3 ubiquitin protein ligase 1 (*Smurf-1*) induces *Runx2* degradation<sup>292</sup> and Smad 6 enhances *Smurf-1*-induced *Runx2* degradation.<sup>293</sup> For example, TNF- $\alpha$  attenuates osteoblast differentiation by promoting *Runx2* proteasomal degradation through upregulation of *Smurf-1* and *Smurf-2* expression.<sup>294</sup> Transgenic overexpression of *Smurf-1* in murine osteoblasts suppresses their differentiation and bone formation, while *Smurf-1*-deficient mice develop age-dependent increases in bone mass.<sup>107,295</sup> HDAC4 and HDAC5 deacetylate *Runx2*, allowing the protein to undergo *Smurf*-mediated degradation.<sup>286</sup>

## 6. Other *Runx2* Interaction Partners

Many transcription factors involved in regulation of the osteoblast differentiation process exert their

action by interacting with *Runx2*. Some provide costimulatory signals, while others directly repress *Runx2* function by affecting its DNA binding activity and/or transactivation potential. DNA binding proteins that interact and cooperate with *Runx2* to activate gene expression include AP1 [FB] osteosarcoma oncogene (*c-Fos*) and *c-Jun*],<sup>296</sup> BMP-responsive Smads (*Smad 1* and *Smad 5*),<sup>89,297,298</sup> E26 avian leukemia oncogene 1, 5' domain (*Ets1*),<sup>299</sup> C/EBP $\beta$  and - $\delta$ ,<sup>300,301</sup> hairy and enhancer of split 1 (*Drosophila*) (*Hes1*),<sup>302</sup> and *Menin*.<sup>303</sup> Most of these proteins interact with either the DNA binding domain or the activation domain of *Runx2*, although the binding sites for some have not been defined. It is generally believed that these transcription factors cooperate with *Runx2* to facilitate the recruitment of coactivators and the assembly of higher-order transactivation complexes. Some proteins, including *Hes1*, may perturb TLE-*Runx* interaction both by competing with TLE corepressors for the binding site on *Runx2* and by titrating TLE away from *Runx2*.<sup>302</sup> Other cooperating transcription factors, such as AP1, *Smad 1*, and *Smad5*, integrate *Runx2* with cell signaling pathways and to the extracellular environment.<sup>304,305</sup>  $\delta$ FosB is a naturally occurring truncated form of AP1 family member *FosB* that is expressed in osteoblasts.<sup>306</sup> Overexpression of  $\delta$ FosB causes increased bone formation, leading to osteosclerosis while down-regulating early markers of adipocyte differentiation, indicating that  $\delta$ FosB transcriptionally regulates osteoblastogenesis. Mutations that affect *Runx2*-Smad interactions are found in cleidocranial dysplasia (CCD) patients and inhibit the ability of *Runx2* to induce osteoblast differentiation after BMP stimulation.<sup>87</sup> *Runx2* null cells do not react to BMP-2 signaling and a triple mutation in the C-terminal domain of *Runx2*, HTY (426–428), disrupts *Runx2*-Smad interaction.<sup>307</sup> The HTY mutation also causes cells to be unable to integrate the BMP-2/TGF- $\beta$  signal on promoter reporter assays, is almost nonfunctional in promoting early stages of osteoblast differentiation, and exhibits reduced subnuclear targeting. Therefore, subnuclear targeting is inseparable from the *Runx2*/Smad osteogenic complex.

Transcription factors that inhibit *Runx2*-dependent activation in mesenchymal cells or osteoblasts include *Dlx3*,<sup>308</sup> *Lef1*,<sup>309</sup> *Msx2*,<sup>255</sup> PPAR $\gamma$ ,<sup>310</sup> *Smad 3*,<sup>311</sup> *Hey1*,<sup>312</sup> and *Stat1*.<sup>313</sup> These proteins

repress Runx2 via several mechanisms, including binding the Runt domain and preventing DNA binding (e.g., Lef1, PPAR $\gamma$ ),<sup>309,310</sup> sequestering Runx2 in the cytoplasm (e.g., C/EBP $\delta$ , Stat1),<sup>313,314</sup> or unknown mechanisms that involve binding in or around the nuclear matrix targeting domain of Runx2 (e.g., Dlx3).<sup>308</sup>

#### D. Osterix

The discovery of a BMP-2-inducible gene, *Osx*, a Kruppel-like specificity protein 1 transcription factor (Sp1) binding factor, identified a second transcriptional regulator for the final stages of bone tissue formation.<sup>100</sup> *Osx* contains a DNA-binding domain consisting of three C2H2-type zinc fingers at its C terminus that share a high degree of sequence identity with similar motifs in Sp1, Sp3, and Sp4. In addition, *Osx* also contains a proline- and serine-rich transactivation domain and activates the *OCN* and *Col1a1* genes. In *Osx*-null mutant mice, no endochondral or intramembranous bone formation occurs.<sup>100</sup> The mesenchymal cells in *Osx*-null mutant mice do not deposit bone matrix, and cells in the periosteum and the condensed mesenchyme of membranous skeletal elements cannot differentiate into osteoblasts, although these cells express normal levels of Runx2. Interestingly, *Osx*-null osteoblast precursors in the periosteum of membranous bones express chondrocyte markers, such as *Sox9* and *Col2a1*, suggesting that Runx2-expressing preosteoblasts are still bipotential cells and *Osx* acts downstream of Runx2 to induce osteoblastic differentiation in bipotential osteochondrogenic cells (Fig. 1).<sup>100</sup> Currently, there is no evidence to indicate whether Runx2 and *Osx* functionally or physically interact. Milona et al. have reported that there is an OSE2 element in the specificity protein-7 (Sp7; the human homologue of the mouse Osterix gene) regulatory region, so the *Osx* promoter may be a direct target of Runx2.<sup>315</sup> Further analysis on the relationship between Runx2 and *Osx* will be the focus of future studies.

Several studies implicate the presence of Runx2-independent mechanisms for ossification.<sup>60,250</sup> These studies implicate that additional signaling pathways may act in parallel to, or

independent of, Runx2 during osteoblast differentiation. It has been shown that MAPK and PKD signaling pathways serve as points of convergence for mediating the BMP-2- and IGF-I-induced effects on *Osx* expression in mesenchymal stem cells.<sup>316</sup> Additionally, Runx2 was required but not sufficient for the BMP-2-mediated *Osx* induction. This result indicated that additional factors (e.g., Dlx5) acting downstream of BMP-2 could induce *Osx* independent of the levels of Runx2 activity. Mechanistic analyses of the effect of FK506 on bone mass showed that NFAT cooperates with Osterix and accelerates osteoblast differentiation and bone formation.<sup>317</sup> Overexpression of NFATc1 stimulates Osterix-dependent activation of the *Col1a1* promoter, but not Runx2-dependent activation of the bone gamma carboxyglutamate protein 1 (*Bglap1*; encoding osteocalcin) promoter.

*En1* expression temporally precedes that of the osteogenic determinant *Osx*, and, in the absence of *En1*, the onset of *Osx* expression is delayed. Since *Osx* is necessary for potentiating the osteogenic fate of the skeletogenic mesenchyme,<sup>100</sup> its perturbed expression provides a mechanistic basis for the delayed calvarial ossification in *En1*<sup>-/-</sup> mice.<sup>318</sup> Furthermore, that *Osx* expression remains impaired in *En1*-null osteoblasts suggests that *En1* also lies upstream of *Osx* during later phases of calvarial osteogenesis, and thus mediates distinct functions in osteoblast differentiation. *En1*<sup>-/-</sup> osteoblasts were deficient in mediating osteoid mineralization and exhibited reduced ALP activity, an enzyme that is essential for this process.<sup>319,320</sup> Moreover, ablation of *En1* results in impaired *OCN* and *BSP* expression, genes that are normally associated with advanced osteoblast differentiation. *OCN* expression has also been shown to be dependent on *Osx*.<sup>100</sup>

#### E. ATF4

Using a combination of human genetic information, analysis of mutant mouse strains, and molecular studies, Yang et al. identified an activating transcription factor (ATF4) as a critical substrate of ribosomal protein S6 kinase polypeptide 3 (RSK2) that is required for the timely onset of osteoblast differentiation, for terminal differentiation of



osteoblasts, for Bsp, and for Osteocalcin expression.<sup>321</sup> Additionally, RSK2 and ATF4 posttranscriptionally regulate the synthesis of type I collagen, the main constituent of the bone matrix. These findings identify ATF4 as a critical regulator of osteoblast differentiation and function, and indicate that lack of ATF4 phosphorylation by RSK2 may contribute to the skeletal phenotype of Coffin-Lowry syndrome (CLS).<sup>321</sup> Elefteriou et al. found that mice lacking neurofibromin in their osteoblasts show increased ATF4-dependent collagen synthesis, RSK2 activity, and bone formation.<sup>322</sup> On the other hand, ATF4 phosphorylation by PKA is also increased in neurofibromin null mice, causing enhanced RANKL expression, osteoclast differentiation, and bone resorption. These changes could be corrected by a low-protein diet, which normalized bone formation and bone mass, showing that ATF4-dependent skeletal dysplasias are treatable by dietary manipulations.

In addition, treatment of nonosteoblastic cells with MG115, a proteasome inhibitor, induced ATF4 accumulation and resulted in activation of an Osteocalcin promoter luciferase construct as well as expression of endogenous Osteocalcin, a molecular marker of differentiated osteoblasts.<sup>323</sup> This study establishes that ATF4, like other osteoblast differentiation factors, such as Runx2 and Osterix, has the ability to induce osteoblast-specific gene expression in nonosteoblastic cells. Cooperative interactions between ATF4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression.<sup>324</sup>

## F. SATB2

The patterning of skeletal elements and bone formation are generally thought to represent distinct pathways; however, evidence is emerging for cross talk between these processes. This is illustrated in studies that establish the functional role of *Hoxa2* in skeletal development, an inhibitor of bone formation and regulator of branchial arch patterning.<sup>267,325</sup> Understanding the mechanisms that mediate these dual roles of *Hoxa2* will provide valuable insight into coordination of pathways governing bone patterning and differentiation.<sup>326</sup> Grosschedl and colleagues make an important stride toward this goal by demonstrating

that the nuclear matrix protein special AT-rich sequence binding protein 2 (Satb2) represses *Hoxa2* expression and is an activator of multiple steps of Runx2-dependent osteoblast differentiation (Fig. 1).<sup>327</sup>

Satb2 is a recently cloned member of the family of special AT-rich binding proteins that binds to nuclear matrix-attachment regions (MARs) and activates transcription in a MAR-dependent manner.<sup>328,329</sup> In humans, translocations that involve the chromosomal region 2q32-q33 and are associated with a cleft palate under conditions of haploinsufficiency have been found to interrupt the *Satb2* gene.<sup>330</sup> Using targeted mutagenesis of *Satb2* in mice, Dobrev et al. provide insight into how the nuclear matrix, chromatin remodeling, and gene activation come together to regulate osteoblast differentiation in development.<sup>327</sup> The most striking phenotypes detected in mice lacking *Satb2* are craniofacial defects in skeletal elements and the inhibition of normal osteoblast differentiation. By combining an impressive series of molecular and genetic approaches, the authors reveal that *Satb2* represses *Hoxa2* expression in osteoblasts through direct recognition of an MAR-like sequence. Chromatin immunoprecipitation (ChIP) and transactivation experiments reveal that *Satb2* can bind to and regulate *BSP* and *OCN* genes, themselves critical components in osteoblast formation. This strongly suggests that *Satb2* has multiple inputs into transcriptional control of osteoblast differentiation. On the basis of genetic synergy between mouse mutants, protein interaction, and transactivation analyses, Dobrev et al. discovered that *Satb2* directly interacts with and enhances the activity of both Runx2 and ATF4.<sup>327</sup> The interaction of *Satb2* with ATF4 and Runx2 augment their binding to the cognate DNA-recognition elements, although *Satb2* does not bind itself to the OSE1 and OSE2 sequences. *Satb2* has been shown to recognize specific sequences, termed MAR sequences,<sup>329</sup> and indeed, *Satb2* was found to regulate the *Bsp* promoter by binding to a site that resembles a bona fide MAR element. Therefore, *Satb2* can act not only as an activating or repressing DNA bound protein, but also as a protein scaffold that enhances the activity of other DNA binding proteins. By its ability to regulate the expression or activity of multiple key determinants of skeletal development, *Satb2* appears to

represent a molecular node of a transcriptional network underlying this process.<sup>327</sup> Distinguishing between the different modes of *Satb2* activity will be important for a detailed understanding of how the nuclear matrix, chromatin structure, and transcriptional activity coordinate the regulation of multiple steps during osteoblast differentiation.<sup>326</sup>

## V. OSTEOCLAST GENE TRANSCRIPTION: TRANSCRIPTION FACTORS AND CYTOKINES THAT REGULATE OSTEOCLAST DIFFERENTIATION

The transcription of eukaryotic genes is regulated by specific DNA binding proteins (transcription factors) that assemble on *cis*-acting DNA sequences in promoters and enhancers. Our lab has cloned osteoclast-specific genes cathepsin K and OC116/ATP6i/a3,<sup>32,331</sup> analyzed their promoters<sup>332</sup> that provided gene models for osteoclast gene regulation, and characterized their function using mouse knockout.<sup>227,333</sup> Many of these DNA binding proteins are ubiquitous in their expression and serve a general role in gene transcription. Others are restricted in expression to one or a few cell types. Cell-specific transcription factors have been identified that activate lineage-specific gene expression in skeletal muscle, neuronal, erythroid, myeloid, and lymphoid lineages.<sup>233,334–337</sup>

Gene-targeted disruption studies have revealed that various transcription factors are essential at different stages of osteoclast differentiation, activation, and survival. PU.1 is thought to be responsible for the earliest established event in osteoclastogenesis. PU.1 disruption in embryonic stem (ES) cells can make ES cells fail to differentiate into macrophages *in vitro*. Several other transcription factors have been found crucial for osteoclast differentiation downstream of M-CSF and RANKL/RANK signaling. Development of macrophages is preserved in NF- $\kappa$ B null mice, suggesting that NF- $\kappa$ B functions later than PU.1 during osteoclast differentiation. A deficiency in *c-fos* causes severe osteopetrosis with lack of osteoclasts.<sup>338,339</sup> NFATc1 is a transcription factor that plays an essential and sufficient role in osteoclastogenesis. It is a master switch for regulating terminal differentiation of OCs, functioning downstream of RANKL.<sup>340</sup> Overexpression of a constitutively active form of

NFATc1 in *c-fos* null cells restores expression of osteoclast-specific genes, demonstrating that NFATc1 is a critical transcriptional regulator downstream of *c-fos* during osteoclast differentiation.<sup>341</sup> Microphthalmia-associated transcription factors (MITFs) and transcription factor E (TFE) 3, TFEB, and TFEC are essential for differentiation of mononuclear precursors into multinucleated osteoclasts.<sup>342</sup> Most of these transcription factors are involved in both the RANK-associated signaling and the expression of typical osteoclastic genes, including calcitonin receptor, TRAP, cathepsin K, osteoclast-associated receptor (OSCAR), and  $\alpha_v\beta_3$  class of integrins.

How a multipotential cell chooses a single pathway of OC differentiation is a central problem in OC biology. Spontaneous and genetically engineered osteopetrotic mutant mice have yielded important insights into the regulation of OC differentiation.

### A. PU.1

PU.1 is an EST-domain transcription factor essential for the development of myeloid and lymphoid lineage cells. Mice homozygous for a null mutation in the PU.1 gene die during fetal development by 18.5 days postcoitum (d.p.c.) and lack lymphoid and myeloid lineages.<sup>343</sup> PU.1<sup>-/-</sup> ES cells fail to differentiate into macrophages *in vitro*.<sup>344</sup> This failure is complemented by a PU.1 transgene, providing evidence that PU.1 promotes macrophage differentiation from pluripotent ES cells.<sup>345</sup> The role of PU.1 in macrophage differentiation was further determined by the discovery that commitment to the monocytic lineage occurs in the absence of PU.1 and a low percentage of monocytic precursors are produced in the PU.1 null mice.<sup>346</sup> The major role of PU.1 in lymphoid and myeloid development is thought to be the regulation of lineage-specific cytokine receptor genes, such as M-CSF receptor (*c-fms*), granulocyte-macrophage colony stimulating factor receptor low-affinity subunit (GM-CSFR $\alpha$ ), G-CSF receptor (G-CSFR), and IL-7R $\alpha$ .<sup>347</sup> The combined data show that PU.1 is absolutely required for macrophage development during the differentiation from promonocyte to the monocyte stage.

OCs are bone-resorbing cells of hematopoietic origin. Hematopoietic transcription factor PU.1 is also critical for osteoclastogenesis; PU.1 expression is detected at all stages of OC differentiation and PU.1 mRNA increases threefold as cells differentiate into OCs.<sup>348</sup> PU.1<sup>-/-</sup> knockout mice are osteopetrotic and are devoid of both OCs and macrophages, indicating a differentiation block at a common MO-CSF precursor stage.<sup>348</sup> PU.1 also binds to the corresponding sequences in the promoters and enhancers of many OC-specific genes. RANK gene, which has been shown to be crucial for osteoclastogenesis, is a transcriptional target of PU.1. The PU.1<sup>-/-</sup> progenitor cells failed to express the RANK gene and reconstitution of PU.1 in these cells induced RANK expression.<sup>349</sup> RANKL-induced cathepsin K gene expression is cooperatively regulated by the combination of PU.1 and NFATc1.<sup>350</sup> The transcription factors Mitf and PU.1 interact with the TRAP gene promoter and activate TRAP gene expression in OCs.<sup>351</sup> Thus, PU.1 appears to be a master regulator, critical for the development of a common progenitor for the lymphoid myeloid cell lineages in the hematopoietic system.

## B. AP-1

AP-1, which is composed mainly of Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) proteins, is important in the osteoclastogenic process.<sup>352</sup> Differentiation of the common precursors into either bone or immune lineages is determined by ligands binding to cell-surface receptors, RANK for OCs or Toll-like receptors (TLRs) for mononuclear phagocytes. Both RANK and TLRs activate the dimeric transcription factors NF- $\kappa$ B and AP-1. Yet, c-Fos/AP-1 plays a positive role in OCs, but a negative role in macrophages and dendritic cells.<sup>353</sup>

Disruption of the *c-fos* proto-oncogene also leads to an osteopetrotic phenotype.<sup>183,338,354,355</sup> However, the number of EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (F4/80) and lectin, galactose binding, soluble 3 (Mac-2) positive cells, presumably macrophages, is increased, and cells positive for the 92-kD type IV collagenase, proposed as a relatively early marker of the OC lineage, are evident at the capillary invasion front of the metaphysis. Thus,

the absence of *c-fos* blocks OC development at the point of divergence from the common MO-CSF precursor, prior to the expression of TRAP-positivity. RANKL signaling to the cell interior also leads to *fos*-dependent transcription of target genes such as *fos*-like antigen 1 (Fra-1) and NFATc1. NFATc1 seems to be the critical downstream target gene of *fos* in osteoclastogenesis, because differentiation of *fos*-deficient bone marrow monocytes (BMMs) into OCs is rescued by ectopic expression of NFATc1.<sup>341</sup> Recently, it was found that the calcium/calmodulin-dependent protein kinase (CaMK)-CREB pathway functions to regulate the transcriptional program of osteoclastic bone resorption by enhancing induction of NFATc1 and facilitating NFATc1-dependent gene regulation once its expression is induced.<sup>356</sup> Genetic ablation of *Camk4* reduced CREB phosphorylation and downregulated the expression of *c-fos*, which is required for the induction of NFATc1. Additionally, PPAR $\gamma$  conditional knockout mice were found to exhibit osteopetrosis and PPAR $\gamma$  was revealed to be a regulator of osteoclastogenesis by directly regulating *c-fos* expression.<sup>357</sup>

Transgenic mice expressing dominant-negative *c-Jun* specifically in the OC lineage manifested severe osteopetrosis due to impaired osteoclastogenesis.<sup>358</sup> *c-Jun* is clearly RANKL activated via TRAF6 by a process involving JNK1, but not JNK2. JNK1 appears to modulate osteoclastogenesis through both *c-Jun* phosphorylation-dependent and -independent mechanisms.<sup>359,360</sup> Recently, *c-Jun* signaling was found to be in cooperation with NFAT in RANKL-regulated OC differentiation.<sup>358</sup> When transcriptionally active, *c-Jun* associates with members of the *fos* family. Several cytokine genes are cooperatively regulated by NFATs and AP-1, which bind to a composite recognition element in their promoter regions.

## C. NF- $\kappa$ B

NF- $\kappa$ B is a family of five transcription factors that are expressed in most cell types and play an essential role in immune and inflammatory responses by regulating the expression of a variety of proinflammatory cytokines and other inflammatory mediators.<sup>361</sup> The five transcription factors p50 (NF- $\kappa$ B1), p52 (NF- $\kappa$ B2), p65 (RelA), c-Rel,

and RelB each contain an Rel homology domain that allows these factors to dimerize and bind DNA. Unlike most transcriptional activators, this family of proteins resides in the cytoplasm and must therefore translocate into the nucleus. Once in the nucleus, they attach to  $\kappa$ B binding sites in the promoter region of a large number of genes including IL-1, IL-6, TNF- $\alpha$ , and GM-CSF and, after stimuli-induced I $\kappa$ B protein degradation in response to extracellular signals, induce their transcription.<sup>362</sup>

Mice lacking NF- $\kappa$ B1 and NF- $\kappa$ B2 (double-knockout mice) developed osteopetrosis because of a defect in OC differentiation, suggesting redundant functions of NF- $\kappa$ B1 and NF- $\kappa$ B2 proteins in the development of this cell lineage.<sup>363</sup> The complete absence of OC precursors in these mice indicates that OC differentiation was arrested at a relatively early stage. Further studies have demonstrated that NF- $\kappa$ B1 and NF- $\kappa$ B2 expression is not required for formation of RANK-expressing OC progenitors but is essential for RANK-expressing OC precursors to differentiate into TRAP<sup>+</sup> OCs in response to RANKL and other osteoclastogenic cytokines.<sup>364</sup>

#### D. NFATc1

Recently, Takayanagi et al. reported that NFATc1 plays an essential and sufficient role in osteoclastogenesis. They demonstrated that RANKL induces and activates NFATc1 through calcium signaling. Both the transient initial release of Ca<sup>2+</sup> from intracellular stores and the influx through specialized Ca<sup>2+</sup> channels control the dephosphorylation of the cytoplasmic components (NFATc1 proteins) and lead to their nuclear localization, which is followed by the activation of osteoclast-specific genes.<sup>365</sup> They reported that inhibiting NFATc1 activity using dominant negative alleles blocks osteoclastogenesis, whereas overexpression of the wild-type protein stimulates osteoclast development from embryonic stem cells in a RANKL-independent manner.

The nuclear factor of activated T-cells cytoplasmic (NFATc) is a family of transcription factors originally identified in T cells. The gene family is currently known to have four members (NFATc1 through NFATc4), which have roles

both within and outside the immune system. NFATc1 is the major induced NFATc in human OCs, with expression greatly exceeding that of NFATc2 through NFATc4.<sup>366</sup> NFATc1-deficient embryonic stem cells fail to differentiate into OCs in response to RANKL stimulation, and ectopic expression of NFATc1 causes precursor cells to undergo efficient differentiation without RANKL signaling, indicating that NFATc1 is a master switch for regulating terminal differentiation of OCs, functioning downstream of RANKL.<sup>340</sup> RANKL induces and activates NFATc1 through calcium signaling, and calcineurin inhibitors such as FK506 and cyclosporin A strongly inhibit osteoclastogenesis.<sup>340</sup> FK506-mediated inhibition of NFATc1 activity results in a defective induction of the mRNA of NFATc1, indicating that NFATc1 induction is dependent on its own activity: NFATc1 autoamplifies its own gene, possibly by binding to its own promoter.<sup>367,368</sup> RANKL/TRAF-6 signaling, rather than CD40/TRAF-6, leads to NFATc1 activation and osteoclastogenesis.<sup>369</sup> Transcriptional activation of NFATc1 in OCs is mediated by a RANKL/TRAF-6/Fos signaling pathway because NFATc1 rescues osteoclastogenesis in precursors lacking c-Fos.<sup>340,341</sup> c-Jun signaling in cooperation with NFAT is crucial for RANKL-regulated OC differentiation. Osteoclastogenic activities of NFAT were abrogated by overexpression of dominant-negative c-Jun and overexpression of NFAT in transgenic mice expressing dominant-negative c-Jun rescues differentiation of OC precursor cells into TRAP-positive multinucleated OC-like cells even in the absence of RANKL.<sup>358</sup> At the final stage of OC differentiation, NFATc1 cooperates with Fos and Jun proteins to induce OC-specific genes such as TRAP, calcitonin receptor, cathepsin K and  $\alpha_v\beta_3$  integrin gene.<sup>350,358,370-373</sup>  $\alpha_v\beta_3$  integrin is crucial to bone resorption and  $\alpha_v\beta_3$  integrin null mice exhibit osteosclerosis and an increased number of dysfunctional osteoclasts, which have an abnormal cytoskeleton and fail to spread in vitro, to form actin rings ex vivo, or to form normal ruffled membranes in vivo.<sup>373</sup>

#### E. Mitf

Mitf is a member of the basic/helix-loop-helix/leucine zipper (b-HLH-ZIP) transcription factor

subfamily, which also includes Tfe3, Tfeb, and Tfec. Mitf is required for the proper development of several cell lineages including OCs, melanocytes, retinal pigment epithelial cells, mast cells, and natural killer cells. Mutations in Mitf result in osteopetrosis in several organisms due to defective OC development.<sup>342</sup> Mononuclear OCs can be detected in mi/mi mice but these cells are incapable of fusing to form multinucleate cells, lack a distinct ruffled border, and are defective in bone resorption.<sup>374-377</sup> Compared with many other osteopetrotic mouse models, such as c-fos, PU.1, and NF- $\kappa$ B mouse knockout models, in which earlier steps of OC differentiation are affected resulting in lower numbers of OCs in vivo,<sup>348,355,378</sup> defects in the OCs of mi/mi mutant mouse models appear to occur late in differentiation.

The interaction of Mitf with either PU.1 or PU.1-interacting protein allows efficient induction of an OC-specific marker of the TRAP gene in a synergistic manner.<sup>351</sup> Through three consensus elements in the cathepsin K promoter, Mitf and Tfe3 could regulate the expression of cathepsin K on the transcriptional level. PU.1 and Mitf transcription factors also synergistically activate the expression of the OSCAR gene, which has two Ig-like domains and functions as a bone-specific regulator of OC differentiation.<sup>379</sup> Furthermore, Mitf is a target for the RANKL signaling pathway in OCs, and phosphorylation of Mitf leads to an increase in OC-specific gene expression.<sup>380</sup> M-CSF could induce phosphorylation of Mitf and Tfe3 via a conserved MAPK consensus site, thereby triggering their recruitment of the coactivator p300.<sup>381</sup>

## F. Myc

Genes of the myelocytomatosis oncogene (Myc) family contribute to the genesis of many human tumors. In mammals, there are four related genes in the Myc family, namely, c-Myc, N-Myc, L-Myc, and S-Myc. They are key regulators of cell proliferation, and their deregulation contributes to the genesis of most human tumors.<sup>382</sup> The number of target genes that are regulated by Myc is surprisingly large. C-Myc was also found to be strongly upregulated in RANKL-induced osteoclast-like cells (OCLs) but was absent in undifferentiated cells. Dominant negative Myc in RAW264.7 cells

was able to block RANKL-induced OCL formation. Therefore, C-Myc is a downstream target of RANKL and its expression is required for RANKL-induced osteoclastogenesis.<sup>383</sup> It was also found that transcription from the TRAP promoter could be negatively regulated by Myc.<sup>384</sup> Compared to the understanding of osteoblast transcriptional regulation, osteoclast transcriptional regulation remains relatively unclear.

## VI. SUMMARY

Bone is constantly being remodeled in a dynamic process where osteoblasts are responsible for bone formation and osteoclasts for its resorption. Formation of skeletal elements during embryogenesis and the dynamic remodeling of bone in the adult involve an exquisite interplay of developmental cues, signaling proteins, transcription factors, and their coregulatory proteins that support differentiation of osteogenic lineage cells from the initial mesenchymal progenitor cell to the mature osteocyte in mineralized connective tissue.<sup>229</sup> Wnt/ $\beta$ -catenin signaling is an enticing target for developing drugs to combat skeletal diseases since the canonical pathway is composed of a series of molecular interactions that offer potential places for pharmacological intervention.<sup>69</sup> Several unresolved issues are offered here in the hopes of stimulating their resolution. Although we have learned much in recent years regarding the role of canonical Wnt signaling in bone formation, some important questions remain to be addressed. For example, we know that most Wnts and FZDs are expressed in bone, but is there a role for Wnt and FZD specificity in the control of osteoblast physiology? Recent evidence has shown that non-canonical pathways also play a role in bone metabolism but there is more research to be done in this area. Finally, canonical Wnt signaling appears to control osteoclastogenesis through actions on osteoblasts, but do Wnts also have direct effects on osteoclasts? This and other questions are likely to be answered in the coming years.

Like all developmentally important proteins, multiple mechanisms actively control Runx2 activity. Further studies are required to clarify the detailed mechanism of the temporal and spatial regulation of Runx2 for the normal development

and homeostatic regulation of the skeletal system. Additional proteins will be added to the list of Runx2-interacting proteins in the next decade. Mechanisms regulating expression and subsequent activity of *Osx*, *ATF*, and *SATB2* at different stages of osteoblast maturation are only beginning to be understood. The role of *Msx2* remains to be defined since some studies have reported that *Msx2* enhances osteoblast differentiation while other studies report that *Msx2* inhibits it (Fig. 1).<sup>385</sup> Finally, insights into the mechanisms by which proteins and developmental signals affect these transcription factors' subcellular localization to the nuclear matrix will be needed to understand how they regulate gene expression.

Recent studies provide evidence that the skeleton plays a role in energy metabolism by exerting an endocrine regulation of sugar homeostasis.<sup>386,387</sup> Lee et al. found that mice lacking *OST-PTP*, a gene expressed in osteoblasts encoding a receptorlike protein tyrosine phosphatase, are hypoglycemic and show an increase in  $\beta$ -cell proliferation, insulin secretion, and insulin sensitivity.<sup>386</sup> On the other hand, mice lacking osteocalcin showed diminished  $\beta$ -cell proliferation, glucose intolerance, and insulin resistance. Osteocalcin was found to improve glucose tolerance in vivo and stimulate *CyclinD1* and insulin expression in  $\beta$  cells and an insulin-sensitizing adipokine in adipocytes ex vivo.<sup>386</sup> Several questions remain to fully demonstrate that the skeleton is a ductless gland secreting osteocalcin from osteoblasts as a hormone.<sup>387</sup> How is the skeletal regulation of glucose and fat connected to the control of bone by leptin through either the hypothalamus or the sympathetic nervous system? Where does osteocalcin sit in the hierarchy of hormones that influence glucose and fat metabolism? How the osteoblast senses the need for metabolic control must be understood, as well as how the cell translates this information into osteocalcin release. The mechanism of osteocalcin signaling will need to be investigated. If osteocalcin deficiency plays a role in human disease such as type 2 diabetes mellitus, there will be several clinical and therapeutic implications.

Osteoclasts are the principal bone-resorbing cells, and their activity has a profound impact on skeletal health. A more complete understanding of the mechanisms by which OCs differentiate from their precursors is therefore critical to devel-

oping therapies for often debilitating diseases. In this review, we have summarized the transcription factors and cytokines that have been shown through genetic studies to be important in bone development. The *PU.1*<sup>-/-</sup> mouse lacks both OCs and macrophages; it represents the earliest developmental form of osteopetrosis yet described. *PU.1* is known to be required for macrophage development during differentiation from promonocyte to monocyte. Recently, it was also reported that *Tal-1* may lie upstream of *PU.1* in a regulatory hierarchy during osteoclastogenesis.<sup>388</sup> Differentiation of mature OCs from committed OC progenitors was blocked by disruption of the *Mitf*, *NF- $\kappa$ B*, *c-Fos*, and *NFATc1* genes. Although these transcription factors play an important role in OC differentiation, the expression of these transcription factors in various cell types suggests that they are unlikely to be the switch that dominates the OC differentiation process. Further study is needed to determine which transcription factors function together with ubiquitous transcription factors (i.e., *c-Fos*, *PU.1*, *NF- $\kappa$ B*, and *NFATc1*) to control OC differentiation. Cytokines including *M-CSF* and *RANKL* also induce and modulate growth and differentiation of the precursors to mature osteoclasts. *M-CSF* binds to its receptor to provide the signals for macrophage survival and proliferation. *RANKL* interaction with *RANK* induces recruitment and activation of *TRAFs*, leading to the activation of multiple signaling cascades. Although much is understood about *RANK* signaling, several questions remain. The in vivo significance of *MAPKs*, along with other genes activated by *RANKL*, has not yet been established. In *ITAM*-mediated costimulatory signaling, the ligands for the *Ig*-like receptors are not identified. The molecules that connect *RANK* with *ITAM* signaling are also unknown. Among the *NFATc1* target genes, the genes that directly promote the differentiation process remain to be elucidated. Additional *RANKL/RANK* signaling questions include: How does *RANKL/RANK* signaling regulate osteoblast function and the anabolic effects of *PTH* or other potential anabolic agents? Does *RANKL/RANK* signaling play an important role in cancer cell growth and interaction with other cells in bone? How exactly do immune cells influence osteoclast and osteoblast/stromal cell function in normal and disease states?

How important are osteoclasts and their precursors in regulating their own formation and function relative to macrophages and other immune cells? Taken together, osteoclastogenesis is likely to be a complicated process, controlled by several regulatory mechanisms at several differentiation transition points. Thus, further detailed studies are necessary for the complete understanding of the osteoclast signaling pathways.

Molecular understanding of these signaling pathways in osteoblasts and osteoclasts will provide unprecedented therapeutic strategies for bone disease. One promising therapeutic target is *Atp6v0d2* in osteoclasts. This gene encodes a subunit of the vacuolar ATPase necessary for osteoclastic bone resorption and the d2 isoform is predominantly expressed in osteoclasts.<sup>389</sup> *Atp6v0d2*-deficient mice exhibit markedly increased bone mass due to defective osteoclasts and enhanced bone formation, revealing that *Atp6v0d2* is required for efficient preosteoclast fusion. In addition, *Atp6v0d2* was shown to inhibit osteoblasts, although the mechanism for this finding has not been revealed. These results raise the possibility of therapeutic intervention for osteoporosis by targeting a single gene product in osteoclasts to inhibit bone destruction and stimulate bone formation.<sup>389,390</sup> The new findings raise hope that the limitations of current drugs for bone disease might be overcome.

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