8 Biological and Molecular Effects of Vitamin D on Bone

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Abstract The physiological activities of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$D], the active hormone of vitamin D_3, in the skeleton are far-reaching and include development and turnover of bone, differentiation and survival of distinct bone cell populations, and maintaining calcium and bone homeostasis through positive and negative control of gene expression. Here we describe these functional activities within the context of the molecular mechanisms established for the bone tissue-specific osteocalcin gene, involving interactions of the vitamin D receptor transcriptional complexes that contribute to various 1,25$(OH)_2$D activities in the skeleton.

Key Words: Bone; VDR; osteocalcin; 1,25-dihydroxyvitamin D; skeleton; osteoblast; osteocyte; alkaline phosphatase; 24-hydroxylase; mineralization

1. INTRODUCTION

Bone is the connective tissue characterized by an extensive extracellular matrix that is mineralized. Calcium and phosphate in the form of hydroxyapatite constitute by weight up to 90% of adult bone. Crystals of hydroxyapatite [$Ca_{10}(PO4)_6(OH)_2$] deposit within the bone, a process which is in part dependent on vitamin D_3 (VD3) for adequate intake, absorption, and retention of dietary calcium and phosphate for producing a mineralized skeleton that functions as a connective tissue. The skeleton provides rigid mechanical support to the body, protects vital organs, and serves as a reservoir of ions, especially for calcium and phosphate required for serum homeostasis. This bone mineral reservoir supports the cellular functions of all cells in the body and releases ions into the circulation for maintaining normal calcium homeostasis. In response to reduced serum calcium levels, calcium transport is stimulated across the gut and from the renal tubular lumen into the bloodstream. At the same time, calcium is mobilized from bone through the coordinated activities of osteoblast and osteoclast lineage cells to resorb

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the bone matrix through regulated expression of genes by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D$]. A broad spectrum of biological activities of VD3 on distinct bone cell populations facilitates this metabolic function of bone tissue, as well as maintaining a balance between bone resorption and bone formation (bone homeostasis). The active hormone ligand, $1,25(OH)_2D$, binds the vitamin D receptor (VDR) in osteoblasts to activate a group of genes for promoting osteoclast differentiation and bone resorption. Replacement of this resorbed bone with new bone tissue synthesized by osteoblasts is mediated by the ability of $1,25(OH)_2D$ to target genes that represent bone matrix proteins. Thus VD3 has a physiological role in the mobilization of mineral ions to support calcium homeostasis and the temporal events of bone remodeling for maintaining bone homeostasis, all through its activities in kidney, intestine, and the skeleton.

This chapter focuses on an expanding knowledge of the multiple cellular activities that are regulated by vitamin D in bone and the molecular mechanisms by which $1,25(OH)_2D$ can regulate bone tissue functions through direct activation or repression of the target genes in bone cells. The many possibilities for interactions of the vitamin D receptor transcriptional complex with other factors for regulating gene expression is provided by the example of $1,25(OH)_2D$ control of the osteoblast-specific osteocalcin gene. Among the factors interacting with the VDR include chromatin remodeling factors, tissue-specific transcription factors, and a group of coregulator proteins that facilitate either positive or negative regulation of the gene in response to physiologic signals and in different cellular contexts.

It has long been appreciated that insufficient calcium levels produce rickets in growing children when normal growth plate maturation of chondrocytes to hypertrophic cells for long bone growth becomes severely impaired (see Chapter 21). In the adult, osteomalacia occurs from impaired mineralization of new bone tissue during bone turnover. In early studies of vitamin D-deficient rodents, supplementation with calcium normalized the rachitic syndrome and direct roles for VD3 on osteoblast activities were not appreciated until discovery of the first vitamin D response element (VDRE) in the bone restricted and developmentally expressed osteocalcin gene. Through characterization of genetic mouse models, cellular pathways, and molecular components of the vitamin D pathway, a new level of understanding of the many facets of $1,25(OH)_2D$ control of bone homeostasis has been reached that impacts on the importance of daily requirements of vitamin D for general health.

2. A SPECTRUM OF CELLULAR ACTIVITIES OF $1,25(OH)_2D$ CONTRIBUTING TO BONE FORMATION

2.1. Bone Tissue Organization, Vitamin D, and Osteoblasts

The cellular composition of bone is quite heterogeneous with two distinct cell lineages giving rise to the bone forming osteoblast from mesenchymal stem cells and the bone resorbing osteoclast which arises from the hematopoietic lineage. Each lineage is represented by subpopulations of cells at different stages of maturation and organized in relation to bone architecture. Progenitor cells or immature osteoblasts are found near the outer bone surface, either in the periosteum or along the endosteum (Fig. 1). Active bone forming surfaces are lined with cuboidal osteoblasts. When bone formation is

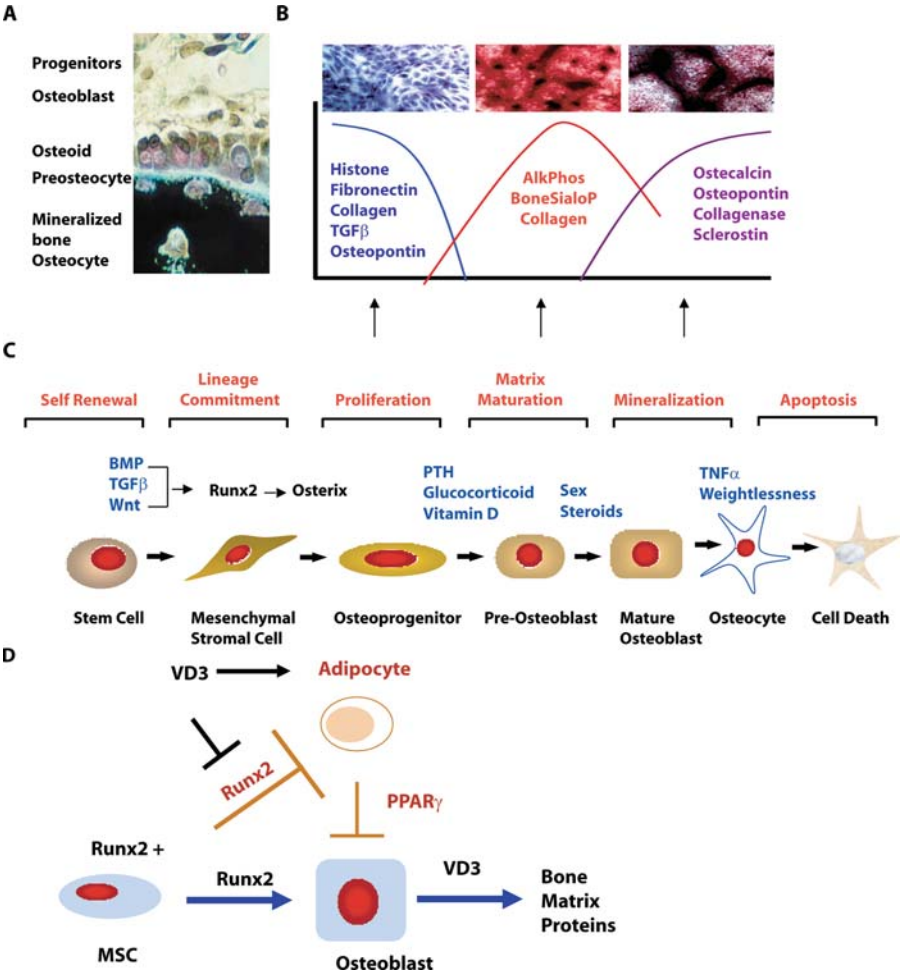

Fig. 1. Stages and markers of osteoblast growth and differentiation. (**a**) Bone surface in vivo shows organization of osteoblast lineage cells as they maturate in relation to the mineralizing bone. (**b**) Primary calvarial osteoblasts cultured for 3 weeks showing cell layers at three principle stages of maturation in vitro: growth (Toluidine blue); matrix maturation (alkaline phosphatase histochemical staining, marker of differentiated osteoblast); mineralization stage (von Kossa stain for phosphate in hydroxyapatite mineral). *Lower panel* – Expression of genes most characteristic of the stages. Note osteopontin is related to proliferation and increases again during mineralization. Histone is a marker of DNA synthesis. (**c**) Schematic of stages of osteoblast differentiation with signaling factors that promote commitment to the chondro- and osteoblast phenotype include BMP2, TGFβ, and Wnt/β-catenin pathways. The transcription factors Runx2 and Osterix are essential for bone formation in vivo (established in knockout mice). Indicated hormones have effects on both cell growth and expression of bone matrix proteins in vitro and can be anabolic at low doses or catabolic at high doses in vivo. (**d**) Illustrates the differentiating promoting properties of VD3 and Runx2 on mesenchymal cells toward the bone and fat cell lineages.

triggered some of the pre-osteoblasts can divide, migrate into the interior of the bone, and differentiate. Bone lining cells, which are inactive osteoblasts, are responsive to elevated PTH and VD3 levels and must retract from the bone surface to facilitate osteoid degradation and exposure of the mineral surface for osteoclast adhesion in order to initiate bone resorption. Finally, when osteoblasts become embedded in the mineralized matrix, their functional and morphologic characteristics change to the osteocyte. Osteocytes have multiple cellular processes that reach out through canaliculi in bone tissue for connectivity and maintaining bone viability. Osteocytes have a mechanosensor function in bone *(1)*.

The bone matrix is composed largely of collagen type I (with associated minor collagens) accounting for as much as 90% of the total protein in adult bone. In addition, numerous non-collagenous proteins reside in the bone matrix that function in either promoting or inhibiting mineralization *(2)*. Many of these are specialized calcium and phosphate binding proteins that are vitamin D regulated and include the bone sialoprotein, osteopontin, which is a protein containing *O*-phosphoserine and osteocalcin, a vitamin K-dependent calcium binding protein characterized by γ-carboxyglutamic acid residues. Also important to the mineralization process are elevated levels of the plasma membrane-bound enzyme alkaline phosphatase. These genes can be negatively or positively regulated by $1,25(OH)_2D$ dependent on multiple factors as will be appreciated throughout this chapter. Considerations for gene regulation must include the physiologic levels of the hormone, the cellular context (e.g., differentiation stage of the cell), and the coregulatory protein interactions with the VDR complex on target genes, as will be described. While physiologic levels of the hormone contribute to normal bone formation, physiologic doses of VD3 that lead to bone resorption can downregulate bone matrix protein synthesis and mineralization-related enzymes until signals for bone formation are activated.

All osteoblast lineage cells express the VDR to respond to paracrine effects of the hormone. However, osteoblasts have the ability to synthesize $1,25(OH)_2D$ and express CYP27B1, the 25D 1α-hydroxylase, and CYP24, the 25(OH) vitamin D-24-hydroxylase, which is a catabolic regulator of the hormones $1,25(OH)_2D$ *(3, 4)*. VD3 modulates expression of many dependent genes in osteoblasts that mediate both bone formation through regulation of bone matrix genes *(5, 6)* and bone resorption genes that facilitate a spectrum of activities *(7, 8)*. Gene profiling studies have led to characterization of factors identified with roles in bone metabolic activities, among which are semaphorin 3B that affects osteoblast and osteoclast activity *(9)*, and FGF2 *(10)*, a key regulator of the Pi axis of mineral homeostasis that are transcriptionally regulated by $1,25(OH)_2D$.

2.2. Skeletal Development

In the last decade, mouse mutations in the VDR or the 1α-hydroxylase enzyme that is essential for the $1,25(OH)_2D$ synthesis have revealed that the vitamin D pathway is not critical for embryonic skeletal development. Mice deficient in VD3 signaling did form a normal skeleton in utero, but after birth developed rickets postnatally which could be corrected by supplementation with calcium. However, other

studies clearly demonstrate direct effects of $1,25(OH)_2D$ on the regulation of genes that mediate key developmental signaling pathways for bone formation. One of these is the well-established canonical Wnt signaling pathway that regulates numerous cell differentiation lineages through multiple Wnt ligands that interact with frizzled receptor complexes *(11)*. Canonical Wnt signaling is transduced through intracellular β-catenin/TCF transcriptional complexes that regulate target genes to promote mesenchymal cell commitment to either chondrogenesis or osteogenesis, dependent on cellular levels of β-catenin. Nuclear hormone receptors commonly interact with Wnt ligands, β-catenin, and TCF complexes. VD3 induces expression of the Wnt frizzled receptor coregulator LRP5 *(12)* and in stromal cells VDR inhibits DKK1 and SFRP2 which are Wnt antagonists. Together these events enhance canonical Wnt signaling which has an anabolic effect on bone formation *(11)*.

The negative regulation of Wnt antagonists by $1,25(OH)_2D$ has also been linked to suppression of adipogenic differentiation in favor of osteoblast differentiation. An interesting observation from the vitamin D receptor null mice was a higher mRNA level of PPARγ, the transcription factor essential for adipocyte differentiation, and these cells expressed higher levels of the Wnt antagonist DKK1, which inhibits the bone promoting properties of the WNT signaling pathway. Thus, in early skeletal development, VD3 might be contributing to the bone forming properties by repressing adipogenesis of mesenchymal stem cells. The Wnt and VD3 pathways are also related to each other for hair follicle development *(13)*. While VD3 promotes differentiation of cells in the absence of VDR, β-catenin can induce tumors resembling basal cell carcinomas *(14, 15)*. Thus, identifying molecular mechanisms by which Wnt signaling converges with $1,25(OH)_2D$ regulation of target genes has important implications for skeletal development and treating tumors arising from deregulated Wnt signaling.

The bone morphogenetic protein 2 (BMP2) is highly osteogenic and a recent global gene profiling study of embryonic stem cells identified a complex temporal interplay on gene effects by VD3, BMP2, and β-catenin activities. BMP2 induction of Wnt signaling is opposed by VD3 blocking β-catenin activity, the intracellular mediator of canonical Wnt signaling that translocates to the nucleus to participate in gene regulation. Depending on the cellular context, there are differences in effects of $1,25(OH)_2D$ in influencing lineage determination to multiple phenotypes. Not until more definitive studies for these changes can be established and in vivo lineage mapping studies carried out in response to VD3 can we obtain a better understanding of the role of VD3 in contributing as a determinant of embryonic skeletal development.

2.3. Vitamin D3 Promotes Cell Differentiation at Multiple Levels

Vitamin D has a key role in influencing lineage direction of a pluripotent stem cell to multiple phenotypes *(13, 16–20)*. Highly relevant to skeletal homeostasis is the ability of $1,25(OH)_2D$ to promote adipocyte differentiation as well as activate genes that are the bone matrix components of osteoblast differentiation. As we age, our marrow becomes more "fatty" and in a way, a contest prevails in directing marrow stromal cells to either the osteoblast or adipocyte lineage. Several studies have shown that subpopulations of multipotential mesenchymal cells or committed fetal rat calvarie-derived

cells can be bipotential forming either osteoblast or adipocyte colonies under appropriate culture conditions. This phenomenon occurs in cultures chronically treated with vitamin D immediately after plating of cells. In early studies, it had been clearly established that short-term (up to 24 h) treatment of osteoblast cells in vitro with $1,25(OH)_2D$, induced expression of vitamin D-regulated genes, including osteopontin and osteocalcin that represent the mineralization stage of osteoblast differentiation. However, other bone matrix-related genes were downregulated as alkaline phosphatase and the bone sialoprotein that represents the pre-mineralization stage. These findings suggest that VD3 may regulate progression through stages of osteoblast differentiation. Not until the precise functions of these bone matrix, structure-related proteins are better defined in relation to bone metabolic activities will we be able to fully interpret these activities of $1,25(OH)_2D$ in osteoblast lineage cells. As will be discussed in Section 4 of this chapter, there are mechanisms to assure that the VDR complex promotes osteogenic lineage gene expression through direct interactions with Runx2, the essential transcription factor for bone formation. Thus, tissue-specific activities of VD3 for driving differentiation appear to reside in the ligand-induced VDR interactions with other cell type phenotypic transcriptional regulators. An understanding of these molecular events mediating these changes is providing new insights for bone renewal.

The relationship between proliferation and differentiation of all skeletal cells must be maintained and is stringently regulated through the activity of cytokines, growth factors, and hormones. It is the potent antiproliferative effects of $1,25(OH)_2D$ that contribute to cell differentiation and are the basis for treatment of certain cancers (see Chapters 12, 40, 47, 50). The mechanisms for VD3 inhibition of proliferation involve $1,25(OH)_2D$-mediated upregulation of two classes of cell cycle inhibitors, p21 and p27 cyclin-dependent kinase inhibitors and the tumor suppressor pRB, thereby impeding cell cycle progression *(21, 22)*. The human p21 (WAF1/CIP1 gene) is regulated by p53 and the VDR gene has multiple p53 and VDR positive regulatory regions *(23)*. VD3 also affects progression through the cell cycle by modulating the cell cycle checkpoint by downregulating expression of the Chk1 and Claspin proteins through interactions of the VDR with E2F recognition motifs in these genes. Thus, VD3 directly interfaces with multiple regulatory mechanisms that inhibit proliferation and stimulate differentiation.

The antiproliferative effects of VD3 in normal osteoblasts are observed in preconfluent cells and can be so potent that they have the effect of inhibiting osteoblast maturation in vitro, as primary osteoblasts in culture require multi-layering to produce bone matrix for mineralization *(24–26)*. However, if VD3 is added to post confluent committed osteoblasts, the induction of osteoblast-related genes is observed. These genes, e.g., osteocalcin and osteopontin, contribute to forming the bone extracellular matrix and become significantly upregulated enhancing further maturation of the osteoblasts and osteoblast mineralization. One must always keep in mind that vitamin D functions as an "enhancer" or "repressor" of genes related to differentiation and does not have the ability to induce a phenotype per se, as the morphogen BMP2. Its cell differentiation properties are coupled to antiproliferative properties and the ability of the vitamin D receptor complex to interact with other transcriptional regulators.

In contrast to inhibitory effects of vitamin D on osteoblasts in vitro at pharmacologic doses due to antiproliferative effects, in vivo studies have identified bone anabolic

effects of vitamin D. A VDR transgene was expressed in mice in mature osteoblast under control of the osteocalcin gene promoter and this resulted in an increase in both cortical and trabecular bone. This study highlights the importance of delineating the effect of the vitamin D ligand on specific subpopulations of osteoblast lineage cells for their selective effects. Our current understanding is that VD3 is an important determinant for regulating distinct activities within cells of the osteoblast lineage, for bone homeostasis which maintains the critical balance between bone resorption, and for bone formation activities *(5, 6, 27, 28)*.

Apoptosis has been proposed to play a key role in controlling osteoblast homeostasis, particularly in the formation of new bone where only a limited number of osteoblasts on the bone surface synthesizing the osteoid will survive as osteocytic cells in the mineralized tissue. Vitamin D has well-known pro-apoptotic properties in cancer cells (see Chapters 41 and 47), but not in normal osteoblasts *(29)*. In human osteoblasts, apoptosis was shown to occur through activation of FAS ligand. Treating the cells with $1,25(OH)_2D$ exerted an anti-apoptotic effect on this pathway through downregulation of components of the mitochondrial- and FAS-related pathways. Expression of the pro-apoptotic BAX protein was decreased with a complimentary increase in the anti-apoptotic BCL protein *(30)*. Thus, vitamin D may also be contributing to osteoblast maturation at the mature osteoblast–pre-osteocyte stage by protecting cells for the transition to osteocytes in a mineralized matrix.

3. THE ROLE OF VITAMIN D IN COUPLING OSTEOBLAST ACTIVITY TO OSTEOCLAST DIFFERENTIATION FOR BONE RESORPTION

An essential calcitrophic hormone function of VD3 is the regulation of bone resorbing osteoclasts indirectly through hormone effects on the osteoblast that secrete factors essential for osteoclastogenesis, as well as directly regulating activity of the differentiated osteoclast (Fig. 2). Vitamin D contributes to the coupling of osteoclast and osteoblast activities at two stages of the remodeling process, thereby mediating completion of the bone remodeling sequence. Initially, VD3 targets osteoblasts and lining cells to retract from the bone surface and secrete VD3-responsive factors which induce osteoclast activity. At the same time, VD3 directly promotes osteoclast formation from mononuclear cells; for example, $1,25(OH)_2D$ regulates carbonic anhydrase II *(31, 32)*. Following the resorption phase, VD3 can stimulate synthesis of cytokines for pre-osteoblast recruitment and growth and expression of osteoblast proteins which form the bone matrix. In this manner, VD3 contributes to the completion of the bone remodeling sequence.

Osteoclasts are of hematopoietic origin and the progenitors can be recruited from marrow, spleen, and blood *(33, 34)*. Immature hematopoietic cells, circulating monocytes, and some tissue macrophages are capable of differentiating into osteoclasts. Characteristic features of the actively resorbing osteoclast include many unique morphological features essential for their bone resorbing functions, the clear sealing zone and ruffled border *(35, 36)*. The clear zone serves to attach osteoclasts to the bone surface and separates the bone resorption area (the Howship lacunae) from the unresorbed bone to create an acidic compartment. A multi-component complex is involved

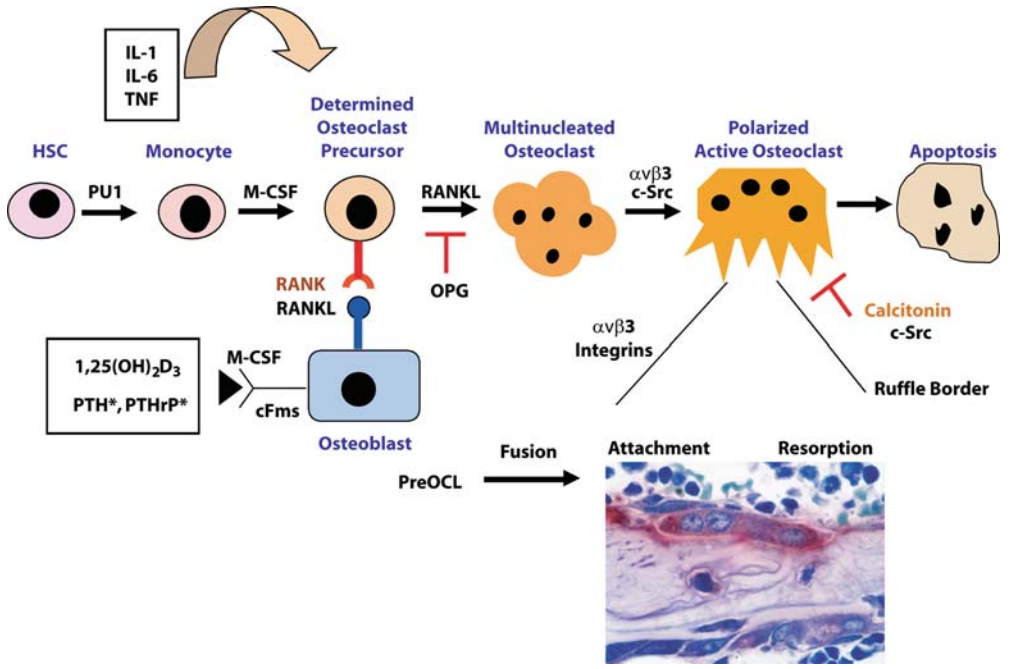

Fig. 2. Osteoclast differentiation, activation, and regulation. Stages of maturation illustrated with regulatory factors driving the differentiation stages and final adhesions and activity of the multinucleated osteoclasts onto the bone surface. Cytokines and hormones contributing to RANKL–RANK interactions between osteoblast lineage cells and mononuclear osteoclast precursors that is the critical step for differentiation. A histologic section of a bone spicule is shown with the osteoclast stained for tartrate-resistant acid phosphatase enzyme activity. On the opposing side of the trabecular bone are cuboidal osteoblasts.

in the attachment of the osteoclast to bone that includes osteopontin and the integrin αvβ3 and synthesis of these proteins are increased in response to vitamin D *(37–39)*. The ruffled border, a structure of deeply infolded plasma membrane, has a large surface area for secretion of protons and enzymes to dissolve the bone and returns ions into the cell for entering the circulation. During bone resorption, besides release of the mineral, chemotactic and mitogenic factors which are stored in the bone matrix become active (for example, TGFβ) *(40)* and are important for the coupling of bone resorption to later bone formation after resorption has normalized calcium levels in the circulation. These released factors can recruit osteogenic precursors and inhibit osteoclast differentiation, thereby providing a negative feedback mechanism for bone resorption *(24–26)*. For osteoclasts to adhere to bone, osteoid on the bone surface must be removed to expose a mineralized surface. Vitamin D increases expression of MMP13, an enzyme secreted from osteoblast to carry out this function *(41)*.

To accomplish this program, $1,25(OH)_2D$ directly regulates genes essential for each stage of osteoclastogenesis. RANKL in osteoblasts and other cells is required for fusion of mononuclear hematopoietic precursors for osteoclast differentiation. RANKL, anchored on osteoblast cell surfaces, interacts with RANK on the surface of osteoclast

precursors, a coupling reaction that leads to the differentiated multinucleated osteoclasts. The RANK receptor expressed in monocytes and osteoclasts is increased by $1,25(OH)_2D$ *(42, 43)*. A well-characterized VDRE and cooperating CCAAT box in the RANKL promoter respond to $1,25(OH)_2D$ by recruitment of the VDR, chromatin remodeling factors, and RNA polymerase II *(44)*. Runx2 also participates $1,25(OH)_2D$-dependent activation of RANKL by remodeling chromatin *(45)*.

Also present in osteoblasts is an inhibitor of osteoclast differentiation, osteoprotegerin (OPG), which is a soluble form of RANKL that binds to RANK to block RANKL interactions *(46)* and thereby prevents fusion of mononuclear precursors. This OPG "decoy receptor" inhibitor of osteoclast differentiation is downregulated by $1,25(OH)_2D$ in mature osteoblasts, thus favoring bone resorption *(47)*. Notably, in MC3T3 pre-osteoblasts, OPG levels are undetectable, further suggesting that vitamin D preferentially regulates the mature osteoblast to resorb bone *(48, 49)*. Interestingly, the OPG gene does not have a consensus negative VDRE, yet is clearly regulated by vitamin D. The suppression is via an AP-1 site which is influenced by $1,25(OH)_2D$ *(46)*.

4. VITAMIN D REGULATION OF GENE EXPRESSION DURING BONE FORMATION

Vitamin D regulation is principally mediated through modulation of transcription. Recent reports indicate that vitamin D controls the expression of at least 913 genes and it may possibly affect, directly or indirectly, the expression of as many as 27,000 genes *(50)*. Vitamin D binds to VDR, which then interacts with specific elements located within the regulatory regions of target genes. Combinatorial- and context-dependent protein–protein interactions with other transcription factors or cofactors bound at specific promoter elements may further modify transcription. Here, physiological control requires that coregulatory proteins determine specificity of biological responsiveness to regulatory cues. It is becoming increasingly evident that organization and assembly of VDR-regulatory complexes are dynamic rather than static *(51, 52)*. Modifications in the composition of these regulatory complexes provide a mechanism for integrating regulatory signals to support positive and negative control through synergism and antagonism, respectively.

4.1. Components of Vitamin D-Dependent Regulatory Complexes

The role that vitamin D plays in bone metabolism provides a paradigm for understanding molecular mechanisms that operate in vitamin D action. Vitamin D directly regulates the expression of genes that support bone formation during development and bone remodeling throughout life. Therefore, osteoblast differentiation is a model for understanding developmental responsiveness to vitamin D *(53, 28)*.

Vitamin D exerts its genomic effects through the VDR which is a member of the superfamily of nuclear receptors *(51, 54)*. As in other nuclear receptors, binding of the ligand induces conformational changes in the C-terminal ligand binding domain (LBD) of the VDR. The changes establish competency for VDR interaction with coactivators

of the p160/SRC family, including SRC-1/NCoA-1, SRC-2/NCoA-2/GRIP/TIF2, and SRC-3/ACTR. These complexes are critical for transcriptional activation *(51, 53, 54)*. p160/SRC coactivators form high molecular weight complexes by interacting with other coactivator proteins including p300, its related homologue CBP, and P/CAF *(55)*. Moreover, p160/SRC coactivators have been shown to recruit CBP/p300 and P/CAF to ligand-bound nuclear receptors. Multiprotein complexes containing different activities are functionally linked to ligand-dependent transcriptional regulation *(51)*. Coactivators such as SRC-3/ACTR, SRC-1/NCoA-1, CBP/p300, and P/CAF contain intrinsic histone acetyl transferase (HAT) activity. Therefore, protein complexes including independent HAT activities can be recruited to gene promoters by nuclear receptors in a ligand-dependent manner *(51)*. Once bound to these promoters, the HAT activities contribute to chromatin remodeling events that increase access of additional regulatory factors to their cognate elements *(56)*. The coactivator NCoA62/SKIP can also interact with VDR in a ligand-dependent manner. However, this protein–protein interaction occurs through a domain of VDR that is different from that recognized by p160/SRC or DRIP205 coactivators *(57)*. Moreover, NCoA62/SKIP can form a ternary complex with VDR and SRC-1 to cooperatively stimulate VDR-mediated transcription activation.

The multisubunit DRIP (VDR-interacting protein) complex also binds to VDR in response to the ligand vitamin D *(58, 59)*. This interaction occurs through the LBD of VDR in the same manner as the p160/SRC coactivators, resulting in transcriptional enhancement *(60)*. In contrast to p160/SRC coactivators, DRIP is devoid of HAT and other chromatin remodeling activities and interacts with nuclear receptors through a single subunit designated DRIP205, which anchors other subunits to the receptor LBD. Several of these subunits are also present in the Mediator complex, which interacts with the C-terminal domain (CTD) of RNA polymerase II, forming the holoenzyme complex *(61)*. Therefore, the DRIP complex appears to function as a transcriptional coactivator by forming a molecular bridge between the VDR and the basal transcription machinery, reflecting the importance of three-dimensional promoter organization to regulatory activity.

An ATP-dependent chromatin remodeling complex that binds to VDR and that is named WINAC (for WSTF [Williams Syndrome Transcription Factor] including nucleosome assembly complex) was recently reported *(62)*. WINAC shares components with two other chromatin remodeling complexes, SWI/SNF and ISWI, and has been proposed to mediate recruitment of unliganded VDR to target genes. Nevertheless, subsequent interaction of the targeted VDR with transcriptional corepressors requires the presence of vitamin D. This complex has been reported to be involved in transcriptional repression *(63, 64)* and in controlling DNA replication *(62)*.

In the last few years various investigators have shown that coactivator complexes including p160/SRC and DRIP are recruited to steroid hormone-regulated genes by nuclear receptors in a sequential and mutually exclusive manner *(65–68)*. The ordered association of transcriptional regulators exhibits binding kinetics with periods of 40–60 min. These results provided the basis for a model in which cyclical association of different coactivator complexes reflects the dynamics of the transcription activation

process of nuclear receptor-regulated genes *(69)*. Alternatively, recent reports also indicate that occupancy at the target gene regulatory regions by nuclear receptor-associated coactivator complexes may also occur gradually and at a significantly lower rate *(70, 71)*. Thus, the molecular mechanisms by which VDR-associated coactivator complexes are recruited to target genes may be context dependent, that is, directly related to the nature of the regulatory factors bound to each particular promoter (see below).

4.2. Vitamin D-Mediated Gene Expression Within the Three-Dimensional Context of Nuclear Structure in Bone Cells

Evidence is accumulating that the architectural organization of nucleic acids and regulatory proteins within the nucleus supports functional interrelationships between nuclear structure and gene expression. There is increasing acceptance that components of nuclear structure are functionally linked to the organization and sorting of regulatory information in a manner that permits utilization (reviewed in Zaidi et al. *(72)*). The primary level of organization, the representation and ordering of genes and promoter elements, provides alternatives for physiological control. The molecular organization of regulatory elements, the overlap of regulatory sequences within promoter domains, and the multipartite composition of regulatory complexes increase options for responsiveness. Chromatin structure and nucleosome organization reduce distances between regulatory sequences, facilitate cross talk between promoter elements, and render elements competent for interactions with positive and negative regulatory factors. The components of higher order nuclear architecture, including nuclear pores *(73)*, the nuclear matrix, and subnuclear domains, contribute to the subnuclear distribution and activities of genes and regulatory factors *(72, 74)*. Compartmentalization of regulatory complexes is illustrated by focal organization of PML bodies *(75)*, Runx bodies *(76, 77)*, the nucleolus *(78)*, and chromosomes *(79)*, as well as by the punctuate intranuclear distribution of sites for replication *(80)*, DNA repair *(81)*, transcription *(82)*, and the processing of gene transcripts *(83–85)*.

There is emerging recognition that nuclear structure and function are causally related. Interestingly, it has been reported that in several mammalian cells, including osteoblastic cells, VDR exhibits a punctate nuclear distribution that is enhanced upon vitamin D stimulation *(86–88)*, raising the possibility that VDR is associated with components of the nuclear architecture. Furthermore, recent results from our group indicate that VDR is bound to the nuclear matrix fraction of several osteoblastic cells in a ligand-dependent manner (Arriagada et al., Unpublished results). Similarly, Zhang et al. *(87)* have reported that the NCoA62/Skip protein, which binds to VDR and functions as a transcriptional coactivator, is also bound to the nuclear matrix in osteoblastic cells. Therefore, association of VDR with components of the nuclear architecture is dynamic rather than static. The bone-specific OC gene and skeletal-restricted Runx2 transcription factor serve as examples of obligatory relationships between nuclear structure and vitamin D-mediated physiological control of skeletal gene expression *(52, 89)*. It appears that there are similar relationships between nuclear organization and other bone-related vitamin D-responsive genes (e.g., osteopontin and 24-hydroxylase).

4.3. Vitamin D Receptor Coregulatory Factors Provide Gene-Specific Regulation

The rat OC gene encodes a 10 kDa bone-specific protein that is induced in osteoblasts with the onset of mineralization at late stages of differentiation *(90)*. Modulation of OC gene expression during bone formation and remodeling requires physiologically responsive accessibility of proximal and upstream promoter sequences to regulatory and coregulatory proteins, as well as protein–protein interactions that integrate independent promoter domains *(91)*. The chromatin organization of the OC gene illustrates dynamic remodeling of a promoter to accommodate requirements for phenotype-related developmental and vitamin D-responsive activity *(92)*.

Transcription of the OC gene is controlled by modularly organized basal and hormone-responsive promoter elements (see Fig. 2a) located within two DNase I-hypersensitive sites (Distal site, positions –600 to –400; proximal site, positions –170 to –70) that are only nuclease accessible in bone-derived cells expressing this gene *(91)*. A key regulatory element that controls OC gene expression is recognized by the VDR complex upon ligand stimulation. This vitamin D-responsive element (VDRE) is located in the distal region (Fig. 3a) of the OC promoter (positions –465 to –437) and functions as an enhancer to increase OC gene transcription *(92)*. Another key regulator of OC gene expression is the nuclear matrix-associated transcription factor Runx2, a member of the Runt homology family of proteins which has been shown to contribute to the control of skeletal gene expression *(89)*. Runx2 proteins serve as a scaffold for the assembly and organization of coregulatory proteins that mediate biochemical and architectural control of promoter activity. The rat OC gene promoter contains three recognition sites for Runx2 interactions, site A (–605 to –595), site B (–438 to –430), and site C (–138 to –130). Mutation of all three Runx2 sites results in significantly reduced OC expression in bone-derived cells *(93)*. The retention of a nucleosome between the proximal and the upstream enhancer domains reduces the distance between the basal regulatory elements and the VDRE and supports a promoter configuration that is conducive to protein–protein interactions between VDR-associated proteins and components of the RNA polymerase II-bound complex (Fig. 2b). Interaction of the VDR at the distal promoter region of the OC gene requires nucleosomal remodeling *(94, 95)*.

We have recently shown that within the OC gene promoter context there is a tight functional relationship between Runx2 and the vitamin D-dependent pathway *(88)*. Runx2 and VDR are components of the same nuclear complexes, colocalize at punctate foci within the nucleus of osteoblastic cells, and interact directly in protein–protein binding assays in vitro *(88)*. Additionally, mutation of the distal Runx2 sites A and B (which flank the VDRE, see Fig. 2a) abolishes vitamin D-enhanced OC promoter activity *(88)*. In contrast to most nuclear receptors, the VDR does not contain an N-terminal AF-1 transactivation domain and thus is unable to interact with coactivators through this region *(51)*. Therefore, Runx2 plays a key role in the vitamin D-dependent stimulation of the OC gene promoter in osteoblastic cells by directly stabilizing binding of the VDR to the VDRE. Runx2 also allows recruitment of the coactivator p300 to the OC promoter (Fig. 3a), which results in upregulation of both basal and vitamin D-enhanced OC gene transcription *(96)*. Based on these results, we have postulated that

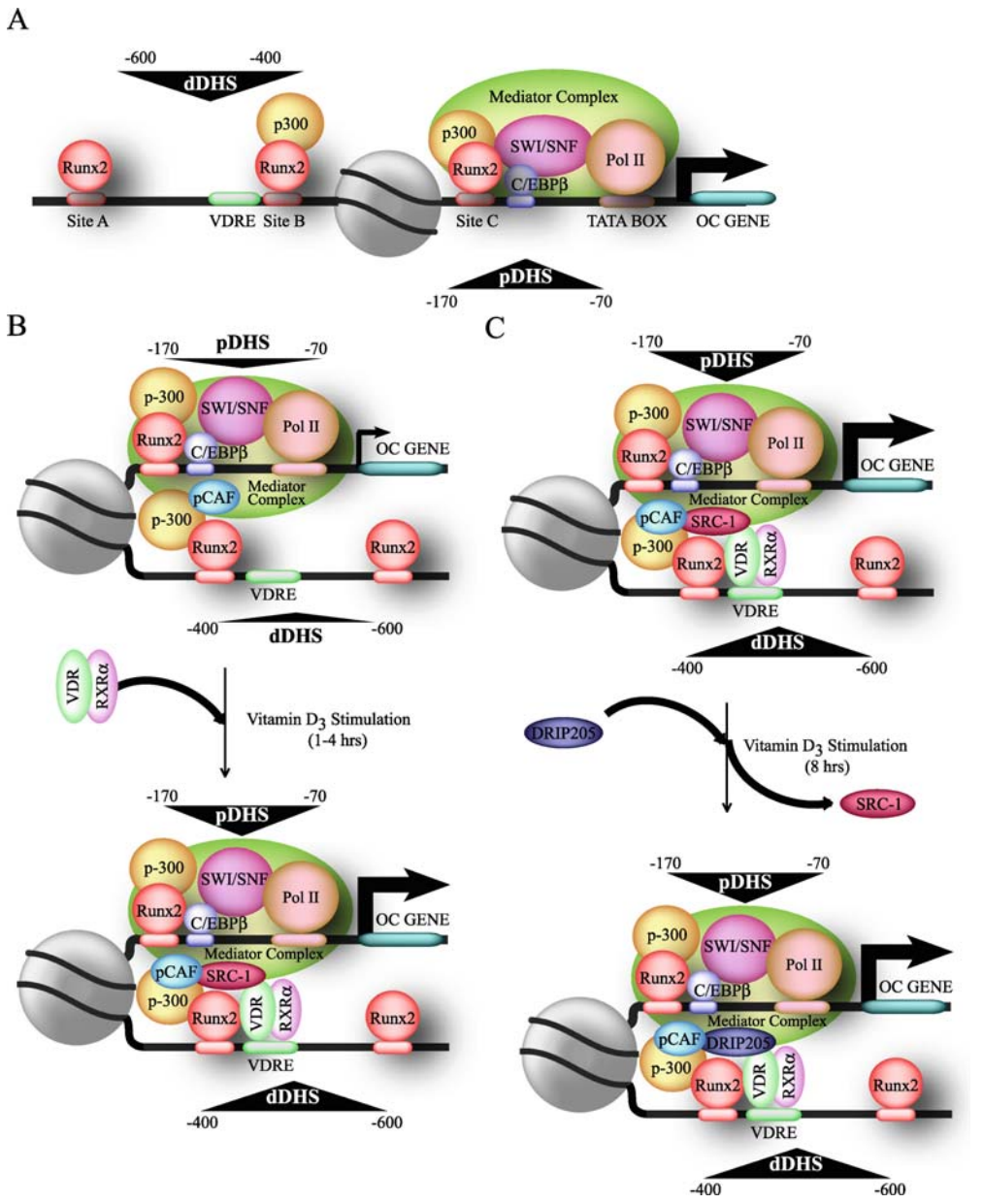

Fig. 3. (**a**) Schematic representation of the rat osteocalcin (OC) gene promoter transcribing at basal levels. The *circle* in the *middle* represents a positioned nucleosome flanked by a distal and proximal DNase I-hypersensitive sites (dDHS and pDHS, respectively). The different regulatory elements and the transcription factors that bind them in osteoblastic cells expressing the OC gene are also indicated. (**b**) Proposed three-dimensional organization of the OC promoter transcribing at basal levels or enhanced by vitamin D. The stimulatory effect of the VDR-associated coactivators on the general transcription machinery is represented by the size of the *arrows* at the transcription start site. The positioned nucleosome facilitates DNA bending and the functional interactions between distal and proximal promoter regulatory elements that are bound by the cognate factors. (**c**) After several hours of vitamin D treatment, DRIP205 is recruited to the OC promoter and SRC-1 is released. VDRE, vitamin D-responsive element; Pol II, RNA polymerase II.

Runx2-mediated recruitment of p300 may facilitate the subsequent interaction of p300 with the VDR upon ligand stimulation *(88)*.

The rate of recruitment of p160/SCR-1 and DRIP coactivators to the OC gene in response to vitamin D has recently been studied *(71)*. It has been found that the VDR and SRC-1 rapidly and stably interact with the distal region of the OC promoter encompassing the VDRE (Fig. 2b). The interaction of SRC-1 and VDR directly correlates with vitamin D-mediated transcriptional enhancement of the OC gene, increased association of the RNA polymerase complex and vitamin D-stimulated histone H4 acetylation *(71, 97)*. Interestingly, DRIP205 was found to bind to the OC promoter only after several hours of continuous treatment with vitamin D, concomitant with release of SRC-1 (see Fig. 3c). Based on these results it has been postulated that this preferential recruitment of SRC-1 to the OC gene promoter is based on the specific distribution of regulatory elements at the distal region of the promoter. This organization may lead to the formation of a stable complex at the distal region that includes Runx2, p300, VDR, and SRC-1. Once established, this complex may directly stimulate the basal transcription machinery bound to an OC promoter actively engaged in transcription. The general relevance of vitamin D-mediated chromatin-based mechanisms of promoter activity, accessibility, and cross talk between regulatory domains is illustrated by the vitamin D responsiveness of the osteopontin and 24-hydroxylase genes.

Recent studies have established that vitamin D induces a rapid and cyclical association of the VDR/RXR heterodimer with the proximal mouse 24-hydroxylase gene promoter in osteoblastic cells *(68)*. Vitamin D treatment also induces a rapid recruitment of coactivators such as p160/SRC and p300/CBP, which leads to acetylation of histone H4. DRIP205/Mediator is also recruited to the proximal promoter region concomitantly with the interaction of RNA polymerase II. Together, these results support a model in which highly dynamic association of the VDR with chromatin occurs during vitamin D-dependent induction of the 24(OH)ase gene in osteoblasts *(68)*.

Carlberg et al. have also monitored the spatio-temporal regulation of the human 24(OH)ase gene *(98)*. They have evaluated 25 contiguous genomic regions spanning the first 7.7 kb of the human 24(OH)ase promoter and found that in addition to the proximal VDREs, three further upstream regions are associated with the VDR upon vitamin D stimulation. Interestingly, only two of these regions contain sequences that resemble known VDREs that are transcriptionally responsive to this hormone. The other VDR-associated upstream promoter region does not contain any recognizable classical VDRE that could account for the presence of the VDR protein. However, simultaneous association of the VDR, RXR, p160/SRC, and DRIP/Mediator coactivators, as well as RNA polymerase, was detected in all four vitamin D-responsive sequences after the addition of the ligand *(98)*. Remarkably, despite participating in the same process, all four chromatin regions displayed individual vitamin D-dependent patterns of interacting proteins. Based on these results, the authors propose that these upstream vitamin D-responsive regions may have a role in the implementation of gene activation, as they raise their vitamin D-dependent histone H4 acetylation status earlier than that of the proximal promoter VDREs *(98)*. It has also been suggested that the simultaneous communication of the individual promoter regions with the RNA polymerase II complex occurs through a particular three-dimensional organization of the chromatin at the 24(OH)ase promoter.

This arrangement could be facilitating close contact between distal and proximal regulatory regions.

On the other hand, Pike et al. have described that upon vitamin D treatment of osteoblastic cells, there is a rapid and cyclical association of the VDR with the OP promoter *(68)*. This increased binding of the VDR parallels vitamin D-mediated transcriptional enhancement of the OP gene and additionally involves cyclical, sequential, and mutually exclusive recruitment of the coactivators p160/SRC, p300/CBP, and DRIP/Mediator. Interestingly and in contrast to the OC and 24(OH)ase genes, p160/SRC-p300/CBP binding does not result in increased histone H4 acetylation. These results further confirm that in osteoblastic cells different promoters are regulated by distinct mechanisms in response to vitamin D.

Recent reports have also described the molecular components of VDR-containing nuclear complexes that repress transcription. Thus, Kato and colleagues have identified a bHLH-type factor, the VDR-interacting repressor (VDIR), which directly binds to an inhibitory VDRE located in the human 1α-hydroxylase gene promoter. Binding of VDIR in the absence of ligand results in transcriptional activation of the 1α-hydroxylase gene *(99)*. Interestingly, vitamin D-induced association between VDR and VDIR leads to ligand-dependent transrepression of the 1α-hydroxylase gene. The mechanism involves an exchange in the VDR-associated coregulatory complex from the coactivator p300 to an HDAC-containing corepressor complex. This exchange is accompanied by a significant decrease in histone acetylation at the 1α-hydroxylase gene promoter and by a release of the RNA polymerase II basal transcriptional machinery *(64)*. The same group has indicated that the complex WINAC functions in this VDR-mediated transrepression of the 1α-hydroxylase gene through its ability to efficiently bind the acetylated nucleosomes present in the 1α-hydroxylase promoter region in the absence of vitamin D *(64)*.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Vitamin D serves as a principal modulator of skeletal gene transcription, thus necessitating further understanding of interfaces between activity of this steroid hormone with regulatory cascades that are functionally linked to regulation of skeletal genes *(53, 52)*. There is growing appreciation for the repertoire of factors that influence gene expression for commitment to the osteoblast lineage. It is well documented that sequentially expressed genes support progression of osteoblast differentiation through developmental transition points where responsiveness to phosphorylation-mediated regulatory cascades determines competency for establishing and maintaining the structural and functional properties of bone cells *(100, 101)*. The catalog of promoter elements and cognate regulatory proteins that govern skeletal gene expression offers essential but insufficient insight into mechanisms that are operative in intact cells. Gene promoters serve as regulatory infrastructure by functioning as blueprints for responsiveness to the flow of cellular regulatory signals. However, access to the specific genetic information requires transcriptional control of skeletal genes within the context of the subnuclear organization of nucleic acids and regulatory proteins. Explanations are required for (i) convergence of multiple regulatory signals at promoter sequences; (ii) the integration of regulatory information at independent promoter domains; (iii) selective utilization of redundant

regulatory pathways; (iv) thresholds for initiation or downregulation of transcription with limited intranuclear representation of promoter elements and regulatory factors; (v) mechanisms that render the promoters of cell growth and phenotypic genes competent for protein–DNA and protein–protein interactions in a physiologically responsive manner; (vi) the composition, organization, and assembly of sites within the nucleus that support transcription, and (vii) the intranuclear trafficking of regulatory proteins to transcriptionally active foci. From a regulatory perspective compartmentalization of components of VD3 control supports the integration of regulatory activities, perhaps by establishing thresholds for protein activity in time frames that are consistent with the execution of regulatory signaling.

It is necessary to understand how the regulatory machinery for vitamin D responsiveness is retained during mitosis to support gene expression in post-mitotic progeny cells. Key components of nuclear architecture are dismantled and must be re-organized to support tissue-specific transcription. In this context, we have recently reported that within the condensed mitotic chromosomes Runx2 is retained in large discrete foci at nucleolar organizing regions where ribosomal RNA genes reside *(102)*. These Runx2 chromosomal foci are associated with open chromatin, colocalize with RNA polymerase I transcription factor UBF1, and transition into nucleoli at sites of rRNA synthesis during interphase. Runx2 forms complexes with UBF1 and SL1 (another RNA pol I factor), cooccupies the rRNA gene promoter with these factors in vivo, and affects local chromatin histone modifications at rDNA regulatory regions as well as competency for transcription. Hence, these findings indicate that Runx2 not only controls lineage commitment and cell proliferation by regulating genes transcribed by RNA pol II, but modulates also RNA pol I-mediated rRNA synthesis *(102)*. A similar focal retention of Runx transcription factors with mitotic chromosomal loci of RNA polymerase II target genes that are functionally consequential has recently been observed *(103)*. Taken together, it appears that in addition to DNA methylation and post-translational modifications of histones, the mitotic occupancy of genes that support proliferation, cell growth, and phenotype by transcription factors and coregulatory proteins is an additional dimension to epigenetic control.

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