

# Pleiotropic Activities of Vitamin D Receptors – Adequate Activation for Multiple Health Outcomes

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## **Abstract**

The vitamin D receptor (VDR), a nuclear transcription factor, elicits physiological regulation of gene transcription following binding of its ligand, 1,25-dihydroxyvitamin D. The major biological activities of vitamin D contribute to regulation of plasma calcium and phosphate homeostasis and bone remodeling, although recent evidence suggests that vitamin D, like other steroid hormone receptors, can regulate a diverse range of biological activities across many tissues. Such properties raise the notion that vitamin D deficiency may not only be detrimental to bone and muscular health, but also a risk factor for a number of adverse health outcomes including increased risk of cardiovascular disease, inflammation, immune system disorders and cancer. Advances in transcriptional research provide data not only on ligand-dependent activities of the VDR, but other activities of vitamin D extending to rapid modulation of intra-cellular signaling pathways as well as apparent ligand-independent interactions between the VDR and other transcriptionally active proteins. In this review, we detail the chief molecular activities of the VDR in regulating gene transcription, intracellular signaling and actions of VDR via binding to transcriptional regulating proteins. The breadth of biological activities attributed to vitamin D informs clinical biochemists and health care professionals on the implications of vitamin D deficiency for health.

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## **Introduction**

The vitamin D receptor (VDR) is a nuclear transcription factor responsible for the biological activity of vitamin D by binding its ligand, the active form of the vitamin D hormone, 1,25-dihydroxyvitamin D (1,25D). Present in a diverse range of tissues, the VDR has the ability to exert an extensive biological response, when activated by ligand-binding, via regulation of gene transcription and stimulation of intracellular signaling pathways. The dominant action of the VDR, as evidenced by genetic knockout models and clinical evidence,<sup>1-4</sup> is to regulate plasma calcium and phosphate homeostasis via stimulation of intestinal calcium absorption, renal tubular reabsorption of calcium and resorption of bone. More recently, vitamin D has been shown to play a role in cell function outside of calcium and mineral homeostasis including inhibition of cellular proliferation and stimulation of cell maturation which may involve various tissues including skin, the immune system and possibly others such as colonic, breast and prostate cells. Moreover, vitamin D deficiency is associated with a number of adverse health outcomes including increased risk of cardiovascular disease,

inflammation, diabetes and disturbed hair follicle cycling.<sup>5-7</sup> Whether vitamin D deficiency exerts a causal role with these extraskeletal systems remains to be established.

Recent advances in transcriptional research, such as the development of chromatin immunoprecipitation (ChIP) analysis, have increased understanding of vitamin D's ability to influence gene transcription via ligand-dependent actions.<sup>8</sup> However, the ability for vitamin D to exert a diverse range of physiological activities extends beyond this classical interpretation of activity to facilitate the more recently discovered functions of 1,25D and VDR including regulation of gene expression via complex intracellular signaling pathways. Since vitamin D plays a key role in fundamental physiological processes and also in the pathophysiology of many disease states, it is important to understand the breadth of biological activities, which are under the control of vitamin D to better inform clinical biochemists and health care professionals of the implications of vitamin D deficiency.

### Regulation of Gene Transcription

Binding of the ligand, 1,25D, to its receptor, VDR, initiates heterodimerisation with the retinoid-X receptor (RXR) at a vitamin D response element (VDRE), a specific DNA sequence located within the promoter region of vitamin D responsive genes.<sup>9</sup> Binding of the liganded, heterodimerised VDR complex (VDR-RXR) to the VDRE directs the formation of a large protein complex that incorporates various co-regulatory molecules. The transcriptional complex activates gene transcription largely through chromatin remodeling and thus represents the specificity and sensitivity of the VDR. Chromatin remodeling is a central function of this transcriptional complex when bound to the VDRE. In the case of gene transcription, the complex recruits steroid

receptor co-activators, such as steroid receptor coactivator-1 (SRC-1),<sup>10</sup> vitamin D receptor interacting proteins (DRIP) such as MED1<sup>11</sup> and NCoA62<sup>12</sup> in addition to histone acetyltransferases to derepress the locally condensed chromatin. The ultimate result is upregulation of a range of genes required for normal calcium and bone homeostasis.<sup>13-18</sup> Furthermore, the VDR-RXR heterodimer may also act in unison with other corepressor molecules and potentiate repression of the expression of genes such as parathyroid hormone.<sup>19</sup> After recruitment of the transcriptional complex, VDR-RXR synergises with vitamin D receptor interacting protein (DRIP) to recruit components of the basal molecular machinery, such as RNA polymerase II, necessary to initiate gene transcription (Figure 1).<sup>20</sup>

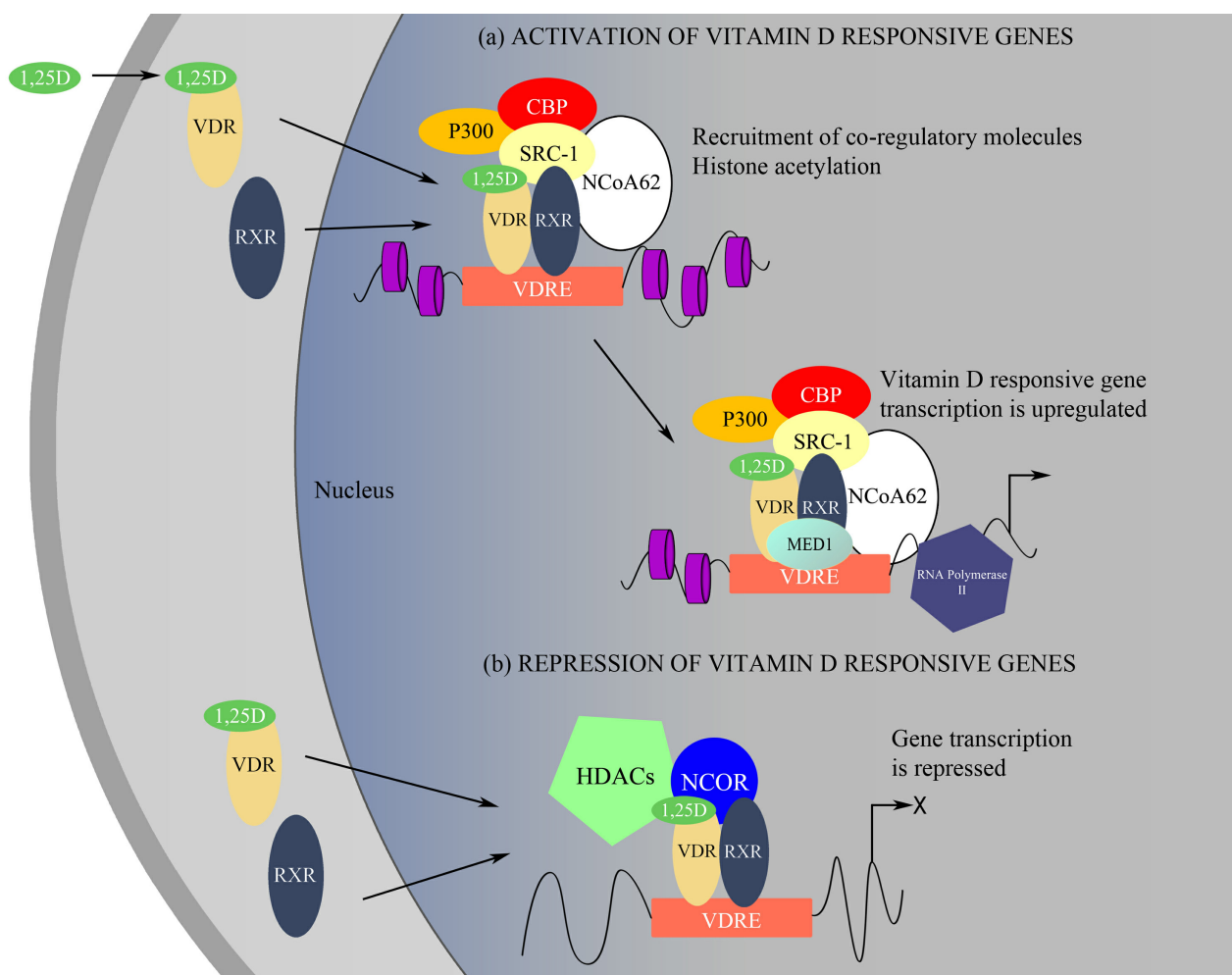


Figure 1. Classical pathway of VDR mediated gene regulation. Exogenous 1,25D enters the cell and binds to the classical VDR, initiating heterodimerisation with RXR. The VDR-RXR complex then binds to a vitamin D responsive element within the genome and directs the formation of a large transcriptional complex. (a) For activation of vitamin D responsive genes, the transcriptional complex recruits co-activators such as NCoA62, steroid receptor co-activator-1 (SRC-1), CREB-binding protein (CBP) and P300 to initiate histone acetylation. Next, VDR-RXR synergises with mediator of RNA polymerase II transcription subunit 1 (MED1) to recruit molecular machinery such as RNA polymerase II for upregulation of gene transcription. (b) VDR-RXR complex can act in unison with co-repressors such as nuclear receptor co-repressor (NCOR) and histone deacetylases (HDACs) to repress gene transcription

It is the totality of the transcriptional complexes which defines the specificity and sensitivity of vitamin D to regulate biological responses through a wide range of tissues. Currently we understand the contribution of at least four elements of the transcriptional complex. In the case of vitamin D, the nuclear receptor ligand, 1,25D, identifies the physiological specificity of the response. The VDRE identifies the genetic specificity of the response. The various co-activators and other proteins complexing to the liganded VDR-RXR heterodimer bound to the VDRE identify the cell specificity and finally, the vitamin D-responsive gene product identifies the biological response.<sup>21</sup>

To regulate the level of 1,25D, the ligand-bound VDR complex must regulate its biosynthesis and degradation. Central to this process are the enzymes 1,25 dihydroxyvitamin D 24-hydroxylase (24-OHase), encoded by the *CYP24* gene and responsible for oxidation of 1,25D to water-soluble catabolites and 25 hydroxyvitamin 1alpha hydroxylase (1 $\alpha$ OHase), encoded by the *CYP27B1* gene and responsible for synthesis of 1,25D from 25-hydroxyvitamin D. As mentioned above, VDR-ligand interaction regulates expression of these genes allowing for exquisite control over the level of intracellular 1,25D. The 1,25D endocrine system arises from plasma levels of 1,25D, which in health are solely regulated through the expression of these genes in the kidney. Expression of these genes occurs across numerous tissues regulating 1,25D within each of these tissues for autocrine/paracrine activities.<sup>22</sup>

*CYP24* is expressed, as far as is known, in every cell which expresses the VDR. It is highly sensitive to 1,25D activation, owing to the fact that it contains two VDREs in its promoter region<sup>23-25</sup> although this varies between species.<sup>26</sup> Intriguingly, ChIP-chip and ChIP-seq analyses have revealed that *CYP24* also contains downstream enhancers some thousands of base pairs from the *CYP24* gene that contribute to VDR gene transcription signalling and protein recruitment.<sup>27</sup> The importance of VDR-ligand binding to 24-OHase levels is most apparent in mice in which the VDR gene has been deleted throughout the whole animal, the global-VDR knockout model, whereby levels of the water-soluble catabolites of 1,25D were found to be extremely low.<sup>28</sup> When the *CYP24* gene was deleted, mice displayed highly elevated plasma 1,25D levels and aberrant calcium homeostasis that greatly affected bone mineralisation processes.<sup>29</sup> Moreover, altered *CYP24* expression has been implicated in a wide variety of cancers, with the overarching suggestion that inhibition of *CYP24* expression may be useful as an anti-cancer therapeutic.<sup>30</sup> Recent data also suggest that 1,25D can also negatively regulate *CYP24* transcription through epigenetic events involving histone methylation.<sup>31</sup>

Regulation of intestinal calcium absorption is one of the most-well recognised aspects of vitamin D activity. Calcium

transport across intestinal epithelium is achieved through the ion channel transient receptor potential vanilloid type 6 (*TRPV6*).<sup>32</sup> Seminal *in vivo* studies by Song et al in 2003 demonstrated that 1,25D regulates mRNA expression of this gene and later work by Meyer and colleagues in 2006 demonstrated that *TRPV6* has multiple VDR binding sites that are necessary for transcriptional regulation of the gene via 1,25D.<sup>18, 33</sup> As is the case with *CYP24*, the upregulation of *TRPV6* requires VDR-RXR binding to several promoter region VDREs enabling histone acetylation to remodel the local chromatin environment, and enhance recruitment of coactivators to stimulate gene transcription. The ultimate physiological outcome has been well demonstrated in transgenic mice where over-expression of *TRPV6* led to significant increases in intestinal calcium absorption and had a marked effect on bone volume.<sup>34</sup> These results provide evidence that manipulation of gene expression is a key component of the physiological activity of the vitamin D hormone, particularly in relation to regulation of intestinal calcium absorption contributing to plasma calcium homeostasis.

Both the vitamin D endocrine and autocrine/paracrine systems support the formation of healthy bones and each of the major bone cell types have the capability to respond to 1,25D to mediate their activities, as well as to synthesise 1,25D.<sup>35-37</sup> Therefore, it is reasonable to suspect that 1,25D can regulate gene transcription of bone cell-specific factors. Indeed, seminal studies demonstrated 1,25D to upregulate expression of the gene coding for the protein receptor activator of nuclear factor kappa-B ligand (RANKL), which is a key controller of osteoclast differentiation.<sup>38</sup> *In vitro* analyses of the gene responsible for RANKL, *TNFSF11*, identified VDR-RXR complex binding to a number of VDREs, including some up to -76kb upstream of the *TNFSF11* promoter region in a portion of the genome termed the Distal Control Region (DCR).<sup>39, 40</sup> *In vivo* evidence illustrated the physiological impact of deletion of the DCR, demonstrably blunting the response of 1,25D and PTH and increasing bone mass and strength in a murine model due to decreased bone resorption.<sup>41</sup> Thus, VDR-ligand binding is important for RANKL gene expression and as such, plays a pivotal role in the regulation of plasma calcium and bone homeostatic processes. Moreover, 1,25D-induced transactivation of RANKL provides a very clear example of the regulatory networks that may be required for physiologically appropriate gene transcription.

Regulation of the calcium economy requires the cooperation of parathyroid hormone (PTH) and plasma 1,25D, with the latter having a powerful effect in decreasing transcription of the *PTH* gene as evidenced by early *in vitro* and *in vivo* studies.<sup>42-44</sup> Notably, mice lacking the VDR demonstrate marked increases in PTH as transcriptional repression is

lost.<sup>1</sup> Although the biological response is quite clear, how the VDREs within the *PTH* gene control a negative molecular response is currently poorly understood, though several groups have proposed sites that may modulate negative regulation.<sup>19,45,46</sup> By contrast, analysis of the VDRE in the avian *PTH* gene demonstrated that there may be several co-regulatory proteins that can in fact mediate a positive regulatory response and thus the VDR-RXR complex alone may not be the key to transcriptional regulation of *PTH*.<sup>47</sup> At present, there is little credible evidence that adequately demonstrates how 1,25D can transcriptionally repress *PTH* and further investigation into this mechanism is necessary to unravel the enigma of 1,25D gene repression.

### **Non-Transcriptional Activities of Vitamin D Membrane-Bound Receptors for 1,25D**

The traditional activities of 1,25D to modulate gene transcription are also complemented by its ability to regulate intracellular, extranuclear pathways and cytoplasmic signalling cascades. As opposed to the genomic activities described in the previous section which act over the course of hours to manifest changes in protein levels, these cytoplasmic actions are generally regarded to be rapid responses, taking place within seconds to minutes. A vast library of studies details the ability for 1,25D to affect intracellular calcium levels as well as intracellular signalling pathways involving phosphate kinases and phosphatases. Of note is the fact that activation of different signalling pathways are dependent on the cell type, thus providing an avenue to explain the pleiotropic effects of vitamin D.<sup>48-50</sup>

Nemere and colleagues have extensively researched the so-called non-genomic activities of 1,25D through investigation of membrane proteins and intracellular signalling. They have revealed that 1,25D can act through a distinct membrane-associated rapid-response steroid-binding (MARRS) protein<sup>51</sup> to facilitate rapid responses. This protein was found to be identical to the multifunctional protein disulfide isomerase family A, member 3 (PDIA3), an enzyme of the endoplasmic reticulum. Antibody blocking this protein prevented calcium and phosphate transport across intestinal epithelial cell membranes.<sup>51-52</sup> Further work also demonstrated PDIA3 can rapidly activate the protein kinase C pathway in chondrocytes and bone-forming osteoblasts.<sup>53-54</sup> Further studies determined that PDIA3 is located in plasma membrane caveolae and that it physically interacts with scaffolding proteins present in this location to activate signalling cascades.<sup>55</sup> *In vivo* ablation of the PDIA3 gene in mice confirmed the necessity of this protein in modulation of the rapid response to 1,25D.<sup>56</sup> Notably, isolation of primary osteoblasts from calvaria of both wild type and global VDR null mice showed rapid activation of this pathway occurred in response to 1,25D in both genotypes,

suggesting that these particular responses do not require the traditional nuclear VDR.<sup>57</sup>

Interestingly, several studies suggest the classical VDR can also associate with caveolae of the plasma membrane, like other steroid hormone receptors such as the estrogen receptor.<sup>58-61</sup> There are several isomers of 1,25D, and to elicit these non-genomic actions through the classical VDR, 1,25D must be in the 6-*s-cis* conformational shape, as opposed to the 6-*s-trans*. The former is responsible for the rapid biological response associated with non-genomic activities, whereas the latter is the preferred conformation for the genomic activities.<sup>62</sup> The importance of these conformation states has become more apparent subsequent to identification that the classical VDR may have two ligand-binding pockets<sup>63</sup> lending credence to the wide variety of biological activities proposed for 1,25D. Moreover, the classical VDR does seem necessary to stimulate some non-genomic responses such as intracellular calcium ion flux, as was demonstrated in ROS17/2.8 osteosarcoma cells.<sup>64</sup> Do the classical VDR and the PDIA3 protein at the plasma membrane work together to achieve biological activity? More recent data suggest that this may be the case as photoprotection of fibroblasts has been demonstrated to be a rapid non-genomic action of vitamin D that requires both proteins.<sup>65</sup> Ultimately, the rapid responses provided by 1,25D regulation of non-genomic pathways demonstrate the extensive variety of actions that the vitamin D system is required to maintain. Coupled with regulation of genomic actions, the extent to which vitamin D can regulate physiological processes is becoming clearer.

### **VDR Binding to Intracellular Proteins**

The VDR also exerts biological activities by directly binding to intracellular proteins to either stimulate VDRE-mediated genomic activity or by influencing the activity of other transcriptional regulating proteins. One of the most extensively researched proteins is  $\beta$ -catenin which has a dual function contributing to cell-cell adhesion when located at adherens junctions of the plasma membrane and to regulation of gene transcription. B-catenin functions in the Wnt signaling pathway as a regulator of gene transcription and has long been implicated in hair follicle cycling, malignancy and more recently, bone mineral homeostasis.<sup>66-68</sup> The activity of Wnt signaling is to mobilise  $\beta$ -catenin from the cytoplasm, where it is bound to the linker protein E-cadherin, to the nucleus allowing it to bind transcription factors of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families to promote gene expression. The vitamin D system can antagonise this B-catenin transcriptional process via two methods. The first is to reduce  $\beta$ -catenin signaling indirectly by genomic regulation of *CDHI*, encoding for E-cadherin. In a human colorectal cancer cell line expressing the VDR (SW480-

ADH), 1,25D markedly increased the level of E-cadherin expression via upregulating transcription of the E-cadherin gene. This coincided with disturbed  $\beta$ -catenin distribution, whereby 1,25D increased nuclear export and localisation to the plasma membrane<sup>69,70</sup> thus reducing proliferation of this cancer cell model. On the other hand, VDR can influence  $\beta$ -catenin signaling independent of E-cadherin upregulation, via direct binding<sup>69</sup> which relies upon the activator function-2 (AF-2) domain of the VDR.<sup>70</sup> Shah and colleagues revealed 1,25D represses  $\beta$ -catenin signaling equally in a colorectal cancer cell line with and without homozygous E-cadherin deletion demonstrating that the biological outcomes are a result of VDR- $\beta$ -catenin interaction.<sup>71</sup>

$\beta$ -catenin-VDR interactions have also been implicated as necessary factors in hair follicle cycling.<sup>5</sup> Mutations in the *VDR* gene giving rise to a defective receptor result in disordered calcium homeostasis and skeletal growth as well as alopecia in both mice and man.<sup>1</sup> While a high-calcium diet prevents the metabolic abnormalities, it does not reverse the alopecia.<sup>72</sup> In contrast, mice with deletion of *CYP27B1* do not display alopecia despite also displaying disordered calcium homeostasis, suggesting that the defect is not a result of loss of 1,25D.<sup>73</sup> When the VDR was specifically reconstituted into keratinocytes of VDR null (-/-) mice, the alopecia was reversed, yet the metabolic abnormalities could not be reversed, demonstrating for the first time that keratinocyte VDR is necessary for normal hair growth<sup>74</sup> and this occurs in an apparent ligand-independent manner.<sup>75</sup> The accumulating evidence indicate that the VDR can act independently of 1,25D however, further studies will be necessary to elucidate whether VDR must bind ligands other than 1,25D to mediate these biological actions.

In contrast to its effects in colorectal cancer cells, activation of the Wnt/ $\beta$ -catenin signaling pathway is responsible for increasing acquisition of bone. This is achieved through stimulation of the canonical Wnt signaling pathway by ligands such as LRP5, while decreasing levels of DKK-1 and SFRP2 which inhibit this pathway, ultimately increasing proliferation of osteoblasts and stimulating bone accrual. Of note, Wnt/ $\beta$ -catenin signaling is inhibited via the actions of sclerostin, a protein produced by osteocytes, demonstrating the importance of the canonical pathway to modulate bone homeostasis. There is potential for VDR and  $\beta$ -catenin to interact within bone cells to modulate bone formation, as preliminary *in vitro* evidence, using a human osteoblast-like osteosarcoma cell line, suggests that  $\beta$ -catenin modulation of gene transcription is affected by the unliganded VDR.<sup>76</sup> The weight of evidence suggests that VDR- $\beta$ -catenin interactions are another activity of the vitamin D system and some of these activities can occur without traditional VDR-1,25D binding. Again it is unknown

whether these 1,25D-independent activities of VDR require another, so far unidentified, vitamin D metabolite as a ligand or are truly ligand independent.

The interplay between the Wnt signaling pathway and vitamin D is just one of several pathways through which vitamin D exerts physiological effects within the body. Several other transcription factors and proteins are now being examined for similar interactions with the VDR lending further evidence to the fundamental role of vitamin D in cell processes such as cell proliferation and differentiation. One of these, the Class O Forkhead box (FoxO) proteins, are important transcription factors that, among their varied roles, control organism longevity and tumour suppression.<sup>77</sup> FoxO transcriptional regulation is activated by both deacetylation and dephosphorylation, ultimately resulting in transcriptional output. It is the activity of the cofactor Sirtuin 1 (Sirt1), a class III histone deacetylase, in addition to the VDR-RXR complex and the catalytic subunit of protein phosphatase 1 (PP1c) which directs this process. Intriguingly, both Sirt1 and PP1c can interact directly with the VDR, independent of 1,25D, though addition of the hormone provides enhancement of Sirt1 recruitment.<sup>78</sup> As such, the ligand-bound VDR is able to rapidly induce deacetylation and dephosphorylation, allowing for FoxO-mediated gene transcription to occur.<sup>79</sup>

## Conclusion

As with other steroid hormone receptors, the VDR exerts powerful and diverse physiological effects due to its capability to influence gene transcription in a number of ways. The vitamin D endocrine system has long been recognised as a major component of the regulation of plasma calcium and phosphate homeostasis which enables adequate muscular function, bone growth and mineralisation. These activities arise from plasma 1,25D activating the VDR in intestinal, renal and bone tissues to regulate gene transcription, predominantly through direct VDR-1,25D-chromatin interactions. More recently in the 21<sup>st</sup> century autocrine/paracrine sources of 1,25D have been confirmed to initiate activities in tissues including skin and bone. The vitamin D effects on gene transcription through direct binding to chromatin can be enhanced through activation of cytoplasmic intracellular or 'second messenger' signaling pathways regulated by the classical nuclear VDR or other membrane bound vitamin D receptors. Furthermore, VDR can directly interact with proteins to modulate gene transcription without directly binding to chromatin. These latter activities appear to be either dependent or independent of 1,25D binding. Whether those activities which are independent of 1,25D binding require another vitamin D metabolite to activate the VDR or are truly ligand independent is unknown at this time. Thus our understanding of the molecular underpinnings

of the vitamin D system has increased exponentially in line with the development of new technologies and methods. This knowledge extends the range of effects we attribute to vitamin D activity into the realms of immunity and tumor prevention.

In light of these findings, the importance of maintaining adequate vitamin D levels is now more pertinent than ever. The full implications of vitamin D deficiency go beyond regulation of vitamin D responsive genes and affect a variety of systems necessary for cell proliferation, growth, maturation and differentiation. Further investigations of the non-classical pathways regulated by vitamin D – whether mediated by liganded- or unliganded-VDR – are necessary for elucidating the vitamin D endocrine and autocrine/paracrine systems contributing to human health.

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