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## Vitamin D receptor signaling mechanisms: Integrated actions of a well-defined transcription factor

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### Abstract

The main physiological actions of the biologically most active metabolite of vitamin D, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>), are calcium and phosphorus uptake and transport and thereby controlling bone formation. Other emergent areas of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> action are in the control of immune functions, cellular growth and differentiation. All genomic actions of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> are mediated by the transcription factor vitamin D receptor (VDR) that has been the subject of intense study since the 1980's. Thus, vitamin D signaling primarily implies the molecular actions of the VDR. In this review, we present different perspectives on the VDR that incorporate its role as transcription factor and member of the nuclear receptor superfamily, its dynamic changes in genome-wide locations and DNA binding modes, its interaction with chromatin components and its primary protein-coding and non-protein coding target genes and finally how these aspects are united in regulatory networks. By comparing the actions of the VDR, a relatively well-understood and characterized protein, with those of other transcription factors, we aim to build a realistic positioning of vitamin D signaling in the context of other intracellular signaling systems.

### Keywords

Chromatin; Gene regulation; Genome-wide view; Nuclear receptor; Vitamin D; Vitamin D receptor

### 1. Introduction

The micronutrient vitamin D is essential for maintenance of health [1]. The most abundant form of vitamin D is 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>), the serum concentrations of which indicate the vitamin D status of a human individual [2]. The most biologically active vitamin D metabolite is the secosteroid 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, which acts as a pleiotropic endocrine hormone and influences many physiological processes [3]. For example, severe vitamin D deficiency leads to rickets, as 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> is essential for adequate Ca<sup>2+</sup> and P<sub>i</sub> absorption from the intestine and hence for bone formation [4].

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An appreciation of the  $1\alpha,25(\text{OH})_2\text{D}_3$  endocrine system precedes the isolation of the VDR by well over 400 years as rickets was first described in the beginning of the 17th century. However, the molecular etiology for rickets remained unresolved until the beginning of the 20th century, when it was discovered that the dietary deficiency that caused rickets could be ameliorated by fish oil extracts and that the active ingredient was identified as vitamin  $\text{D}_3$  [1]. Moreover, it was found that rickets could be cured by exposure to UV radiation. The analysis of  $1\alpha,25(\text{OH})_2\text{D}_3$  metabolism and the identification of  $25(\text{OH})\text{D}_3$  in the 1960's [4] was followed by the identification of vitamin D-binding proteins in the 1970's [5,6] and the cloning of the VDR (also referred to as NR1H1 in the generic nuclear receptor terminology) in 1988 [7]. All this leads to a functional understanding of the vitamin D endocrine system.

In the subsequent decades remarkable strides have been made in describing the diverse biology that the VDR participates in. Researchers accommodated this diversity of biological actions by separating functions into the so-called "classical" actions, i.e. the regulation of serum calcium levels [8], and "non-classical" actions, i.e. everything else that includes control of metabolism, cellular growth and immune functions [9]. In particular, immunoregulatory properties of  $1\alpha,25(\text{OH})_2\text{D}_3$  may be important, as low  $25(\text{OH})\text{D}_3$  levels are associated with poor immune function and increased disease susceptibility [10]. Perhaps now these views are beginning to be consolidated into more unified views of the actions of the VDR.

Although a number of rapid and non-genomic actions of  $1\alpha,25(\text{OH})_2\text{D}_3$  have been described [11], the vast majority of the effects of the hormone are mediated by the VDR, which is the only protein that binds  $1\alpha,25(\text{OH})_2\text{D}_3$  effectively at sub-nanomolar concentrations [12]. This simplifies the understanding of vitamin D signaling, since the physiological effects of the hormone largely overlap with the actions of the transcription factor VDR.

Taken together, the VDR system can be viewed as a comprehensively understood transcription factor in terms of both mechanistic insight and phenotypic consequences. In this review, we therefore focus on VDR and its actions from multiple perspectives. We will (i) illuminate VDR as a transcription factor and member of the nuclear receptor superfamily, (ii) describe VDR's genome-wide locations and DNA-binding modes, (iii) analyze VDR's dynamic interactions with chromatin modifiers and other nuclear co-factors, (iv), address VDR's primary protein-coding and non-protein coding target genes and (v) delineate these roles and actions of VDR as a modular component in a regulatory network. Finally we will consider these regulatory networks integrated with the actions of other transcription factors, and thereby position the VDR, and its ligand  $1\alpha,25(\text{OH})_2\text{D}_3$ , into the complex signaling system of human tissues and cell types.

## 2. Perspective 1: VDR is a member of a transcription factor family

In humans there are approximately 1900 classical transcription factors, i.e. proteins that sequence-specifically contact genomic DNA [13]. VDR is one of these DNA-binding transcription factors, but has an important additional property, which it shares only with some other members of the nuclear receptor superfamily: VDR can get specifically activated by low nanomolar concentrations of a small lipophilic molecule in the approximate size and

molecular weight of cholesterol [14]. This property is shared with the nuclear receptors for the steroid hormones estradiol (ER $\alpha$  and ER $\beta$ ), testosterone (AR), progesterone (PR), cortisol (GR) and mineralocorticoids (MR), for the vitamin A derivative all-*trans* retinoic acid (RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$ ) and for the thyroid hormone triiodothyronine (TR $\alpha$  and TR $\beta$ ). Moreover, also a number adopted orphan members of the nuclear receptor superfamily, such as retinoid X receptors (RXRs)  $\alpha$ ,  $\beta$ , and  $\gamma$ , peroxisome proliferator-activated receptors (PPARs)  $\alpha$ ,  $\delta$ , and  $\gamma$ , liver X receptors (LXR)  $\alpha$  and  $\beta$  and farnesoid X receptor (FXR), show a similar mode of action, but their natural ligands, for example, 9-*cis* retinoic acid, fatty acids, oxysterols and bile acids, respectively, to date have not been considered as classical endocrine hormones and are in most cases bound by their respective receptors with far lower affinity and specificity [15].

The 48 human members of the nuclear receptor superfamily are characterized by a highly conserved DNA-binding domain (DBD) and a structurally conserved ligand-binding domain (LBD) [16]. The lower part of the LBD of all ligand-activated nuclear receptors contains a ligand-binding pocket of 400–1400 Å<sup>3</sup> in volume, in which the respective ligands are specifically bound [17]. The interior surface of these pockets is formed by the side chains of mostly non-polar amino acids and thereby complements the lipophilic character of the ligands [18].

All nuclear receptors have a similar mode of action. Therefore, a number of mechanisms that were identified, for example with ERs, apply also for the VDR. For example, ligand specificity is achieved through a limited number of stereo-specific polar contacts that include the so-called anchoring points and the actual shape of the pocket. Nuclear receptors that bind their specific ligand with high affinity, such as VDR and ERs, have a relatively small ligand-binding pocket, which is filled to a high percentage by ligand, while adopted orphan nuclear receptors, such as PPARs and LXRs, have a significantly larger ligand-binding pocket, which is filled to a far lower percentage by their ligand molecules [17].

As observed with other transcription factors, the DBD of the VDR cannot contact more than six nucleotides within the major groove of genomic DNA. Binding sites of monomeric nuclear receptors are therefore hexameric sequences and most members of the superfamily share consensus on the sequence RGKTSA (R = A or G, K = G or T, S = C or G). However, the DNA-binding affinity of monomeric VDR is insufficient for the formation of a stable protein–DNA complex and therefore the VDR has to complex with a partner protein, in order to achieve efficient DNA binding. The predominant partner of VDR is the nuclear receptor RXR [19].

Steric constraints allow dimerization of nuclear receptor DBDs only on DNA-binding sites that contain properly spaced hexameric binding motifs; these sequences are also referred to as response elements (REs). An asymmetric, direct repeat arrangement of two motifs spaced by three nucleotides (DR3) provides an efficient interface of the DBDs of VDR and RXR (Fig. 1A, top). This fits with the so-called “3-4-5 rule” of Umesono et al. [20], in which VDR–RXR heterodimers show optimal binding to DR3-type REs, while other nuclear receptors, reflecting different structures and steric constraints, prefer altered spacing, such as DR4 for TRs and DR5 for RARs.

Genome-wide analyses for VDR binding sites (see Section 4) confirmed the preferential binding of VDR to DR3-type REs (Fig. 1A, bottom), but only for approximately one third of all genomic binding sites. Therefore, there must be additional mechanisms for how the VDR can associate with genomic loci, in order to control its primary target genes. These mechanisms include partnering with presently undefined partner proteins (Fig. 1B, middle) or the tethering to other DNA-binding transcription factors (Fig. 1B, bottom). Independent of the exact mechanism, the VDR recruits to these regions in complexes that include positively and negatively regulating proteins, referred to as co-activators (CoAs) [21] and co-repressors (CoRs) [22], respectively. CoA proteins build a bridge to the basal transcriptional machinery, which is assembled on the transcription start site (TSS) of the primary VDR target gene, and stimulate in this way the transcription of the target gene (more details in Section 4). This process is known as transactivation.

In contrast, transrepression is a process whereby transcription factor actions include gene repression. In the context of nuclear receptors this may include direct mechanism associated with co-repressor recruitment or repression of the activity of a second transcription factor through a protein–protein interaction, such as tethering (Fig. 1B, bottom). With nuclear receptors ligand-dependent transrepression is well established for PPAR and LXR [23], and appears to apply also for other members of the superfamily, such as VDR. The net result of transrepression is a down-regulation of gene transcription and is considered as one of the mechanisms by which VDR down-regulates some of its primary target genes.

The cell specificity of the actions of VDR and its ligand  $1\alpha,25(\text{OH})_2\text{D}_3$  can be explained in part by VDR's recognition mode for its genomic binding sites (see Section 4) and the tissue-specific differences in the expression of VDR and its key co-factors. The *VDR* gene shows highest expression in metabolic tissues, such as kidneys, bone and intestine, but at least low to moderate expression is found in nearly all other of the approximately 250 human tissues and cell-types [24]. Moreover, in contrast to GR and AR, the VDR can bind its genomic targets also in the absence of ligand, i.e. in this respect the functional profile of the VDR is larger than that of its ligand [25]. This relates to both repression and activation events and involves the action of CoAs and CoRs (more details in Section 5). Such a phenotype is also displayed by other members of the nuclear receptor superfamily, such as RARs and TRs [26].

### 3. Perspective 2: Genome-wide binding of VDR

For a detailed analysis of enhancer and promoter regions of primary transcription factor target genes in living cells, the method of chromatin immunoprecipitation (ChIP) [27] became very popular. This technique uses mild chemical cross-linking, for example, with 1% formaldehyde, to fix nuclear proteins to genomic DNA in living cells or tissues at any chosen time point. After sonication of chromatin into fragments of 200–400 bp in size, immunoprecipitation with an antibody against the chosen nuclear protein, such as the VDR, enriches those chromatin regions that had been in contact with the protein at the moment of cross-linking. After a reverse cross-linking reaction, the resulting chromatin fragments can either be amplified by quantitative PCR using primers specific for the chosen genomic region (ChIP-qPCR) or are directly applied to massive parallel sequencing (ChIP-seq).

When a significant enrichment in relation to a control (which mostly is ChIP with unspecific IgGs) is observed for a given genomic region, this is taken as an indication that the nuclear protein had been in contact with the investigated genomic region. For example, by ChIP-qPCR approximately 10 kb of the regulatory regions of the primary VDR target genes *CYP24A1* [28], *CYP27B1* [29], *CCNC* [30] and *CDKN1A* (also called *p21*) [31,32] were screened for genomic VDR-binding sites and per gene 2–4 specific sites were identified. Alternatively, the complete human *ALOX5* gene sequence (some 85 kb) was first screened *in silico* for regions comprising putative vitamin D response elements (VDREs) and then studied by ChIP-qPCR [33]. From 22 investigated regions, two were shown to be functional in living cells, one of which is located far downstream (+42 kb) of the TSS of the *ALOX5* gene.

To date, three VDR ChIP-seq studies have been published. In human lymphoblastoids, which were treated for 36 h with  $1\alpha,25(\text{OH})_2\text{D}_3$ , Ramagopalan et al. [34] reported 2776 genomic VDR-binding sites. In human monocytes (THP-1), Heikkinen et al. [35] observed after 40 min ligand stimulation 1820 VDR ChIP-seq peaks, 1171 of which occur only in the presence of  $1\alpha,25(\text{OH})_2\text{D}_3$ . For comparison, in the absence of ligand in lymphoblastoids and monocytes only 623 and 520 genomic VDR sites were found. Finally, in human colorectal cells (LS180), which were stimulated for 180 min with  $1\alpha,25(\text{OH})_2\text{D}_3$ , Meyer et al. [36] showed that 1674 VDR-binding sites co-locate with those of the VDR partner protein RXR. Importantly, the ChIP-seq studies confirmed a number of previously reported VDR-binding sites on known primary  $1\alpha,25(\text{OH})_2\text{D}_3$  targets, such as that of the genes *MYC* [37], *VDR* [38], *CCNC* [30] and *ALOX5* [33]. In addition, they reported some extra sites for known  $1\alpha,25(\text{OH})_2\text{D}_3$  target genes and also indicated a large number of previously unknown targets of VDR.

Despite different cellular models and large differences in ligand treatment times, the three ChIP-seq studies revealed a comparable number of VDR-binding sites of approximately 1600–2700 specific peaks. However, only 20% of these genomic sites are identical in all three investigated cell lines, such as in the case of *CYP19A1* gene (Fig. 1C). The latter case codes for the estrogen synthesizing enzyme aromatase, which was previously established to be an up-regulated  $1\alpha,25(\text{OH})_2\text{D}_3$  target gene [39]. Interestingly, the VDR-binding site of this gene is located within an intron some 110 kb downstream of the TSS. In all three cellular models it is bound in a ligand-dependent fashion by the VDR.

Although the majority of VDR binding across the genome is both time and cell background specific, it can reasonably be anticipated that the shared 20% of VDR-binding sites are conserved and represent important functions in all VDR expressing tissues. This implies that data, such as shown in Fig. 1C, may be extrapolated to other human cell types.

Another result, on which the three VDR ChIP-seq studies are in accordance with findings of the ENCODE project [40], is that the distribution of the VDR binding sites has a Gaussian shape, i.e. VDR binding sites are found both up- and downstream of the TSS region of the primary target genes. The likelihood of detecting a functional VDR binding site decreases by distance from the TSS, but there is no maximal distance limiting the interaction between a VDR carrying enhancer region and a TSS region. However, the functionality of the most

newly identified genomic VDR binding sites needs to be validated by assays that monitor the three-dimensional interaction of genomic regions, ideally by a genome-wide method, such as chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) [41].

Furthermore, most of the ChIP-seq studies with other members of the nuclear receptor superfamily indicated some 5000–10,000 genome-wide binding sites [42,43], i.e. the numbers reported for VDR is relatively low. However, nuclear receptor binding appears modest compared to other transcription factors, such as FOXA1, for which up to 80,000 ChIP-seq peaks were found [44]. Transcription factors that show such a high number of genomic binding sites are assumed to have greater binding promiscuity and/or diversity of interactions. In this manner they may act more as “pioneer factors”, i.e. as transcription factors that bind regulatory genomic regions at first and start the opening of these loci via the interaction with chromatin modifying enzymes. This then allows “following factors” to bind a subset of these accessible regions and to execute their regulatory actions. Viewed in this manner it is most likely that the VDR is most likely a following than a pioneer factor.

Single gene studies support this model whereby modulation of VDR-binding appears determined by the transcription factors AP1 [45] or RUNX2 [46] suggesting that there are pioneer processes that influence and determine VDR function. So far, however, no genome-wide study of possible pioneer factors cooperating with VDR has been published. However, although a negative result, Meyer et al. [36] showed that in human colorectal cells the transcription factor TCF7L2 does not act as a pioneer factor for VDR. Nevertheless, in analogy to studies with ER $\alpha$  [47], it can be assumed that ubiquitously expressed transcription factors, such as FOXA1, AP1, SPI1 or SP1, may act as pioneer factors for the VDR.

#### 4. Perspective 3: Genomic DNA-binding modes of the VDR

Central to ChIP-seq data studies is the analysis of the sequences below the identified peaks (mostly within  $\pm 100$  bp of the peak summit) for any enriched sequence motif, the idea being that this sequence will reflect a transcription factor-binding site. In all three VDR ChIP-seq studies [34–36], such agnostic binding site searches identified the well-established DR3-type RE consensus sequence for VDR–RXR heterodimers as being the most highly enriched (Fig. 1A, bottom). Strikingly, using the narrow observation window of  $\pm 100$  bp either side of the peak height, only 31.7% (742) of all 2340 VDR peak summits in monocytes include one or more DR3-type REs [35]. Similar numbers apply for the datasets from lymphoblastoids and colorectal cancer cells.

When focusing only on  $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent VDR peaks and plotting the percentage of DR3-type RE content over the quality of the VDR ChIP-seq peak, the three ChIP-seq datasets provide similar results. That is, the higher the fold enrichment/value of a VDR peak, the higher is the chance that it contains a high-quality DR3-type RE [48]. In contrast, from the 520 genomic VDR-binding locations that uniquely occur in monocytes in the absence of ligand, only 14% contain a DR3-type VDRE [35]. This observation suggests that after ligand activation, the VDR shifts from genomic regions without a DR3-type RE to those with a DR3-type RE. This suggests that either the VDR becomes more specific in focusing

upon its regulated genomic targets, or the binding sites associated with the basal state are more nuanced and less well explored. An intriguing implication of this discovery is that the non-DR3 locations may serve as a nuclear store of VDR to be utilized rapidly upon the introduction of the ligand, partly substituting for the need to transport VDR into the nucleus from outside.

The processes that drive the VDR to re-distribute to these locations remain unresolved. The lack of a DR3-type RE consensus sequence, even in the ligand stimulated state, in the majority of the VDR ChIP-seq peaks suggests that VDR either (i) has far more promiscuous or relaxed DNA binding specificities than previously assumed, probably by forming a complex with a presently undefined transcription factor (Fig. 1B, middle) or (ii) tethers to another DNA-binding transcription factor, such as a pioneer factor, rather than directly contacting DNA (Fig. 1B, bottom). Searches for other VDRE types with either different spacing or relative orientations of the core binding motifs have not provided any statistically significant enrichment within  $\pm 100$  bp of the peak summit. Although it is still possible that a few individual regions carry such alternative VDRE types, in the published datasets there is no genome-wide evidence for their widespread use.

## 5. Perspective 4: VDR in dynamic interactions with chromatin components

The complex of genomic DNA and nucleosomes, referred to as chromatin, *per se* prevents access of transcription factors to their genomic targets [49]. This intrinsic repressive potential of chromatin is essential for long-lasting regulatory decisions, such as terminal differentiation of cells [50]. However, the epigenetic landscape can also be highly dynamic and lead to short-lived states, such as a response of chromatin to extra- and intracellular signals, for example, an exposure to  $1\alpha,25(\text{OH})_2\text{D}_3$  [51]. One major component of epigenetic changes is the reversible post-translational modification of histone proteins, such as acetylation and methylation, that is directed by a large group of chromatin modifying enzymes, with either histone acetyltransferase (HAT), histone deacetylase (HDAC), histone methyltransferase (HMT) or histone demethylase (HDM) activity [52]. Some of these histone modifications are associated with genes that are actively transcribed, whereas others are a sign of repressed genes [53], i.e. the post-translational modifications of histones correlate with either active or inactive chromatin regions. A second class of nuclear enzymes have ATP-dependent chromatin remodeling activity and induces plasticity of chromatin by rearranging the organization of nucleosomes [54].

Nuclear receptors in general and the VDR in particular are amongst the first and most well described examples of the dynamic nature of transcriptional regulation in the context of chromatin [55–58]. In the so-called deactivation phase, i.e. in the absence of ligand, nuclear receptors that have a nuclear location, including the VDR, interact with CoR proteins, which in turn associate with HDACs leading to a locally more compact chromatin packaging [59]. In the activation phase, ligand binding induces the dissociation of CoRs and the association of CoAs [60]. Some CoAs have HAT activity or are complexed with proteins harboring such activity, which in net effect results in local chromatin relaxation [61]. In the initiation phase, nuclear receptors interact with another class of CoAs, which are members of the Mediator complex, that build a bridge to the basal transcriptional machinery and initiate a burst of

mRNA synthesis by RNA polymerase II [62] (Fig. 2, top). In this way, gene activation by a nuclear receptor, such as VDR, can be separated into three phases, in each of which the transcription factor interacts with a different class of nuclear proteins.

Using time-resolved ChIP, Shang et al. [63] demonstrated that several CoA proteins were recruited in a cyclical fashion to an estrogen responsive chromatin region of the human *TFF1* gene. Metivier et al. [64] showed on the same genomic region the sequential and ordered recruitment of ER $\alpha$ , RNA polymerase II and many chromatin-associated proteins, such as CoAs, CoRs, HATs, HDACs and HMTs. Similar observations were made with AR on the human *KLK3* gene [65], with TRs on the human *DIO1* gene [66] and with VDR on the human genes *CYP24A1* [28,67], *CDKN1A* [32], *IGFBP3* [68] and *MYC* [37]. All these examples show cyclical association of co-regulator proteins and, in part, also of the respective nuclear receptor with a periodicity of 30–60 min. Interestingly, the more recently published reports on *CDKN1A* and *IGFBP3* also demonstrate the cycling of mature mRNA [32,68] or even protein [58]. Cycling in the abundance of mature mRNA can be observed only with those genes, whose half-life of the induced mRNA transcript is shorter than the periodicity of cyclical association of transcription factors and their co-regulators, i.e. in average 60 min or less. It is only under this condition that there is enough mRNA degradation within one transcription cycle in order to observe cycling of transcript levels [69]. This reduces the list of genes that show transcriptional cycling to those that encode short-lived regulatory proteins, such as transcription factors and kinases.

The cellular basis for this control most likely reflects the fact that transcriptional dynamics allows a better control of protein expression than controlling protein stability. A gene can be silenced far quicker, when it has to confirm every 60 min, if its transcription is still required. For example, pulsatile exposure of cells with cortisol stimulates transcriptional dynamics of GR [70], but these dynamics are not observed, when the synthetic GR ligand dexamethasone is used. The latter stabilizes the receptor for longer periods than the natural ligand cortisol. A similar observations was made with the synthetic VDR agonist Gemini, which failed to induce transcriptional dynamics of the human *IGFBP3* gene, while 1 $\alpha$ ,25(OH) $_2$ D $_3$  does [68]. These observations may have implications for the therapeutic application of synthetic nuclear receptor ligands and may explain some of their side effects.

## 6. Perspective 5: Primary VDR target genes

Each eukaryotic gene is under the control of a large set of transcription factors that bind up- and downstream of its TSS. An essential prerequisite for a direct modulation of transcription by 1 $\alpha$ ,25(OH) $_2$ D $_3$  is the interaction of activated VDR with the basal transcriptional machinery. This is achieved through the specific binding of VDR to a genomic binding site, which via DNA looping gets into vicinity of a core promoter region of a primary 1 $\alpha$ , 25(OH) $_2$ D $_3$  target gene [71]. The effect of 1 $\alpha$ ,25(OH) $_2$ D $_3$  on gene expression, i.e. 1 $\alpha$ , 25(OH) $_2$ D $_3$ -induced changes of the transcriptome, has been investigated by multiple mRNA microarrays and more recently also by miRNA microarrays [72] in various cellular models (either established cell lines or primary cells) or in *in vivo* models (mostly rodents). However, there is a large variation in the microarray platforms used for these transcriptome studies and also the experimental conditions, such as treatment time and ligand

concentration, have been rather divergent. Moreover, the application of a next-generation sequencing technology method for the detection of RNA transcripts, called RNA-seq, has not yet been reported for VDR target genes. Similar to ChIP-seq, this technique is based on the sequencing of all RNA transcripts of all cells and is supposed to be more sensitive than hybridization-based microarrays [73].

Some studies focused on the identification of primary VDR target genes and used rather short incubations with the ligand (2–6 h), while others were more interested in the overall physiological or consequential effects of  $1\alpha,25(\text{OH})_2\text{D}_3$  and used far longer treatment times (24–72 h). In the past, cDNA arrays with an incomplete number of genes were used and rather short lists of VDR target genes from colon [74], prostate [75–78], breast [79] and osteoblasts were obtained [80,81]. However, despite these limitations many genes appear to respond to  $1\alpha,25(\text{OH})_2\text{D}_3$  activation. For example, in squamous cell carcinoma cells more than 900 genes responded within 12 h to a stimulation with  $1\alpha,25(\text{OH})_2\text{D}_3$  [82]. Unfortunately, the results of many of the earlier microarray studies with  $1\alpha,25(\text{OH})_2\text{D}_3$  were not placed in public data repositories, such as the Gene Expression Omnibus (GEO) of NCBI [83], which made a direct comparison of the results difficult.

Also more recent microarray analyses in various tissues and cells from different species have suggested long lists of VDR target genes. For example, in human monocytes (THP-1) 638 genes responded to a 4 h treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  [35], while a 36 h stimulation of human lymphoblastoids let only 229 genes move [34]. However, the overlap between these two  $1\alpha,25(\text{OH})_2\text{D}_3$  target gene lists is only 5.6%. This confirms the overall impression that most VDR target genes respond to  $1\alpha,25(\text{OH})_2\text{D}_3$  in a very tissue- and time-specific fashion and some of them show only a rather transient response to the ligand. Although a number of these genes may not be primary VDR targets, they nevertheless contribute to the physiological effects of  $1\alpha,25(\text{OH})_2\text{D}_3$ . Although there are far fewer studies to date on VDR regulation of miRNAs, the numbers regulated and the time-dependent patterns appear comparable to mRNA targets in terms of the proportion of the total number regulated and the kinetics [58,72].

The combination of  $1\alpha,25(\text{OH})_2\text{D}_3$  microarray data with VDR ChIP-seq data from the same cellular model allows a more detailed exploration of the mechanisms of VDR target gene regulation. This was possible in particular for the study in monocytes [35], where a 40 min ligand stimulation for VDR location mapping and a 4 h  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment for mRNA expression studies was used. Due to the short stimulation time most of the 638 regulated genes can be assumed to be primary  $1\alpha,25(\text{OH})_2\text{D}_3$  targets, i.e. that their mRNA expression changes are a direct consequence of the binding of VDR to genomic regions looping to their respective core promoter region. Plotting the positions of the  $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated VDR ChIP-seq peaks in relation to the TSS of the  $1\alpha,25(\text{OH})_2\text{D}_3$  target genes showed a clear peak at the TSS region and symmetrical decline towards both the upstream and downstream flanking regions [48]. This emphasizes again that VDR binds as likely upstream as downstream of the core promoter region of its target genes. This fits with insights of the ENCODE project [84] and indicates that the pre-genomic focus on the upstream region only addressed half of the regulatory regions of a gene.

The gene regulatory scenarios of up-regulated VDR target genes vary considerably. In monocytes there are only about 20 genes, such as *SP100* or *CAMP*, where VDR binds close to their core promoter region [35]. More common are situations where one target gene has multiple VDR-binding sites in various distances to its TSS region. Alternatively, a pair of closely located VDR target genes share one or more VDR-binding sites, as shown for the members of the *IGFBP* gene family [85]. From the 638  $1\alpha,25(\text{OH})_2\text{D}_3$  target genes in monocytes, 408 are up-regulated and for 93 of the latter (22.8%) the largest  $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated VDR peak is within 30 kb from their TSS. For another 201 genes (49.3%), the most prominent VDR-binding site is in a distance of 30–400 kb from the core promoter region. For comparison, in pre-genomic studies a distance of 30 kb between a VDRE and the TSS was already considered large [71], while 400 kb was practically unimaginable.

Interestingly, only 99 (43.0%) out of the 230 down-regulated genes in monocytes have a  $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated VDR peak in the  $\pm 400$  kb region [35]. This observation emphasizes that the mechanisms of down-regulation of VDR target genes seem to be different from that of up-regulation. They may require gene-specific investigations as demonstrated for the genes *CYP27B1* [29] and *MYC* [37]. In the case of the *CYP27B1* gene, the repressive function of VDR results from indirect interaction with genomic DNA, via transcription factor 3, also known as VDR-interacting repressor [86].

Another mechanism of gene regulation is de-repression, which was first described for the nuclear receptors TR and LXR [87,88]. In this regulatory process the nuclear receptor actively represses genes via the interaction with CoR and HDAC proteins. The addition of ligand induces a dissociation of the nuclear receptor from its binding site and a release of the repression. In monocytes, only six up-regulated genes meet the de-repression criteria that they have a VDR peak in the unstimulated sample and no peak in the  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated sample [35]. An additional 21 up-regulated genes can be called dominantly de-repressed, since their main peak is found only in the unstimulated sample. This indicates that for some 10% of all up-regulated  $1\alpha,25(\text{OH})_2\text{D}_3$  target genes, a de-repression mechanism may apply.

Nevertheless, for some 25% of the up-regulated and more than the half of the down-regulated  $1\alpha,25(\text{OH})_2\text{D}_3$  target genes in monocytes the ChIP-seq approach does not identify any VDR binding within 400 kb of their core promoter region, i.e. for these genes there is no obvious explanation for their regulation by VDR [35]. However, gene regulation by VDR is a very dynamic process (see Section 5) with rapid changes of VDR-binding site occupancy [32,37,68], which a single, short time point at 40 min may have not fully captured. The time points chosen in each study represent only snap-shots of the actions of the VDR and it is likely that without time-course data, a considerable proportion of transient VDR-binding sites remain unknown.

## 7. Perspective 6: VDR as a module component

Much of the activity of a cell depends on gene regulatory networks, which are built of interacting regulatory pathways, also referred to as modules. A module is represented by a set of co-regulated genes (both protein and non-protein coding) that respond to different conditions [89]. In such modules, transcription factors and epigenetic modifications serve as

inputs, while the output is a gene expression pattern representing a physiological situation, such as a differentiation stage. Transcription factors show two different types of inputs, as they determine the expression of the target genes and serve as functional drivers, which come into play only during specific situations during development or cell fate decisions. Additionally, the regulation of chromatin structure and nuclear organization also play a role in determining and controlling the function of these modules, for example, by regulating the amplitude and magnitude of gene expression periodicity.

Understanding the central control of architectural modules in these gene circuits may yield insight into predicting cellular responses and thus therapeutic targets. For example, nuclear receptors regulate CYP enzymes in negative feedback loops that degrade ligand and signal output [15]. These metabolic enzymes are frequently altered in expression, and equally provide therapeutic targets in various syndromes.

In this context, the regulation of miRNA genes by VDR may be of special importance. After processing of its precursor the active part of a miRNA is a single-stranded RNA molecule of 21–23 nt in length, which associates with cytosolic proteins that use the miRNA for a sequence-specific recognition of the 3'-UTR of mRNA molecules and their consequent degradation [90]. In this way miRNAs control the half-life of their target mRNAs and regulate the level of translated proteins. Like transcription factors, each miRNA can have up to hundred targets [91], i.e. the regulation of a miRNA gene by VDR may have larger impact than the regulation of, for example, a metabolic enzyme. Some VDR regulated modules include feed forward loops that are crucial for the precise regulation of target genes, in terms of signal amplitude and magnitude. These loop motifs often include roles for miRNAs to fine-tune transcriptional signals [92] (see also Fig. 2, bottom). Studies with VDR combined with an emerging literature [93,94] suggest that these motifs are common in normal human biology and disrupted in cancer. For example, VDR regulates the *MCM7* gene that encodes the *MIR106b* cluster. VDR also regulates *CDKN1A* that in turn is targeted by *MIR106b*. These members thereby form a VDR feed forward loop that governs cell cycle progression in human prostate epithelial cells [58]. The balance of these interactions appear disrupted in cancer cells compared to non-malignant models with selective attenuation and repression of VDR transcriptional responses of target genes such as *CDKN1A*. The suppressed transcriptional responses in PC-3 human prostate cancer cells were associated with gene-specific VDR-induced enrichment of the CoR NCOR1 leading to gene silencing. Other cyclin-dependent kinase inhibitors appear to be regulated in a similar manner. VDR represses *MIR181a*, which targets the *CDKN1B* gene (encodes for p27) and thereby establish another feed forward loop that promotes hematopoietic differentiation [95].

The architecture of these modules also appears to provide enough flexibility and information to generate spatial and temporal patterns of gene expression, for example, during cellular differentiation. Again, this can be studied best in the hematopoietic system. Hematopoiesis is believed to be controlled by a hierarchy of a relatively small number of critical transcription factors that are sequentially expressed, are largely restricted to a specific lineage and can interact directly to mediate and reinforce cell fate decisions [96]. However, genome-wide studies suggest amore complex architecture in regulatory circuits involving

larger numbers of transcription factors that control different combinations of modules of co-expressed genes [97,98].

Novershtern et al. [99] measured the transcriptome profiles of a large number of hematopoietic stem cells, multiple progenitor states and terminally differentiated cell types. They found distinct regulatory circuits in both stem cells and differentiated cells, which implicated dozens of new regulators in hematopoiesis. They identified 80 distinct modules of tightly co-expressed genes in the hematopoietic system. One of these modules is expressed in granulocytes and monocytes and includes genes encoding enzymes and cytokine receptors that are essential for inflammatory responses. Major players in this module are VDR together with the pioneer factors CEBPA and SPI1 (Fig. 2). Further contributors are the proteins ATF3, CREB5, PPARGC1A, VENTX and MYCL1. This indicates that VDR works together with this small set of transcription factors, in order to regulate granulocyte and monocyte differentiation.

These findings also fit with previously obtained information about potent effects of  $1\alpha, 25(\text{OH})_2\text{D}_3$  both on the innate and the adaptive immune system. For example,  $1\alpha, 25(\text{OH})_2\text{D}_3$  enhances the differentiation of monocytes into functional macrophages with increased phagocytic capacity and altered cytokine-secreting capacity, but impairs the differentiation of monocytes into dendritic cells [100]. The main  $1\alpha, 25(\text{OH})_2\text{D}_3$  targets in differentiating monocytes are anti-microbial peptides, such as cathelicidin, co-stimulatory molecules, such as CD14 [35], and cytokines, such as interleukins 10 and 12b [101,102]. The new insight of the dominant role of VDR in the granulocyte/monocyte module now allows more specific investigations on the functional interplay of VDR with its partner transcription factors, for example with the pioneer factors CEBPA and SPI1.

These provocative studies also reflect a very powerful light on much earlier and translational studies on the role of  $1\alpha, 25(\text{OH})_2\text{D}_3$  and its analogs to drive so-called differentiation therapy in myeloid malignancies [103–107]. However, clinical exploitation of these studies was ultimately equivocal and perhaps required more accurate analyses of individual patient responsiveness to such therapies. The new modular understanding of the VDR may ultimately provide this insight.

## 8. Conclusions

The different perspectives presented here for the VDR reflect the pleiotropic molecular actions of the receptor and its natural ligand  $1\alpha, 25(\text{OH})_2\text{D}_3$ . In this context the parameter time has emerged to be very critical due to the dynamic response of tissues and cell types, especially in the early phase of their treatment with  $1\alpha, 25(\text{OH})_2\text{D}_3$ . Therefore, further time-course experiments for VDR ChIP-seq and  $1\alpha, 25(\text{OH})_2\text{D}_3$  microarrays will provide a more detailed understanding of this aspect.

Genome-wide the actions of VDR and  $1\alpha, 25(\text{OH})_2\text{D}_3$  to date have best been understood in cells of the hematopoietic system. Modular studies have started to demonstrate with which other partner transcription factors VDR forms integrated units that offer up windows of potent transcriptional actions to determine cell fate. These modular actions may also shed light on the targeted effects of the VDR in physiology. Part of this range of targeted effects

and sensitivity is in part determined by the intrinsic epigenetic states and shared expression of co-factors and histone modifying complexes. For example, VDR is important for the differentiation of mesenchymal stem cells to bone and fat cells. The large datasets obtained from genome- and transcriptome-wide investigations on VDR and on related transcription factors and epigenetic modifications provide new insight and will allow the integration of the actions of VDR with that of other signaling systems, such as that of other nuclear receptors or of pioneer factors, such as CEBPA and SPI1. This will allow a more generalized understanding of VDR and  $1\alpha,25(\text{OH})_2\text{D}_3$  in the control of the whole body's physiology.

This may also illuminate the discrepancies observed on responsiveness of the VDR in disease states, such as cancer, where responsiveness of cells towards VDR actions, ranging from sensitivity to recalcitrance. Given that miRNA regulation by the VDR appears common, this can be exploited to define individual cell or patient responsiveness to the vitamin D-based therapies. Tumor-specific miRNA patterns are emerging as highly attractive biomarkers, for example, of cancer risk and progression. Given miRNAs are secreted into body fluids [108] and can be reliably extracted and measured [109], they offer significant clinical potential as highly sensitive serum-borne prognostic indicators [110,111]. Using serum-borne miRNAs as prognostic markers is highly attractive for several reasons. First, they can overcome the limitations of inaccurate sampling for the presence of cancer. Second, they can encapsulate the effects of heterotypic cell interactions within the tumor microenvironment. Third, they form a non-invasive test procedure. Therefore understanding miRNA regulation, within critical VDR modules, offers up the real opportunity of tailoring and monitoring vitamin D therapies to the individual.

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## Abbreviations

<b><math>1\alpha, 25(\text{OH})_2\text{D}_3</math></b>	$1\alpha,25$ -dihydroxyvitamin $\text{D}_3$
<b><math>25(\text{OH})\text{D}_3</math></b>	25-hydroxyvitamin $\text{D}_3$
<b>ALOX5</b>	arachidonate 5-lipoxygenase
<b>AR</b>	androgen receptor
<b>CAMP</b>	cathelicidin anti-microbial peptide
<b>CCNC</b>	cyclin C
<b>CDKN1A</b>	cyclin-dependent kinase inhibitor 1A
<b>CoA</b>	co-activator

<b>CoR</b>	co-repressor
<b>ChIP</b>	chromatin immunoprecipitation
<b>ChIP-seq</b>	ChIP coupled with massive parallel sequencing
<b>CYP</b>	cytochrome P450
<b>DBD</b>	DNA-binding domain
<b>DIO1</b>	thyroxine deiodinase type I
<b>DR3</b>	direct repeat spaced by 3 nucleotides
<b>ER</b>	estrogen receptor
<b>FXR</b>	farnesoid X receptor
<b>GLDN</b>	gliomedin
<b>GR</b>	glucocorticoid receptor
<b>HAT</b>	histone acetyltransferase
<b>HDAC</b>	histone deacetylase
<b>HDM</b>	histone demethylase
<b>HMT</b>	histone methyltransferase
<b>IGFBP</b>	insulin-like growth factor binding protein
<b>KLK3</b>	kallikrein 3
<b>LBD</b>	ligand-binding domain
<b>LXR</b>	liver X receptor
<b>miRNA</b>	micro RNA
<b>MR</b>	mineralocorticoid receptor
<b>PR</b>	progesterone receptor
<b>RAR</b>	retinoic acid receptor
<b>RE</b>	response elements
<b>RXR</b>	retinoid X receptor
<b>SP100</b>	SP100 nuclear antigen
<b>TFF1</b>	trefoil factor 1
<b>TR</b>	thyroid hormone receptor
<b>TSS</b>	transcription start site
<b>VDR</b>	vitamin D receptor
<b>VDRE</b>	vitamin D response element

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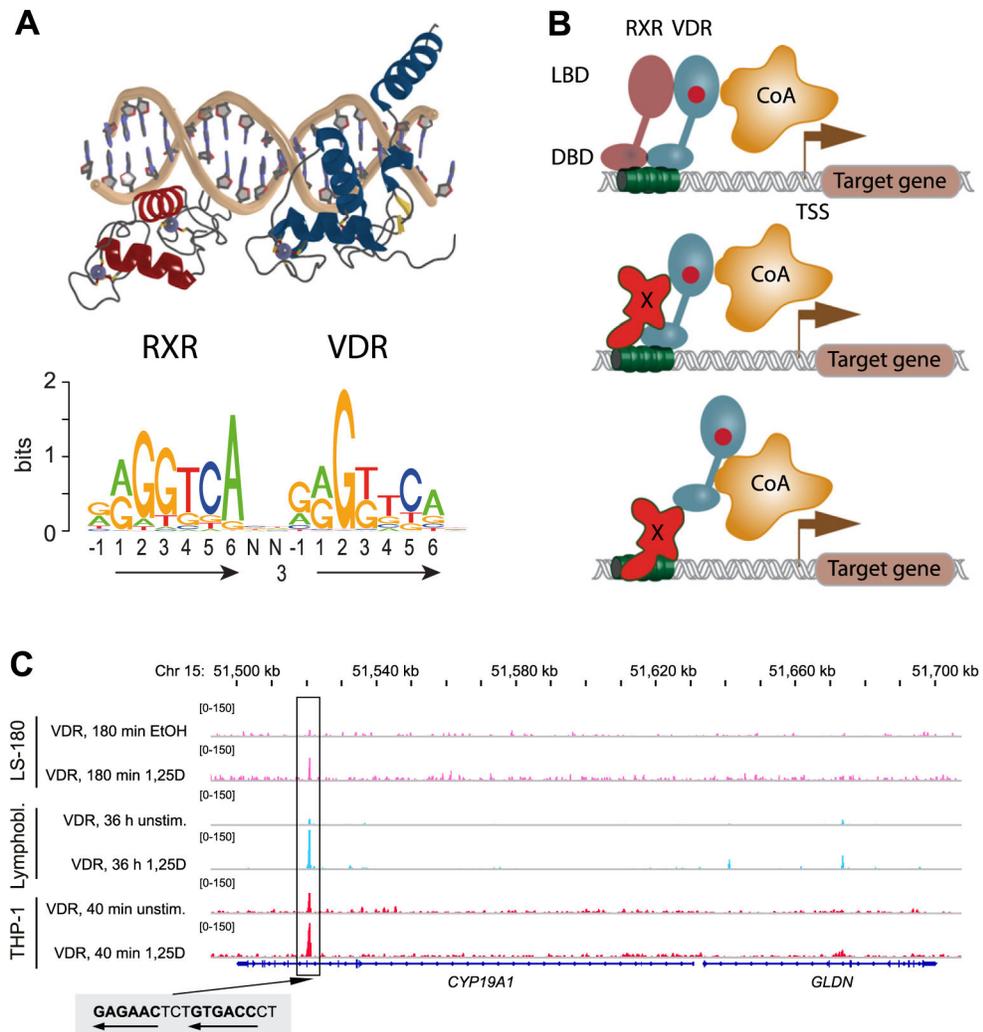
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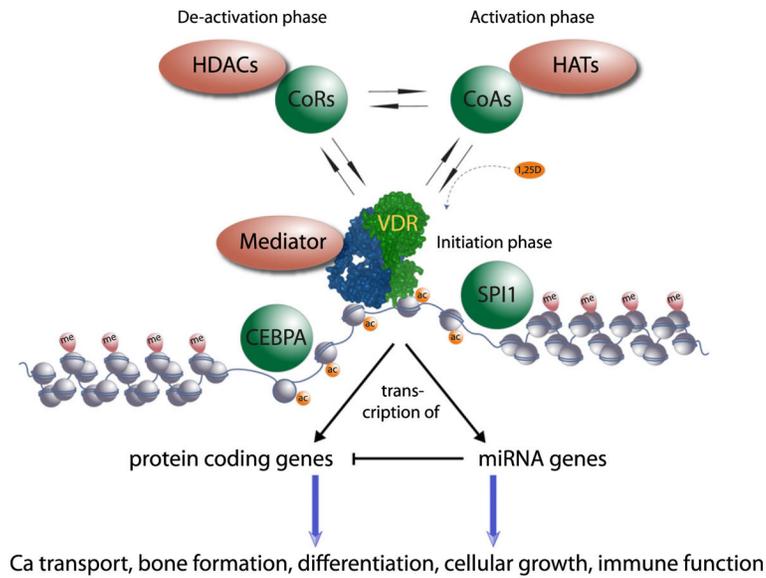
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**Fig. 1.** VDR binding sites and target genes. (A) The crystal structure (protein data bank identifier 1YNW [112]) of the heterodimer of the DBDs of VDR (blue) and RXR (red) bound to a DR3-type RE (top) is aligned with the *de novo* DR3-type sequence motif found below 742 of 2340 VDR peaks (31.7%) in THP-1 cells [35] (bottom). (B) Three modes of VDR regulating its primary target genes are indicated: VDR–RXR heterodimers preferentially binding to a DR3-type RE (top), VDR partnering with undefined protein X bound to DNA (middle) and VDR tethering undefined protein X bound to DNA (bottom). In all three cases it is assumed that the contact of ligand (red)-activated VDR leads to an association with CoA proteins and the activation of primary target genes. (C) The genome view of one primary VDR target gene, *CYP19A1*, is shown. The peak tracks on top show data from VDR ChIP-seq in LS-180 cells (pink [36]), lymphoblastoids (blue [34]) and THP-1 cells (red [35]) comparing genomic VDR binding at the *CYP19A1* locus in unstimulated or vehicle-stimulated cells with that after  $1\alpha,25(\text{OH})_2\text{D}_3$  (1,25D) treatment for indicated times. The structure of *CYP19A1* gene and its direct neighbor *GLDN* is shown in blue and the sequence of the DR3-type VDRE at the summit of the VDR ChIP-seq peak is indicated.



**Fig. 2.**

Integration of VDR actions. Together with the pioneering factors the VDR is the central part of a differentiation module. Putative pioneer factors such as CEBPA and SPI1 appear to help the VDR to access to its genomic binding sites, but may not be found at all VDR binding loci. At these genomic VDR binding regions there is a cyclical alternation of proteins representing the deactivation phase (for example, CoRs and HDACs), the activation phase (for example, CoAs and HATs) and the initiation phase (for example, VDR and Mediator proteins). The outcome of the dynamic interaction of VDR with its binding sites and partner proteins is the modulation of the transcription of its primary target genes. The latter are either protein coding genes or non-coding genes, such as miRNA genes. Some of the miRNAs are involved in the fine-tuning of the mRNA expression of the protein-coding genes. Together with secondary target genes they mediate the physiological actions of  $1\alpha, 25(\text{OH})_2\text{D}_3$  and its receptor VDR.