REVIEW

Genome-wide effects of chromatin on vitamin D signaling

Andrea Hanel, Henna-Riikka Malmberg and Carsten Carlberg

School of Medicine, Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland

Correspondence should be addressed to C Carlberg: carsten.carlberg@uef.fi

Abstract

Molecular endocrinology of vitamin D is based on the activation of the transcription factor vitamin D receptor (VDR) by the vitamin D metabolite $1\alpha,25$ -dihydroxyvitamin D_3 . This nuclear vitamin D-sensing process causes epigenome-wide effects, such as changes in chromatin accessibility as well as in the contact of VDR and its supporting pioneer factors with thousands of genomic binding sites, referred to as vitamin D response elements. VDR binding enhancer regions loop to transcription start sites of hundreds of vitamin D target genes resulting in changes of their expression. Thus, vitamin D signaling is based on epigenome- and transcriptome-wide shifts in VDR-expressing tissues. Monocytes are the most responsive cell type of the immune system and serve as a paradigm for uncovering the chromatin model of vitamin D signaling. In this review, an alternative approach for selecting vitamin D target genes is presented, which are most relevant for understanding the impact of vitamin D endocrinology on innate immunity. Different scenarios of the regulation of primary upregulated vitamin D target genes are presented, in which vitamin D-driven super-enhancers comprise a cluster of persistent (constant) and/or inducible (transient) VDR-binding sites. In conclusion, the spatio-temporal VDR binding in the context of chromatin is most critical for the regulation of vitamin D target genes.

Key Words

- ▶ vitamin D
- ▶ VDR
- ▶ vitamin D target genes
- ▶ chromatin
- ▶ epigenome
- ▶ transcriptome
- ▶ gene regulation
- ▶ super-enhancer
- ▶ VDR binding sites
- ▶ monocytes

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Introduction

The micronutrient vitamin D_3 is known best as a key regulator of calcium homeostasis, which is essential for proper bone formation (van de Peppel & van Leeuwen 2014, Carmeliet *et al.* 2015). Interestingly, the nuclear receptor for vitamin D, VDR, evolved some 550 million years ago in a boneless fish, i.e. even before vertebrates developed a stable skeleton for locomotion (Whitfield *et al.* 2003, Bouillon & Suda 2014). Thus, in analogy to other members of the nuclear receptor superfamily, VDR's evolutionary oldest function was the control of energy metabolism, supporting the evolving immune system of ancestral vertebrates (Hanel & Carlberg 2019). Vitamin D supports the immune system in its fight against infections,

such as tuberculosis (Chun *et al.* 2014), and in parallel prevents autoimmune disorders, such as multiple sclerosis (Lu *et al.* 2019).

Vitamin D_3 can be synthesized in human skin *via* an UV-B-dependent, non-enzymatic reaction that uses the cholesterol precursor 7-dehydrocholesterol as a substrate (Holick 2011). The energy of UV-B opens the B-ring and creates a biologically inert secosteroid. However, hydroxylations first at carbon 25 of the side chain (producing 25-hydroxyvitamin D_3) and then at carbon 1 within the A-ring create the most active vitamin D metabolite $1,25(\text{OH})_2\text{D}_3$ ($1\alpha,25$ -dihydroxyvitamin D_3) (Fig. 1A). The molecule easily passes through biological

membranes and binds with a remarkably high affinity of 0.1 nM to the VDR (Haussler *et al.* 2013). There are no other cellular proteins that bind vitamin D and/or its metabolites with comparable affinity. However, the endocytosis receptor megalin in the plasma membrane supports the uptake of vitamin D compounds, in particular in the kidneys (Nykjaer *et al.* 1999), and the membrane-associated endoplasmatic protein disulfide isomerase PDIA3 has been shown to be affected in its function by vitamin D (Khanal & Nemere 2007). Nevertheless, neither megalin nor PDIA3 affects the functions of vitamin D on gene expression in the nucleus. Since most human tissues and cell types express the *VDR* gene (www.proteinatlas.org/ENSG00000111424-VDR/tissue), 1,25(OH)₂D₃ is a nuclear hormone that is able to modulate gene expression throughout the whole human body (Sutton & MacDonald 2003).

In general, the expression of a gene requires that its regulatory regions, such as TSSs (transcription start sites) and transcription factor-binding sites located within accessible chromatin (Haberle & Stark 2018). Chromatin is a complex of genomic DNA and histone proteins forming nucleosomes, a major purpose of which is to restrict the access of transcription factors to regulatory regions, such as enhancers (Rando & Chang 2009). The simplest form of an enhancer is a single transcription factor-binding site, while super-enhancers contain multiple single enhancers (Whyte *et al.* 2013). Well-known markers for active chromatin are acetylated histone H3 proteins at position lysine 27 (H3K27ac) (Creyghton *et al.* 2010). Interestingly, within a terminally differentiated cell only some 100,000 genomic regions are accessible, i.e. they can act as enhancers (ENCODE-Project-Consortium 2012).

The genome-wide information included in covalent and structural modifications of chromatin is referred as the epigenome (Rivera & Ren 2013). Chromatin is the molecular representation of the epigenome on the level of post-translational modifications of histone proteins, DNA methylation and 3D organization, such as TADs (topologically associated domains) (Carlberg & Molnár 2016). In contrast to the genome, the epigenome is dynamic and can be modulated by environmental signals, such as 1,25(OH)₂D₃. Thus, different attributes of the epigenome, such as histone modifications and the binding of transcription factors, are vitamin D sensitive (Carlberg 2017).

In summary, this review discusses the epigenome- and transcriptome-wide response of vitamin D target tissues in the context of chromatin. We will present regulatory scenarios of vitamin D target genes that are based on the VDR binding to different types of enhancers.

Figure 1

Vitamin D signaling in the context of chromatin. Via CYP enzyme-driven hydroxylation reactions vitamin D₃ is converted to 25-hydroxyvitamin D₃ (25(OH)D₃) and 1,25(OH)₂D₃ (A). The latter vitamin D metabolite binds with high affinity to VDR (red) bound to thousands of accessible genomic regions (B). VDR binding and placement of histone marks (H3K27ac) at respective enhancer regions as well as changes in chromatin accessibility are supported by pioneer transcription factors, such as PU.1 (dark blue) and CEBPA (green). Looping of VDR-bound enhancers to TSS regions leads to changes to histone marks at promoters (H3K4me3 and H3K27ac) and the activation RNA polymerase II. This finally leads to mRNA transcription of respective vitamin D target genes.–

Vitamin D signaling in the context of chromatin

At present, the most comprehensive set of epigenome- and transcriptome-wide data on the actions of vitamin D are available from cells of the hematopoietic system (Carlberg 2014). Accordingly, the chromatin model of vitamin D signaling (Fig. 1B) is primarily based on data (Carlberg 2017, 2019) obtained in the human monocytic cell line THP-1, which was derived from the peripheral blood of a 1-year-old human male with acute monocytic leukemia

(Tsuchiya *et al.* 1980). The chromatin model describes the sequential process of vitamin D target gene activation, the central event of which is the high-affinity binding of $1,25(\text{OH})_2\text{D}_3$ to the VDR, when the receptor is located in the nucleus. The VDR cistrome, i.e. the receptor's genome-wide binding pattern, had been determined by the method ChIP-seq (chromatin immunoprecipitation combined with massive parallel sequencing) in a number of human cell culture models, such as GM10855 and GM10861 B lymphocytes (Ramagopalan *et al.* 2010), LS180 colorectal cancer cells (Meyer *et al.* 2012), RWPE1 prostate epithelial cells (Fleet *et al.* 2019), LX2 hepatic stellate cells (Ding *et al.* 2013) and lipopolysaccharide-polarized THP-1 cells (Tuoresmäki *et al.* 2014), but most intensively it was studied in undifferentiated THP-1 cells (Heikkinen *et al.* 2011, Neme *et al.* 2017). In the presence of ligand, in these *in vitro* model systems some 5000–20,000 genomic VDR-binding sites had been detected, which represents a 2- to 10-fold increase compared to cells not stimulated with $1,25(\text{OH})_2\text{D}_3$.

In THP-1 cells, ChIP-seq detected 11,657 VDR-binding sites (Neme *et al.* 2017). This largely exceeds the number of primary vitamin D target genes in the same cell line, i.e. genes that are significantly up- or downregulated within the first 4 h after ligand stimulation. Interestingly, out of this large pool of VDR loci only 510 are persistent, i.e. they are occupied at all conditions. Nevertheless, the binding of VDR to its persistent sites can change significantly over time after ligand stimulation. Thus, persistent VDR loci are the primary contacts of the genome with $1,25(\text{OH})_2\text{D}_3$ and in addition coordinate the functional consequences of vitamin D stimulation over time. The machine learning algorithm SOM (self-organizing map) (Kohonen 1995) reduced the number of these persistent loci to 339 sites (Neme *et al.* 2017). Persistent VDR loci are positioned strategically over the whole human genome, which fits with the pattern of the genomic location of primary vitamin D target genes (Neme *et al.* 2016b). In addition, there are 2109 transient VDR-binding sites, i.e. loci where the binding of VDR is significantly induced by stimulation with $1,25(\text{OH})_2\text{D}_3$. Transient VDR sites are not always occupied, but this allows these loci to modulate the response of the epigenome to vitamin D either alone or in coordination with persistent VDR sites, in order to regulate primary vitamin D target genes. However, the large majority of VDR-binding events (9038 sites) are observed only after a ligand stimulation over 24 h. Thus, the $1,25(\text{OH})_2\text{D}_3$ -stimulated, genome-wide binding of VDR to its preferred loci is the primary epigenome-wide effect of vitamin D.

There are a number of secondary epigenome-wide effects of $1,25(\text{OH})_2\text{D}_3$ stimulation, such as a significant modulation in the genomic binding of the pioneer factors PU.1 (purine-rich box 1) (Seuter *et al.* 2017), CEBPA (CCAAT/enhancer-binding protein alpha) (Nurminen *et al.* 2019) and GABPA (GA-binding protein transcription factor subunit alpha) (Seuter *et al.* 2018) to vitamin D-responding chromatin regions. Pioneer factors bind rather promiscuously to genomic DNA and interact with a large number of other proteins (Zaret & Carroll 2011). In the process of monocyte and granulocyte differentiation PU.1 cooperates with VDR (Novershtern *et al.* 2011). For example, in THP-1 cells PU.1 is present at nearly 2/3 of all VDR-binding sites (Seuter *et al.* 2017).

TADs are segments of genomic DNA in a size range from 30 kb to 3 Mb that define at least 2000 functionally independent domains of our genome (Dixon *et al.* 2012). The anchors of TAD-forming chromatin loops act as insulators and show high conservation between tissues and cell types (Dixon *et al.* 2015, Vietri Rudan *et al.* 2015). The transcription factor CTCF (CCCTC-binding factor) binds to TAD anchors and is one of the key proteins organizing the 3D-structure of chromatin (Ong & Corces 2014). Interestingly, the binding of CTCF to 1300 of its genomic sites (5% of all) is modulated by $1,25(\text{OH})_2\text{D}_3$ (Neme *et al.* 2016a). Approximately 600 of these vitamin D-dependent CTCF sites function as TAD anchors. Thus, the $1,25(\text{OH})_2\text{D}_3$ sensitivity of CTCF binding to TAD anchors implies that also on the level of 3D chromatin organization vitamin D affects the epigenome.

Importantly, also the accessibility of chromatin, such as determined by the method FAIRE-seq (formaldehyde-assisted isolation of regulatory elements sequencing) (Giresi *et al.* 2007), is significantly affected at some 9000 genomic loci by $1,25(\text{OH})_2\text{D}_3$ stimulation (3300 loci after 2 h, 4500 sites after 24 h and 2400 regions after 48 h only) (Seuter *et al.* 2016). This observation was confirmed on the level of histone markers. Accordingly, 550 of 22,998 genomic regions with H3K4me3 marks, i.e. primarily promoters, and even 2473 of 45,578 regions with H3K27ac modifications, i.e. mainly enhancers, respond significantly to vitamin D stimulation (Nurminen *et al.* 2018).

The contacts between VDR-bound enhancer regions and the TSS of vitamin D target genes occur primarily within the same TAD, i.e. the borders of a TAD limit the reach of the effects of an activated VDR locus. Thus, a given VDR-binding site can only affect the expression of vitamin D target genes within the same TAD. For example, in THP-1 cells there are 425 TADs that contain at least one VDR-binding site and one vitamin D target gene

(Neme *et al.* 2016b, 2017). Machine learning algorithms, such as K-means, random forests and SOM, were used for the analysis and implementation of time-dependent data on accessible chromatin and mRNA expression. This approach suggested that the relative amounts of persistent and transient VDR-binding sites are the main attribute for describing the regulatory scenario of a given gene within its TAD (Carlberg & Neme 2019). For example, TADs comprising vitamin D target genes, which are important for immune function, are primarily regulated by transient VDR sites, i.e. by genomic loci that respond in an 'on/off' modus. In the following, we will present a new analysis relating the occurrence of persistent and transient VDR sites within super-enhancers to the regulation of primary upregulated vitamin D target genes.

Taken together, the chromatin model of vitamin D signaling (Fig. 1B) suggests that $1,25(\text{OH})_2\text{D}_3$ binds specifically to VDR molecules, which in a spatio-temporal order settle down on thousands of genomic loci. The contact of VDR with enhancers is supported by pioneer factors at these loci leading to an increase in chromatin accessibility as well as in changes in the marking of histones, i.e. in chromatin activity. VDR-activated enhancers then loop to TSS regions, where RNA polymerase II is stimulated to increase mRNA production. In its present version the model can explain the regulation of primary vitamin D target genes. In contrast, secondary target genes do not directly require VDR as a central regulator, but may be controlled by a transcription factor, such as BCL6, NFE2, POU4F2 and ELF4 (Nurminen *et al.* 2015), or a chromatin modifier, such as the lysine demethylase KDM6B (Pereira *et al.* 2011) or the chromatin remodeler BRD7 (Wei *et al.* 2018), which themselves are primary vitamin D target genes.

Transcriptome-wide responses to vitamin D

The most comprehensive description of the vitamin D-modulated transcriptome, as determined by RNA-seq (RNA sequencing) (Wang *et al.* 2009), had been performed with THP-1 cells that were stimulated for 2.5, 4 and 24 h with $1,25(\text{OH})_2\text{D}_3$ (GSE69284, www.ncbi.nlm.nih.gov/geo). Initially this dataset was aligned to the reference genome (hg19, representing in total 23,487 genes) using the algorithm Bowtie (Langmead *et al.* 2009). Statistics for differential expression applying the algorithm Cuffdiff (Trapnell *et al.* 2012) indicated 1284 vitamin D target genes (Seuter *et al.* 2016). A re-analysis (Neme *et al.* 2017) of this dataset used STAR alignment (Dobin *et al.* 2013) for mapping the sequence reads to the genome and the algorithm DESeq2 (Love *et al.* 2014) for calculating

differential expression. This approach even suggested 3650 target genes, i.e. one in four expressed genes, which is an unrealistically high number, indicating a large number of false positives due to weakly regulated genes. Therefore, SOM analysis was performed with the restriction that the average change in gene expression should be at least 2. This resulted in a list of 587 genes, all of which were vitamin D responsive after 24 h stimulation (Neme *et al.* 2017). Interestingly, 311 of these 587 genes (53%) are primary vitamin D targets, only 12 of which are downregulated.

In the past, lists of differentially expressed genes were filtered either by the highest fold change or lowest *P* value (Palmer *et al.* 2003, Heikkinen *et al.* 2011, Verway *et al.* 2013). However, highly expressed genes, which are statistically significantly up- or downregulated but do not show very impressive inducibility (fold change) are often overlooked by the traditional approach. Moreover, most gene filtering approaches are quite sensitive to threshold settings. We demonstrate this with the new filtering of the THP-1 time course experiments of Seuter *et al.* (2016) as an example. In order to include very low expressed but highly induced genes, such as *CYP24A1*, we defined a gene as 'expressed' when its basal expression times the fold change 24 h after $1,25(\text{OH})_2\text{D}_3$ stimulation exceeded the value 10. This condition applied to 14,011 genes, 3803 of which showed at least at one of the three stimulation time points a significant ($P < 0.05$) change in expression (Fig. 2A). Thus, compared with the first re-analysis of Neme *et al.* (2017) this less restricted condition increased the number of possible target genes even by 153 candidates. In order to work with a more condensed set of vitamin D genes, we focused on the 100 vitamin D target genes with highest basal activity in combination with the top 100 lists concerning fold change (highest) and *P* value (lowest) (Fig. 2B). Due to overlaps within the lists of fold change and *P* value, this led to a total of 264 genes (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). Only 119 of these 264 genes (45%) are primary vitamin D targets, six (5%) of which are downregulated (Supplementary Table 1). Moreover, only to 11% of the top 100 genes concerning highest basal activity are primary vitamin D targets, while this applies to 57% and even 86% of the top 100 lists concerning fold change and *P* value, respectively (Fig. 2C). For comparison, 1.4, 4.3 and 10.2% of the 587 vitamin D target genes identified after 2.5, 4 and 24 h after onset of $1,25(\text{OH})_2\text{D}_3$ stimulation, respectively, are downregulated (Neme *et al.* 2017).

In summary, the gene filtering approach used here as an alternative example (i.e. the restriction to the top 100

Figure 2

Vitamin D-triggered transcriptome. Venn diagrams were used for displaying different analyses of RNA-seq datasets obtained from THP-1 cells treated in three biological repeats for 2.5, 4 and 24 h with 10 nM 1,25(OH)₂D₃ in reference to solvent (final concentration 0.1% ethanol)-treated cells (Seuter *et al.* 2016). Raw data were analyzed using STAR alignment and DESeq2 statistics and provided 3803 significantly ($P < 0.05$) vitamin D-responsive genes (A). An alternative gene filtering approach is the restriction to the top 100 target genes concerning highest basal expression (i.e. absolute expression after solvent treatment), inducibility (highest fold change) and significance (lowest P value), respectively, leading to in total 264 genes (B). Based on the data assembly of Supplementary Table 1, the number of primary and secondary target genes as well as of down- and up-regulated genes is indicated (C). Genes were considered as primary vitamin D targets when their expression changed significantly ($P < 0.05$) within 4 h after ligand treatment.

genes of each basal expression, fold change and P value) highlights 99 genes as possibly functionally important vitamin D targets that have not been considered before. Interestingly, the vast majority of these genes are down-regulated secondary vitamin D target genes, which is in clear contrast to genes defined by highest inducibility (i.e. fold change) or sensitivity (i.e. P value).

Regulation of primary vitamin D target genes

The chromatin model of vitamin D signaling outlines the regulation of induced primary vitamin D target genes. In contrast, there is neither a general model for secondary target gene regulation nor an explanation, how vitamin D target genes are downregulated. Both secondary and downregulated genes have individual mechanisms of regulation that cannot be generalized (Bourdeau *et al.* 2008). Thus, we will restrict the discussion of gene regulatory scenarios to the 113 primary upregulated target genes (shaded green in Supplementary Table 1).

Top ranking in the list of vitamin D targets with highest basal expression is the *SRGN* (serglycin) gene, which encodes for a unique intracellular proteoglycan secreted by hematopoietic and endothelial cells (Iozzo & Schaefer 2015). The IGV (Integrative Genomics Viewer) browser (Thorvaldsdottir *et al.* 2013) was used for displaying data obtained in THP-1 cells, which had been stimulated for 24 h with 1,25(OH)₂D₃ or solvent (EtOH). These are ChIP-seq data for VDR (Neme *et al.* 2017), the histone markers H3K27ac and H3K4me3 (Nurminen *et al.* 2018) and the pioneer factors PU.1 (Seuter *et al.* 2017) and CEBPA (Nurminen *et al.* 2019) as well as FAIRE-seq data (Seuter *et al.* 2016) (Fig. 3). We define vitamin D-driven super-enhancers as genomic regions of maximal 20 kb in size that comprise a cluster of at least three VDR binding loci, one of which should be either a persistent or transient VDR site. The *SRGN* gene is driven by a super-enhancer that spans over 20 kb (11–31 kb upstream of the TSS) and contains five VDR-binding loci (one prominent persistent (P) site and four weaker sites that occur only after 24-h stimulation (24)) (Fig. 3A). Interestingly, the five VDR loci are associated with vitamin D-dependent chromatin opening. However, at the TSS regions no VDR binding was detected. This is most likely due to the moderated upregulation of the gene (fold change 1.7), in the context of which enhancer-TSS looping events were not frequent enough, in order to cross-link during ChIP-seq experiments sufficient amounts of the receptor to the TSS.

The gene *CD14* (cluster of differentiation 14), which encodes for a Toll-like receptor co-receptor (Zhang *et al.*

Figure 3

Epigenomic profiles of vitamin D target genes. The IGV browser was used to display the epigenomic profiles at enhancer and TSS regions of the genes *SRGN* (serglycin, A), *CD14* (cluster of differentiation 14, B) and *CYP24A1* (cytochrome P450 family 24 subfamily A member 1, C). The peak tracks display merged data from three biological repeats of ChIP-seq experiments with antibodies against VDR (red) (Neme *et al.* 2017), H3K27ac (light brown) (Nurminen *et al.* 2018), H3K4me3 (purple) (Nurminen *et al.* 2018), PU.1 (dark blue) (Seuter *et al.* 2017) and CEBPA (green) (Nurminen *et al.* 2019) as well as FAIRE-seq data (light blue) (Seuter *et al.* 2016) obtained in THP-1 cells that were treated for 24 h with 1,25(OH)₂D₃ (1,25D) or vehicle (EtOH). The gene structures are shown in blue and vitamin D target genes are indicated in red. VDR binding sites (P (persistent), T (transient) and 24 (24 h only)) at enhancer regions are shaded in gray, while TSS regions are highlighted with a purple shade.

1994), is the most significantly regulated vitamin D target, i.e. it is top ranking in the p-value list. *CD14* is another example of a gene being regulated by a super-enhancer. This VDR cluster region is shorter and only spans over 6 kb (21–27 kb downstream of the TSS). It contains three VDR binding loci (two transient (T) sites and one '24' site) (Fig. 3B). Moreover, the gene is strongly induced (fold change 83.9) so that VDR can also be detected on the TSS region of *CD14* and the neighboring gene *TMCO6* (transmembrane and coiled-coil domains 6), although only after 24-h 1,25(OH)₂D₃ stimulation.

The most induced vitamin D target gene is *CYP24A1* (cytochrome P450 family 24 subfamily A member 1), which encodes for a mitochondrial monooxygenase specialized on the degradation of 1,25(OH)₂D₃ (Kerry *et al.* 1996). Single gene ChIP-qPCR studies had identified a strong VDR binding enhancer only 300 bp upstream of the gene's TSS (Väisänen *et al.* 2005) and a cluster of additional VDR loci 50–69 kb downstream of the TSS (Meyer *et al.* 2010). Genome-wide ChIP-seq studies confirmed these VDR-binding sites (Fig. 3C), although the strong persistent VDR site close to TSS cannot be distinguished from TSS occupancy. Similarly, 29 other primary vitamin D target genes are driven by enhancers carrying either a persistent or a transient VDR site close (within 2 kb) to their TSS region (Supplementary Table 1). The three VDR-binding sites ('24') downstream of the *CYP24A1* gene represent individual weaker enhancers but do not form a super-enhancer. Thus, the most likely scenario for *CYP24A1* regulation by vitamin D is the prominent persistent VDR site close to the TSS. This scenario applies to 50 primary upregulated vitamin D target genes.

Taken together, primary upregulated vitamin D target genes are regulated *via* an enhancer carrying at least one prominent VDR-binding site (persistent or transient) that is located within the same TAD (Carlberg & Neme 2019, Nurminen *et al.* 2019). However, the gene regulatory scenarios differ in detail, such as the linear distance between the enhancer and the target gene's TSS as well as in the number of VDR-binding sites per enhancer. The majority of primary upregulated vitamin D target genes are driven by super-enhancers and will be discussed below in more detail.

Impact of super-enhancers on vitamin D signaling

In their classical definition, super-enhancers contain binding sites for different types of transcription factors

(Whyte *et al.* 2013). Since transcription factors are activated by a multitude of different signal transduction pathways, a super-enhancer is able to respond to a number of different signals and serves as their integrator (Hnisz *et al.* 2017). However, in the context of vitamin D signaling we focus on super-enhancers that carry three or more VDR-binding sites. This concept was first reported for the vitamin D response of the *TNFSF11* (TNF superfamily member 11) gene in mouse osteoblastic cells (Kim *et al.* 2007). We assume that such super-enhancers mediate an enhanced response to vitamin D. For this purpose, we inspected manually each of the 113 primary upregulated vitamin D target genes in THP-1 cells (Supplementary Table 1) for its VDR-binding profile at enhancer regions in a distance of 1 Mb (for a few genes even further) to its TSS. Interestingly, 29 genes (25.7% of all) have a prominent vitamin D response element (11 persistent and 18 transient VDR-binding sites) within 2 kb of their TSS (Fig. 4). Some of these, such as the one of the *CYP24A1* gene (Fig. 3C), have already been identified by single-gene studies during the past 30 years (Pike 1991, Haussler *et al.* 1997, Carlberg & Polly 1998).

Figure 4

Regulatory scenarios of 113 primary vitamin D target genes. Less than half of the primary vitamin D target genes are regulated by single strong VDR sites close (<2 kb) or distant (>2 kb) from their TSS (A). The majority of target genes are associated with VDR clusters forming super-enhancers that are either more distant from the TSS than the single strong VDR sites (B) or comprise the latter site (C).

Fifty of the 113 primary upregulated genes (44.2%) do not contain a super-enhancer in a distance of 1 Mb from their TSS, i.e. they are driven by a single vitamin D response element (Fig. 4A). Eleven of these single strong VDR-binding sites are located within the TSS region. In total, 63 of the 113 genes (55.8%) carry a super-enhancer in a distance of up to 955 kb from the TSS. Interestingly, 21 of the 63 genes with a super-enhancer have a prominent single vitamin D response element closer to their TSS than the VDR cluster (Fig. 4B). For these 21 genes the single vitamin D response element may be the more prominent regulator of the gene's expression than the VDR cluster. Nine of these 21 genes carry their single strong VDR-binding site even within 2 kb of the gene's TSS. In contrast, for 42 of the 63 genes with a VDR cluster the prominent single VDR site is part of a super-enhancer, nine of which comprise the TSS region (Fig. 4C).

Vitamin D-driven super-enhancers contain VDR-binding sites, which were classified as persistent (P), transient (T) and '24 h only' (24) (Neme *et al.* 2017). For example, the constellation T-24-24, such as observed for the *TSPAN18* (tetraspanin 18) gene (Fig. 5C), is found in 26 of the 63 VDR clusters followed by 11 representatives of the P-24-24 configuration, as found for the *SRGN* gene (Fig. 5A). Other super-enhancer constellations are P-T-24 (8-times), P-T-T (5-times), T-T-24 (5-times, as exemplified for the *CD14* gene (Fig. 5B)), T-T (4-times), T-T-T (3-times) and P-T (once). All super-enhancer constellations (summarized in Supplementary Table 1) are based on a manual inspection of vitamin D target genes using the IGV browser displaying genome-wide data as described in Fig. 3 (including datafiles of the respective original publications) as well as the VDR-binding site classification of Neme *et al.* (2017). We do not count the 13 examples of VDR clusters in a 24-24-24 configuration, such as observed for the *CYP24A1* gene (Fig. 3C), as super-enhancers, since these VDR sites most likely have far lower impact than the strong single VDR sites.

The rather low number of genes with VDR clusters (63) did not allow the detection of statistically significant correlations between super-enhancer correlations and the functional profile of primary up-regulated vitamin D target genes. However, there is a tendency that genes that are strongly up-regulated, i.e. genes of high significance (Fig. 5B) and/or high inducibility (Fig. 5C), are dominated by clusters containing transient VDR sites. These genes often show low basal expression, so that they are highly sensitive to transcriptional 'noise', i.e. random fluctuations in gene transcription (Kellogg & Tay 2015). In contrast,

Figure 5

Classification of vitamin D target genes. Based on data assembled in Supplementary Table 1 primary vitamin D target genes can be classified by highest basal activity, inducibility (fold change) and significance (*P* value) (left). The expression of the genes *SRGN* (serglycin, A), *CD14* (cluster of differentiation 14, B) and *TSPAN18* (tetraspanin 18, C) are used as examples. Representative VDR-binding patterns (P (persistent), T (transient) and 24 (24 h only)) to super-enhancers are related to these three classes of vitamin D target genes (right) and are based on Seuter *et al.* (2016). 1,25D = 1,25(OH)₂D₃.

genes with high basal activity show only minor variations, i.e. they provide more stable results (Fig. 5C). For these genes a regulation *via* VDR clusters with persistent sites is more appropriate.

In summary, there are three main gene regulatory scenarios for vitamin D target genes: single strong VDR sites alone as well as in overlap or distance to a super-enhancer. There are different types of vitamin D-driven super-enhancers, but their correlation with gene function could not yet be established.

Functional profile of vitamin D target genes

A central question of the transcriptome-wide analysis of vitamin D target genes is, how their differential regulation after 1,25(OH)₂D₃ stimulation translates into physiological functions of the investigated cell type or tissue. Gene ontology analysis offers the statistically significant overrepresentation of vitamin D target genes in biological processes. Using the webtool *Enrichr* (Chen *et al.* 2013) (<https://amp.pharm.mssm.edu/Enrichr>) for

the transcriptome of THP-1 cells demonstrated that on the basis of the 3803 genes of the raw analysis (Fig. 2A) suggests that the prime function of vitamin D is energy metabolism, such as 'mitochondrial ATP synthesis coupled electron transport', the 587 SOM filtered genes (Neme *et al.* 2017) have a major role in signal transduction, such as 'phosphatidylinositol 3-kinase signaling', while the 264 genes based of the three top 100 lists (Fig. 2C) confirmed the expected function of monocytes. The top processes, such as 'neutrophil activation', 'neutrophil

mediated immunity', 'neutrophil degranulation' and 'positive regulation of viral entry into host cell' relate to innate immunity, while 'cellular response to lipid', 'regulation of alcohol biosynthetic process', 'cellular response to oxygen-containing compound', 'regulation of cholesterol biosynthetic process' and 'regulation of steroid biosynthetic process' indicate functions in the context of metabolism (Table 1). This fits with the immunometabolism concept (Vanherwegen *et al.* 2017, Munoz Garcia *et al.* 2018), which was suggested as main

Table 1 Gene ontology analysis.

	Gene ontology term	Adjusted P value	Overlap
Top 100 filtered list (264 genes)	Neutrophil activation involved in immune response (GO:0002283)	1,67E-04	23/484
	Neutrophil mediated immunity (GO:0002446)	1,85E-04	24/488
	Cellular response to lipid (GO:0071396)	2,00E-04	14/179
	Neutrophil degranulation (GO:0043312)	2,88E-04	23/480
	Regulation of alcohol biosynthetic process (GO:1902930)	3,23E-04	7/35
	Cellular response to oxygen-containing compound (GO:1901701)	7,14E-04	16/275
	Regulation of cholesterol biosynthetic process (GO:0045540)	7,20E-04	7/41
	Regulation of cholesterol metabolic process (GO:0090181)	7,48E-04	7/42
	Positive regulation of viral entry into host cell (GO:0046598)	0,001016823	4/8
	Regulation of steroid biosynthetic process (GO:0050810)	0,00108109	7/45
SOM-filtered list (587 genes)	Regulation of phosphatidylinositol 3-kinase signaling (GO:0014066)	0,003231095	13/81
	Positive regulation of phospholipid metabolic process (GO:1903727)	0,003333861	8/29
	Regulation of cell migration (GO:0030334)	0,003351463	26/317
	Positive regulation of phosphatidylinositol 3-kinase signaling (GO:0014068)	0,003352442	10/54
	Regulation of phosphatidylinositol 3-kinase activity (GO:0043551)	0,004129987	8/34
	Podosome assembly (GO:0071800)	0,004161661	5/9
	Positive regulation of phosphatidylinositol 3-kinase activity (GO:0043552)	0,005339282	7/26
	Positive regulation of lipid kinase activity (GO:0090218)	0,007103159	7/28
	Regulation of platelet activation (GO:0010543)	0,007781412	6/19
	Positive regulation of intracellular signal transduction (GO:1902533)	0,007862047	32/480
Raw analysis list (3803 genes)	Mitochondrial ATP synthesis coupled electron transport (GO:0042775)	2,59E-15	54/86
	Respiratory electron transport chain (GO:0022904)	1,96E-14	56/95
	Neutrophil degranulation (GO:0043312)	2,94E-08	151/480
	Neutrophil activation involved in immune response (GO:0002283)	3,64E-08	152/484
	Mitochondrial respiratory chain complex I assembly (GO:0032981)	4,40E-08	36/65
	Neutrophil-mediated immunity (GO:0002446)	4,80E-08	153/488
	Mitochondrial respiratory chain complex I biogenesis (GO:0097031)	5,03E-08	36/65
	NADH dehydrogenase complex assembly (GO:0010257)	5,87E-08	36/65
	Mitochondrial electron transport, NADH to ubiquinone (GO:0006120)	7,87E-08	29/47
	Mitochondrial transport (GO:0006839)	2,84E-06	55/136

Lists of vitamin D target genes (Fig. 2) were analyzed using the web-tool *Enrichr* for most significant biological processes.

function of vitamin D-responsive cells of the innate immune system, such as monocytes and their derivatives, dendritic cells and macrophages.

The most prominent locations of the proteins encoded by the 264 vitamin D target genes (Supplementary Table 1) are the plasma membrane (34.5%), the nucleus (22.0%), the cytosol (12.5%), the cytoskeleton (5.3%) and the endoplasmic reticulum (5.3%). Moreover, 9.8% of the proteins are primarily secreted. Since proteins with an important role in immunity are primarily found at the plasma membrane or are secreted, this observation confirms the link of proteins encoded by vitamin D target genes in the immune system.

Taken together, the approach of the restricted list of vitamin D target genes including genes with high basal activity demonstrated its reliability by highlighting functionally important vitamin D target genes involved in immunometabolism.

Conclusion

In this analytical review, we extended the chromatin model of vitamin D signaling by the concept of VDR clusters. More than half of all primary upregulated vitamin D target genes are regulated by super-enhancers containing strong VDR-binding sites. In this context, we demonstrated that vitamin D target genes should not only be classified according to their significance and inducibility, but that also the basal expression of the genes should be taken into account. This approach highlighted some 100 genes that previously have been neglected. The inclusion of these genes as well as a focus on the genes with highest significance and inducibility provided a reliable description of the functional response to vitamin D.

The analysis presented here is primarily based on an *in vitro* model (THP-1 cells), which had been treated with a super-physiological concentration of $1,25(\text{OH})_2\text{D}_3$ (10 nM) in a de-lipidized cell culture medium context. Thus, the up to 400-fold inductions of genes, such as *CYP24A1*, will not be observed under *in vivo* conditions. Recently, we developed an experimental system, in which human volunteers were treated with a bolus of vitamin D_3 (2000 µg) and changes of gene expression with peripheral blood mononuclear cells (PBMCs), which were isolated before and 24 h after the bolus, was measured by RNA-seq (Neme *et al.* 2019). Although far lower fold changes were observed, 702 genes significantly ($P < 0.05$) changed their expression. Interestingly, 181 (25.8%) of these vitamin D target genes had already been described in THP-1 cells (Seuter *et al.* 2016). Thus, the here presented extension of

the chromatin model of vitamin D signaling will be soon applied for primary human cells, such as PBMCs.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-19-0246>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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