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Molecular endocrinology of vitamin D on the epigenome level

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ABSTRACT

The molecular endocrinology of vitamin D is based on the facts that i) its metabolite $1\alpha,25$ -dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is the high affinity ligand of the nuclear receptor vitamin D receptor (VDR) and ii) the transcription factor VDR is the unique target of $1,25(OH)_2D_3$ in the nucleus. Short-term alterations of the epigenome are primarily changes in the post-translational modification status of nucleosome-forming histone proteins, the consequences of which are i) a local increase or decrease in chromatin accessibility and ii) the activation or repression of gene transcription. Vitamin D has via VDR a direct effect on the expression of several hundred primary target genes implying numerous effects on the epigenome. Next-generation sequencing methods, such as ChIP-seq and FAIRE-seq, were applied to cellular model systems of vitamin D signaling, such as THP-1 human monocytes, and provided data for a chromatin model of vitamin D signaling. Key points of this model are that i) in the absence of ligand VDR binds to a limited number of loci within accessible chromatin, ii) a stimulation with ligand increases the number of DNA-bound VDR molecules, iii) VDR's access to genomic DNA is supported by pioneer factors, such as PU.1 in monocytes, iv) VDR binding leads to local opening of chromatin and v) the binding strength of topologically associating domain anchor forming CCCTC-binding factor sites upstream and downstream of prominent VDR binding sites is changing in response to ligand stimulation. This model provides the present basis of the molecular endocrinology of vitamin D and will be in future refined by the integration of vitamin D-sensitive chromatin markers and other genome-wide data, such as the $1,25(OH)_2D_3$ -sensitive binding of co-factors, chromatin modifying enzymes and chromatin remodeling proteins.

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1. Introduction

The term “epigenetics” was first defined by the embryologist Conrad Waddington as the genetic process behind development and cellular differentiation (Waddington, 1942). In a more general

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Abbreviations

1,25(OH) ₂ D ₃	1 α ,25-dihydroxyvitamin D ₃
25(OH)D ₃	25-hydroxyvitamin D ₃
CAMP	cathelicidin antimicrobial peptide
ChIA-PET	chromatin interaction analysis by paired-end tag sequencing
ChIP	chromatin immunoprecipitation
ChIP-seq	ChIP coupled with massive parallel sequencing
CTCF	CCCTC-binding factor
CYP	cytochrome P450
CYTH4	cytohesin 4
DNMT	DNA methyltransferase
DR3	direct repeat spaced by 3 nucleotides
ELFN2	extracellular leucine rich repeat and fibronectin type III domain containing 2

ENCODE	Encyclopedia of DNA elements
FAIRE-seq	formaldehyde-assisted isolation of regulatory elements sequencing
GTEX	Genotype-Tissue Expression
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
HMT	histone methyltransferase
HOMER	Hypergeometric Optimization of Motif EnRichment
IGV	Integrative Genomics Viewer
RNA-seq	RNA sequencing
RXR	retinoid X receptor
TAD	topologically associating domain
TET	ten-eleven translocation
TSS	transcription start site
VDR	vitamin D receptor

definition epigenetics comprises all functionally relevant changes of the genome that do not involve any alteration in the nucleotide sequence but are still potentially heritable (Wu and Morris, 2001). This view emphasizes that epigenetics is the basis of far more physiological processes than cellular differentiation. Nevertheless, the first epigenetic change identified was cytosine methylation of genomic DNA during development (Holliday and Pugh, 1975). In today's clinical research, such as in oncology, measuring changes in DNA methylation, preferentially within so-called "CpG islands", is still the "gold standard" for monitoring epigenetic effects (reviewed in (Dawson and Kouzarides, 2012)). Post-translational modifications, such as acetylation and methylation, of nucleosome-forming histone proteins are also considered as epigenetic events (Carlberg and Molnár, 2016b). This extends the focus of epigenetics from genomic DNA to its complex with nucleosomes, referred to as chromatin. These epigenomic modifications, also called "histone marks", represent a kind of chromatin indexing. Some of these marks stay stable during cell divisions and can last for multiple generations, i.e. they can be inherited. In contrast, other epigenomic marks are very transient, i.e. they last only for a short time (Zentner and Henikoff, 2013). The latter leads to the broadest definition of epigenetics that comprises all factors involved in gene expression (reviewed in (Deans and Maggert, 2015)). In a genome-wide perspective this definition implies that all nuclear proteins interacting with genomic DNA and chromatin, such as transcription factors, co-factors and chromatin modifiers, are part of the epigenome.

The endocrinology of vitamin D is based on the fact that already at sub-nanomolar concentrations the biological active form of vitamin D₃, 1,25(OH)₂D₃, binds and activates the nuclear hormone receptor superfamily member VDR (Haussler et al., 2008). VDR is the exclusive nuclear target of 1,25(OH)₂D₃, i.e. the molecular endocrinology of vitamin D can be well explained by the actions of this unique nuclear receptor. VDR is one of more than 1600 human genes that encode for transcription factors, i.e. for proteins that i) associate sequence-specifically with genomic DNA and ii) are able to modulate the activity of RNA polymerase II in the gene expression process (Carlberg and Molnár, 2016d; Vaquerizas et al., 2009). A harmonized meta-analysis of genome-wide VDR binding in six cellular models indicated that in the presence of 1,25(OH)₂D₃ the number of DNA-bound VDR proteins increases in average by a factor of 2.5 compared to the absence of ligand (Tuoresmäki et al., 2014). Vitamin D-triggered genomic VDR binding is already an epigenetic event, the functional consequences of which will be

discussed here. Thus, the theme of this review, the epigenomics of vitamin D endocrinology, is directly linked to the genome-wide effects of 1,25(OH)₂D₃ and its receptor VDR.

2. The human epigenome

In a repeat of every 200 bp some 75% of genomic DNA (147 bp) are wrapped around nucleosomes, each of which is formed of two copies of the histone proteins H2A, H2B, H3 and H4. The macromolecular complex of DNA and histones is referred to as chromatin and provides the scaffold for the packaging of the entire human genome (and that of other eukaryotes). This implies that even within highly accessible chromatin the simplistic scheme of "naked" DNA does not correctly describe the status of genomic DNA within the nucleus of a cell. Chromatin can largely differ in its degree of packaging with the extreme states of open euchromatin comprising most of the active genes and highly condensed heterochromatin primarily containing inactive genes (Fig. 1) (Carlberg and Molnár, 2016c).

Histones are small proteins that in particular at their protruding amino-terminal tails are rich in the basic amino acids lysine and arginine. The electrostatic attraction of positively charged histones and negatively charged phosphate groups in the backbone of genomic DNA supports the wrapping of DNA around nucleosome cores (Helin and Dhanak, 2013). Cytosine methylations as well as histone methylations and acetylations can alter chromatin structure by affecting the non-covalent interactions within and between nucleosomes. Many histone tail residues are subject to these post-translational modifications that are performed by a large variety of chromatin modifying enzymes. Histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases (HDMs) regulate covalent histone modifications, while genomic DNA gets methylated by DNA methyltransferases (DNMTs) and further modified and finally demethylated by ten-eleven translocation (TET) proteins. Moreover, in an ATP-dependent fashion chromatin remodeling protein complexes are able to shift the position of nucleosomes or even evict them completely at the positions of heavily used transcription start site (TSS) regions.

Less than 10% of the chromatin of an average terminally differentiated cell of the human body is in the euchromatin stage, i.e. the vast majority of the human genome is covered by intrinsically repressive heterochromatin (Smith and Meissner, 2013). Epigenome-wide analysis, such as performed by the Encyclopedia

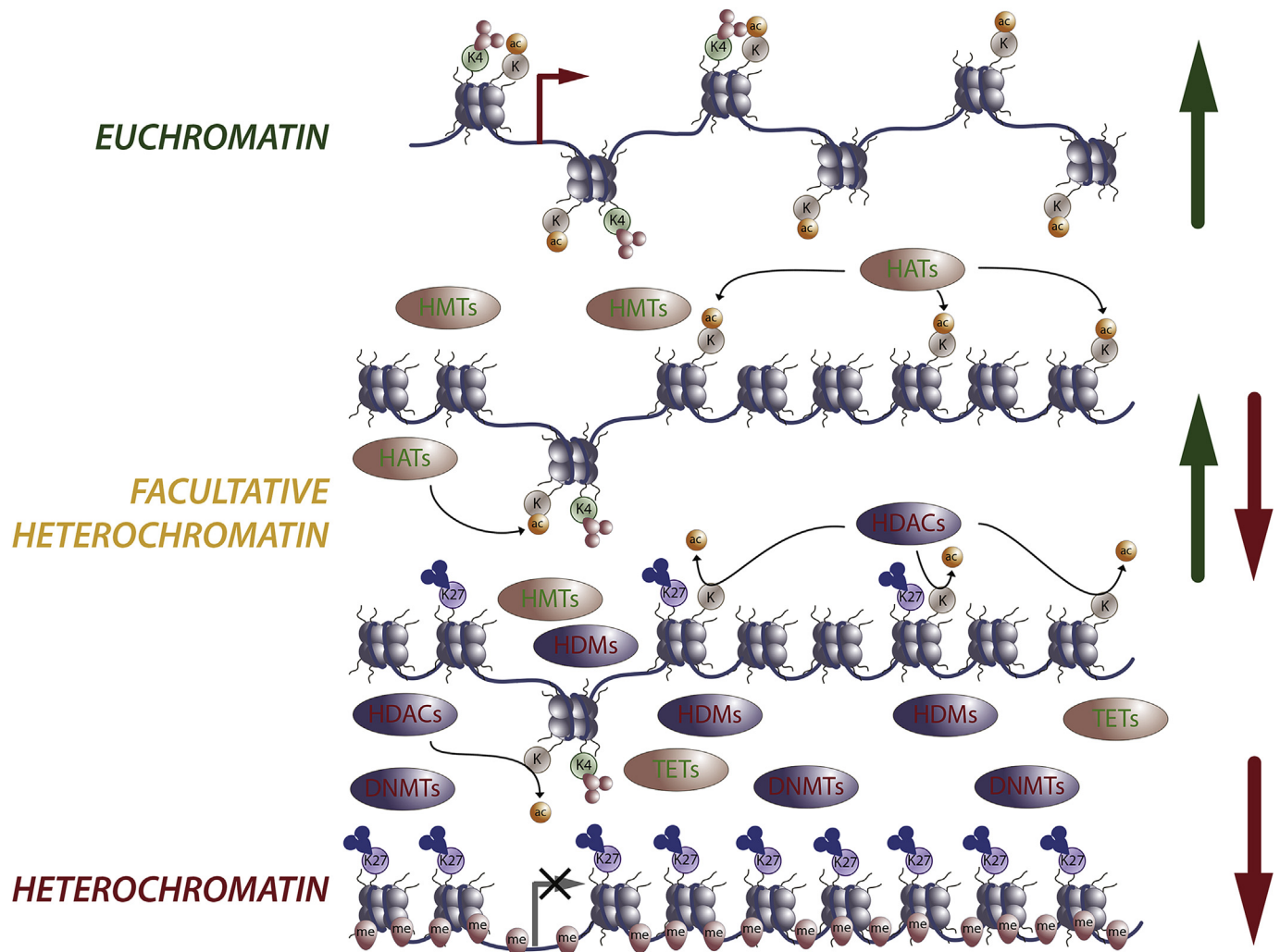


Fig. 1. Stages of chromatin. Association of gene activating chromatin modifying enzymes (HATs, HMTs and TETs) and gene silencing enzymes (HDACs, HDMs and DNMTs) with euchromatin (top) and heterochromatin (bottom) and two of many intermediary stages of facultative heterochromatin (center). Only a few representative chromatin marks are indicated. Histone acetylation can happen at a variety of lysine residues at the histone tails. Please note that methylated histones can either be marks of active chromatin (e.g., H3K4me3) or inactive chromatin (e.g., H3K27me3). ac, acetylation; me, methylation.

of DNA elements (ENCODE) project (www.encodeproject.org), demonstrated that a differentiated cell contains only some 50–100,000 accessible chromatin loci that primarily contain TSS and enhancer regions (ENCODE-Project-Consortium et al., 2012). In contrast, the remaining genomic DNA is protected from unintentional activation of its gene content by being located within far more densely packed and largely inaccessible heterochromatin. However, via the activity of chromatin modifying enzymes some of these chromatin regions, referred to as facultative heterochromatin, can be opened and again closed (Carlberg and Molnár, 2016a). More than hundred human genes encode for chromatin modifiers that add (“write”), interpret (“read”) or remove (“erase”) post-translational histone modifications. Chromatin acetylation is generally associated with transcriptional activation, while it seems to be less critical which amino acid residues of the histone tails are acetylated (Fig. 1). The acetylation state of histones within a given chromatin locus is controlled by two classes of antagonizing histone modifying enzymes, HATs and HDACs. In analogy, also for histone methylation there are two classes of enzymes with opposite functions, HMTs and HDMs. Although histone methylation mainly mediates chromatin repression, such as H3K27me3, at certain residues, such as H3K4me3, it results in activation (Fig. 1).

Therefore, for histone methylation the exact residue in the histone tail and its degree of methylation (mono-, di- or tri-methylation) is of critical importance. Most histone marks are assigned to functional regions of the chromatin, such as TSS regions, enhancers or heterochromatin (Jenuwein and Allis, 2001).

At a given chromatin locus the activities of the modifying enzymes are controlled by local transcription factor binding and signal transduction cascades originating from intra- and extracellular signal transduction pathways (Badeaux and Shi, 2013). VDR and its ligand 1,25(OH)₂D₃ belong to the factors influencing the activity of chromatin modifiers and thus are modulators of the human epigenome.

3. Interaction of vitamin D with the epigenome

The first level of interaction of vitamin D with the epigenome concerns the expression of the key proteins required for vitamin D endocrinology, such as VDR and the vitamin D metabolizing enzymes CYP2R1 (hydroxylation at C25), CYP27B1 (hydroxylation at C1α) and CYP24A1 (hydroxylation at C24) (reviewed in (Fetahu et al., 2014)). The regulatory regions of the genes encoding for these proteins contain large CpG islands that can be silenced by

DNA methylation. The human body is composed of approximately 400 tissues and cell types, 53 of which are represented in the current version (January 2017) of the Genotype-Tissue Expression (GTEx) portal (www.gtexportal.org) (GTEx-Consortium, 2013). Harmonized RNA-seq-based gene expression analysis of primary samples from more than 500 donors indicated that the *VDR* gene is expressed in approximately half of the tested human material, including adrenal glands, bladder, colon, fibroblasts, kidney, lymphocytes, pituitary gland, small intestine, skin and whole blood. This suggests that in these tissues and cell types the enhancer and TSS regions of the *VDR* gene are within accessible chromatin, so that the gene can be transcribed and most likely the protein will be expressed. Accordingly, the respective tissues should be responsive to vitamin D, i.e. they are part of the vitamin D endocrine system. Similarly, the *CYP2R1* gene is expressed in most human tissue samples tested by the GTEx consortium, while the expression of the genes *CYP27B1* and *CYP24A1* is restricted to kidney or kidney and bladder, respectively. This suggests that the first step in the activation of vitamin D₃, its conversion to 25-hydroxyvitamin D₃ (25(OH)D₃), may take part in many human tissues, while the second step, the production of 1,25(OH)₂D₃, is restricted to the kidney. The latter information is well-established knowledge in vitamin D endocrinology, while so far the liver was indicated as the prime site for 25(OH)D₃ synthesis (reviewed in (Bikle, 2014)). Interestingly, cellular transformation during tumorigenesis often increases chromatin access at the *CYP24A1* locus via a reduction of DNA methylation of the gene's regulatory region (Luo et al., 2010). In addition, the genomic region of *CYP24A1*, 20q13, is often overexpressed in cancers, such as colon cancer (Wang et al., 2016). Accordingly, the *CYP24A1* gene is expressed in many primary tumor samples and established cancer cell lines (reviewed in (Cross et al., 2003; Fetahu et al., 2014)). This epigenetic event decreases the concentration of available 1,25(OH)₂D₃ within the tumor tissue.

The second level of interaction of vitamin D endocrinology with the epigenome comprises direct protein-protein interaction of VDR with chromatin components, such as members of the Mediator complex (Rachez et al., 1999) or co-activators of the NCOA family (Herdick and Carlberg, 2000), which have HAT activity leading to local chromatin opening. In turn, the interaction of VDR with co-repressor proteins, such as NCOR1 (Polly et al., 2000), mediates the contact with HDACs that close chromatin at the respective genomic loci. So far, more than 50 nuclear proteins have been reported to interact with VDR (reviewed in (Molnár, 2014)). Together with the above-mentioned effect of ligand on the number of genome-wide VDR binding events, this suggests that the chromatin around VDR-associated genomic loci is sensitive to stimulation with vitamin D. The result of these epigenomic changes is a looping of vitamin D-inducible enhancers towards accessible TSS regions in their relative vicinity (reviewed in (Carlberg and Campbell, 2013)). Thus, ligand-activated VDR assembles not only with enhancers, but also with TSS regions, nuclear adaptor proteins and (at least indirectly) RNA polymerase II resulting in the modulation of the transcription of primary vitamin D target genes. The transcription factor VDR binds DNA sequence specifically, while HDACs are chromatin modifiers that recognize all acetylated chromatin regions. Therefore, vitamin D is able to act as a far more specific chromatin modulator than HDAC inhibitors, such as trichostatin A (Gaschott et al., 2001; Marks et al., 2001; Monneret, 2005). Nevertheless, as shown in THP-1 human monocytes, 1,25(OH)₂D₃ and trichostatin A can act synergistically in their epigenome-wide effect on gene expression (Pan et al., 2010; Seuter et al., 2013a).

In addition, vitamin D can influence the epigenome via chromatin modifying and remodeling proteins, the encoding genes of which are primary VDR targets. For example, in colon cancer cells the gene encoding for the HDM Jumonji C-domain containing

protein 3 (encoded by the gene *KDM6B*) is a primary VDR target (Pereira et al., 2011) suggesting that vitamin D contributes to the control of the histone methylation level. Furthermore, vitamin D was demonstrated to stimulate the demethylation of genomic DNA at the regulatory regions of VDR target genes, such as *CDKN1A* (encodes for the cyclin-dependent kinase inhibitor p21) but the exact mechanism of this effect is not yet understood (Doig et al., 2013).

4. Genome-wide monitoring of VDR binding

Numerous studies have demonstrated that VDR binds *in vitro* most efficiently to DNA when it forms a heterodimer with the nuclear receptor retinoid X receptor (RXR) (Carlberg et al., 1993; Liao et al., 1990; Sone et al., 1991) on a direct repeat of the sequence RGKTS(A/R = A or G, K = G or T, S = C or G) spaced by three nucleotides (DR3) (Ozono et al., 1990; Shaffer and Gewirth, 2004; Umehono et al., 1991). The application of the genome-wide method chromatin immunoprecipitation (ChIP) combined with tiled microarrays (ChIP-Chip, reviewed in (Pike, 2011)) or massive parallel sequencing (ChIP-seq, reviewed in (Carlberg, 2014)) demonstrated below the summits (± 100 bp) of VDR peaks a significant enrichment of DR3-type binding motifs.

In human cellular systems VDR ChIP-seq had been reported for B cells (GM10855 and GM10861) (Ramagopalan et al., 2010), monocytes (THP-1) (Heikkinen et al., 2011), macrophages (lipopolysaccharide-differentiated THP-1) (Tuoresmäki et al., 2014), colon cancer cells (LS180) (Meyer et al., 2012) and hepatic stellate cells (LX2) (Ding et al., 2013). In these experiments the time of ligand treatment ranged from 40 min (monocytes) up to 36 h (B cells). A harmonized re-analysis of these VDR ChIP-seq datasets resulted in more than 23,000 unique VDR binding loci throughout the whole human genome (Tuoresmäki et al., 2014). However, 70% of these VDR sites occurred in only one cellular model, i.e. VDR has a very cell type-specific genome-wide binding profile. Thus, also the epigenome-wide effects of vitamin D can be assumed to have similar tissue specificities.

The screening for DR3-type binding sites below VDR peaks using bioinformatic tools, such as HOMER (Heinz et al., 2010) at a highly stringent score of 9.1, identified such a motif only at 11.5% of all sites (Tuoresmäki et al., 2014). Although both the total number of identified VDR sites as well as their rate of DR3-type motifs critically depend on the threshold settings of the applied bioinformatic methods, it is obvious that by far not all VDR binding sites contain a DR3-type sequence. On none-DR3 sites VDR may form alternative complexes than heterodimers with RXR, such as indirect DNA binding “backpack” to other transcription factors (reviewed in (Carlberg and Molnár, 2015)). Interestingly, additional HOMER motif screening below VDR peaks showed a significant enrichment for binding sites for the transcription factor PU.1 (Tuoresmäki et al., 2014). PU.1 is a well-known pioneer transcription factor of the hematopoietic system (Zaret and Carroll, 2011) and works together with VDR in the process of monocytes and granulocyte differentiation (Novershtern et al., 2011). In monocytes PU.1 ChIP-seq demonstrated the co-location of VDR and PU.1 at nearly 2/3 of all VDR binding sites (Seuter et al., 2017) (see Fig. 2 for examples). Thus, at least in monocytes, VDR seems to use at the majority of its binding loci the support of PU.1, in order to locally keep the respective chromatin sites accessible. Nevertheless, there is no evidence that PU.1 has, comparable to RXR, a direct physical interaction with VDR.

Interestingly, at some 5% of all its genomic loci 1,25(OH)₂D₃ significantly increases PU.1 binding (Seuter et al., 2017) suggesting that there is also an inverse relationship on which VDR supports PU.1 binding. Furthermore, this observation is another example of

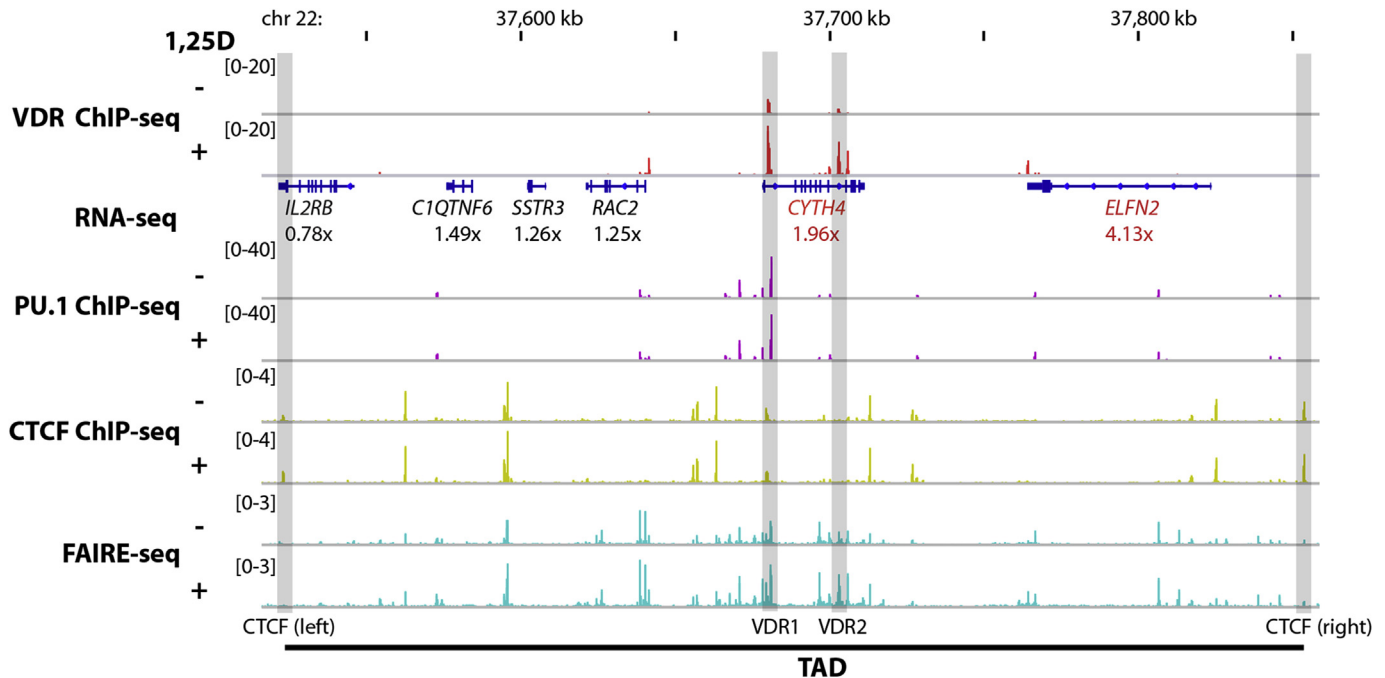


Fig. 2. Vitamin D response of the *CYTH4/ELFN2* locus in human monocytes. THP-1 cells were treated for 24 h with 1,25(OH)₂D₃ (1,25D) or vehicle (EtOH) and ChIP-seq, FAIRE-seq and RNA-seq were performed in three biological repeats. The Integrative Genomics Viewer (IGV) browser (Robinson et al., 2011) was used to visualize the TAD around the vitamin D target genes *CYTH4* and *ELFN2*. The peak tracks display data from ChIP-seq for VDR (red), PU.1 (purple), CTCF (light green) and FAIRE-seq (light blue). Gene structures are shown in blue. The change in expression of the six genes within the TAD was measured by RNA-seq. The genes *CYTH4* and *ELFN2* (red) are primary 1,25(OH)₂D₃ targets, since they were already significantly ($p < 0.05$) up-regulated after 2.5 h ligand stimulation (not shown, see (Seuter et al., 2016)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the epigenome-wide effects of vitamin D.

5. CTCF as organizer of vitamin D-dependent chromatin domains

The multi-zinc finger protein CCCTC-binding factor (CTCF) (reviewed in (Ghirlando and Felsenfeld, 2016; Phillips and Corces, 2009)) is the key transcription factor within genomic insulator regions. Genomic DNA loops in the size of hundreds of kilobases to a few megabases, referred to as topologically associating domains (TADs) (Ali et al., 2016), organize the human genome into at least 2000 active and inactive regions (Dixon et al., 2012). CTCF has the unusual property that its binding is highly conserved between tissues and cell types. Genome-wide every cell contains at least 20,000 CTCF loci, some 15% of which are involved in forming TAD anchor regions. In monocytes, at more than 1300 loci genomic CTCF binding was found to be significantly enhanced by 1,25(OH)₂D₃ (Neme et al., 2016b). More than half of these vitamin D-sensitive CTCF loci mark the anchors of 427 putative TADs containing at least one VDR binding site and one 1,25(OH)₂D₃ target gene. The assumption that these CTCF sites are involved in DNA looping and form TAD borders is based on data of the ENCODE tier 1 cell line K562 (human monocytes). Chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) assays with CTCF monitored all genome-wide 3-dimensional interactions of the chromatin organizing protein (ENCODE-Project-Consortium et al., 2012). As the genome-wide CTCF binding profile between K562 and THP-1 cells is to 95% identical (Neme et al., 2016b), the loops mediated by CTCF are most likely largely identical between both types of monocytic cell models (Oti et al., 2016). More than 95% of vitamin D-sensitive, TAD anchor-forming CTCF sites are up-regulated by ligand treatment (Neme et al., 2016b). Moreover, there is no obvious clustering of down-regulated vitamin D target genes

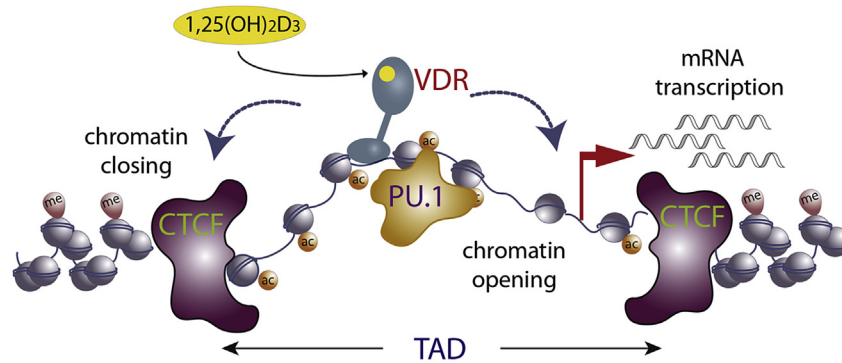
within the same TADs. Therefore, TADs seem not to provide any general mechanism for gene repression by vitamin D.

Fig. 2 shows an example TAD being formed by two vitamin D sensitive CTCF sites in a distance of 331 kb (Neme et al., 2016b). The TAD contains two prominent VDR binding sites and the two primary vitamin D target genes cytohesin 4 (*CYTH4*) and extracellular leucine rich repeat and fibronectin type III domain containing 2 (*ELFN2*). More than 400 similar TADs can explain the regulatory scenarios of up to 70% of the approximately 600 vitamin D target genes in the THP-1 model system (Neme et al., 2016b). This suggests that vitamin D-sensitive CTCF sites are critical elements in VDR signaling and represents another example of epigenome-wide effects of vitamin D. CTCF functionally interacts also with thyroid hormone receptors, which are, like VDR, members of the nuclear hormone receptor superfamily (Arnold et al., 1996). On a few example sites, but not genome-wide, this association has been shown to be thyroid hormone dependent (Lutz et al., 2003).

6. Vitamin D-triggered changes in chromatin accessibility

The functional readout of the activity of chromatin modifying enzymes and the resulting distribution of histone marks are different levels of accessibility of a given chromatin region (Fig. 1). In this way, transcription factors and their associated co-factors can bind more (or less) efficiently to a given genomic region (Bell et al., 2011). Chromatin accessibility is experimentally monitored by the methods DNase I hypersensitivity sequencing (DNase-seq) (Song and Crawford, 2010), Formaldehyde-Assisted Isolation of Regulatory Elements sequencing (FAIRE-seq) (Giresi et al., 2007) or Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) (Buenrostro et al., 2013).

For example, FAIRE-seq determined in THP-1 cells 62,000 accessible chromatin loci (Seuter et al., 2016). Most of the VDR



1. Absence of ligand: VDR binds to a limited number of loci within accessible chromatin.
2. Presence of 1,25(OH)₂D₃: The number of DNA-bound VDR molecules increases
3. Pioneer factors (e.g., PU.1 in monocytes): VDR's access to genomic DNA further increases.
4. Chromatin accessibility: Local increase after VDR binding
5. TAD anchors: CTCF sites upstream and downstream of prominent VDR binding sites increase in strength.
6. Vitamin D target genes: Located together with a prominent VDR site in a TAD flanked by vitamin D-sensitive CTCF sites.

Fig. 3. Chromatin model of vitamin D signaling.

binding sites in this cellular model co-localize with these loci of open chromatin (Seuter et al., 2013b) (see Fig. 2 for examples). Interestingly, a stimulation with 1,25(OH)₂D₃ leads not only to an enhanced binding of VDR to pre-occupied sites and the association of the receptor with a huge number of additional genomic loci (Tuoresmäki et al., 2014), but also to a significant increase in chromatin accessibility at many genomic regions. In monocytes, the accessibility of nearly 9000 chromatin loci is significantly modulated by 1,25(OH)₂D₃ (Seuter et al., 2016). Although the exact molecular mechanisms of these 1,25(OH)₂D₃-triggered epigenome changes are not yet understood, it is very likely that they are secondary consequences of ligand-sensitive genome-wide VDR binding. Interestingly, top-ranking motifs below the summits of vitamin D-sensitive chromatin regions are those for CTCF and PU.1 (Seuter et al., 2016). This suggests that both transcription factors are involved in the process of 1,25(OH)₂D₃-stimulated chromatin opening.

7. Transient and long-lasting epigenome-wide effects of vitamin D

A subset of the 9000 vitamin D-sensitive chromatin sites in THP-1 cells, such as those at TSS regions, are already fully accessible 2 h after onset of ligand stimulation. However, the majority of the chromatin regions reach their maximal accessibility after 24 h (Seuter et al., 2016). This indicates that the process of vitamin D-triggered chromatin opening involves multiple steps. Chromatin opening observed after 24 h after onset of ligand stimulation is most likely the secondary effects of primary vitamin D target genes, such as *BCL6*, that encode for chromatin modifiers, co-factors of transcription factors (Nurminen et al., 2015). Interestingly, after 48 h most vitamin D-sensitive chromatin sites return back to basal levels, i.e. many epigenome-wide effects of 1,25(OH)₂D₃ seem to be only transient.

Mouse differentiation models are an alternative way to follow vitamin D-triggered effects on the epigenome. In mice, VDR ChIP-seq had been performed in i) 3T3-L1 pre-adipocytes (Siersbaek et al., 2014), ii) IDG-SW3 osteocytes (St John et al., 2014), iii) MC3T3-E1 undifferentiated and differentiated osteoblasts (Meyer

et al., 2014a), iv) the proximal half of the small intestine (duodenum and jejunum) (Lee et al., 2015) and v) bone marrow-derived mesenchymal stem cells differentiating into bone and fat cells (Meyer et al., 2016). In contrast to VDR ChIP-seq in human cellular systems that studied rather short-term ligand effects, the main question of most of the experiments in mouse systems was the change of VDR binding during cellular differentiation over many days to weeks. For example, changes in gene expression over the 35 days of the osteocyte differentiation process were accompanied by post-translational modifications to histones H3 and H4, i.e. in alterations of the epigenetic landscape, that in turn alter the transcriptomic response to 1,25(OH)₂D₃ (St John et al., 2014). Similarly, in the mouse bone marrow-derived mesenchymal stem cell model the differentiation of 7 and 15 days into adipocytes and osteocytes, respectively, is a multi-step process that is epigenetically modulated by 1,25(OH)₂D₃ (Meyer et al., 2016). Genome-wide analysis in this model indicated that VDR is a key factor in the epigenetic programming of mesenchymal stem cell into osteocytes and has a different role for the differentiation into adipocytes. In pre-osteogenesis VDR regulates the expression of genes that are essential for osteoblastic identity (Meyer et al., 2014a, 2014b). Interestingly, during the differentiation process the genome-wide number of VDR binding sites is drastically reduced from some 10,000 to the order of 1000 (Meyer et al., 2016). However, a concluding meta-analysis of the VDR ChIP-seq data from different mouse cellular systems has not yet been reported.

8. Conclusions

The data that were collected in human cellular systems, in particular in THP-1 monocytes, can be summarized in a chromatin model of vitamin D signaling (Fig. 3). The model is based on the following key observations:

1. In the absence of ligand VDR already binds to a limited number of loci within accessible chromatin.
2. A stimulation with 1,25(OH)₂D₃ increases the number of DNA-bound VDR molecules as well as the percentage of binding sites carrying a DR3-type motif.

3. VDR's access to genomic DNA is supported by pioneer factors, such as PU.1 in monocytes.
4. VDR binding leads to changes in chromatin accessibility, the vast majority of which are opening of chromatin.
5. In parallel with VDR binding and chromatin opening the binding strength of TAD anchor forming CTCF sites upstream and downstream of prominent VDR binding sites is changing in response to ligand stimulation.
6. In monocytes the borders of more than 400 TADs that contain at least one VDR sites and one vitamin D target gene are formed by ligand sensitive CTCF loci.

In future, this emerging model will be further refined by data on vitamin D-sensitive chromatin marks, such as H3K27ac and H3K4me3, and other nuclear proteins, such as the 1,25(OH)₂D₃-sensitive binding of co-factors, chromatin modifying enzymes and chromatin remodeling proteins. In addition, the model needs to be integrated with the observations made in differentiation models. It will be a challenge to differentiate primary from secondary effects of vitamin D- and VDR-triggered epigenomics changes.

This and other models of vitamin D signaling are primarily based on experiments, in which a high dose of 1,25(OH)₂D₃ was applied once to a cell culture model and in parallel the reference sample was fully depleted from VDR ligands. In such experimental systems more than 100-fold changes in the expression of some genes, such as *CYP24A1*, cathelicidin antimicrobial peptide (*CAMP*) and *CD14*, can be observed in a time frame of up to 24 h (Neme et al., 2016a). However, one should always be mindful of the fact that in the *in vivo* endocrinology of vitamin D rapid responses to very high concentrations of hormone are not the norm (DeLuca, 2004; Norman, 2008).

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