Review

The First Genome-wide View of Vitamin D Receptor Locations and Their Mechanistic Implications

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Abstract. *The transcription factor vitamin D receptor (VDR) is the nuclear sensor for the biologically most active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$). The physiological actions of the VDR and its ligand are not only the well-known regulation of calcium and phosphorus uptake and transport controlling bone formation, but also their significant involvement in the control of immune functions and of cellular growth and differentiation. For a general understanding of the mechanisms of $1\alpha,25(OH)_2D_3$ signaling, it is essential to monitor the genome-wide location of VDR in relation to primary $1\alpha,25(OH)_2D_3$ target genes. Within the last months, two chromatin immunoprecipitation sequencing (ChIP-Seq) studies using cells of the hematopoietic system, lymphoblastoids and monocytes, were published. The reports indicated the existence of 2776 and 1820 $1\alpha,25(OH)_2D_3$-stimulated VDR-binding sites, comparable numbers, of which, however, only 18.2% overlapped. The two studies were very different in their $1\alpha,25(OH)_2D_3$ treatment times (36 h versus 40 min), but showed consensus in identifying response elements formed by a direct repeat of two hexameric binding sites with three intervening nucleotide (DR3) as major DNA contact sites of the VDR. Interestingly, when analyzed in the same way, both reports indicated that within 100 bp of their VDR ChIP-Seq peak summits only fewer than 40% contain a DR3-type response element. This review provides a detailed comparison of the insights obtained from both ChIP-Seq studies.*

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Physiological Impact of Vitamin D in the Immune System

Vitamin D is a micronutrient which under ultraviolet (UV) radiation can also be produced in the skin (1). The most abundant form of vitamin D is its liver hydroxylation product 25-hydroxyvitamin D_3 ($25(OH)D_3$), serum concentrations of which indicate the vitamin D status of the human individual (2). The biologically most active vitamin D metabolite is obtained from further hydroxylation of $25(OH)D_3$ in the kidney to $1\alpha,25(OH)_2D_3$ (3). Interestingly, the hydroxylation of vitamin D can also take place in other tissues and a few of them, such as keratinocytes and macrophages, have the capacity for the full conversion of vitamin D to $1\alpha,25(OH)_2D_3$, *i.e.* they are most sensitive to vitamin D. Initially vitamin D (and its hydroxylation products discovered later) was considered to be important primarily for adequate Ca^{2+} and P_i absorption from the intestine and hence for bone formation (4). Fittingly, the most striking effect of severe vitamin D deficiency is rickets. However, during the past 30 years it has become more and more obvious that $1\alpha,25(OH)_2D_3$ is a pleiotropic hormone that is involved in many physiological processes, such as the control of metabolism, cellular growth and immune functions (5).

In particular the immunoregulatory properties of $1\alpha,25(OH)_2D_3$ are gaining attention, as vitamin D deficiency is associated with poor immune function and increased disease susceptibility. The benefits of moderate UV radiation exposure and the positive effect of the latitude of residence (the closer to the equator, the better) observed for some immune-related diseases may therefore reflect the activities of UV-induced vitamin D production (6). $1\alpha,25(OH)_2D_3$ has potent effects both on the innate and the adaptive immune system. This nuclear hormone 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 (7). Main $1\alpha,25(OH)_2D_3$ targets in differentiating monocytes are antimicrobial peptides, such as cathelicidin, co-stimulatory molecules, such as CD14 (8), and cytokines, such as interleukins (ILs) 10 and 12b (9, 10). In order to modulate adaptive immune responses, $1\alpha,25(OH)_2D_3$ regulates the differentiation of regulatory T (T_{reg}) cells and of T helper (T_H) cells. Although $1\alpha,25(OH)_2D_3$ enhances the differentiation and suppressive capacities of T_{reg} cells, it reduces those in T_H1 and T_H17 cells.

Genomic Effects of $1\alpha,25(OH)_2D_3$

$1\alpha,25(OH)_2D_3$ binds the transcription factor VDR with an affinity of less than 1 nM, and the VDR is the only nuclear protein that binds this nuclear hormone with high affinity (11). VDR is a member of the nuclear receptor superfamily, which is the largest transcription factor family in humans (12). The members of this family are characterized by a highly conserved DNA-binding domain (DBD) and a structurally conserved ligand-binding domain (13). The vast majority of the actions of $1\alpha,25(OH)_2D_3$ are genomic and are directly correlated with the actions of the VDR. However, in contrast to a few other members of the nuclear receptor superfamily, such as the glucocorticoid receptor, the VDR can also bind its genomic targets in the absence of ligand, *i.e.* the functional profile of the VDR is larger than that of its ligand (14).

Similar to other transcription factors, the VDR interacts with the complex of genomic DNA and nucleosomes, referred to as chromatin, which *per se* prevents access of DNA-binding proteins to their genomic targets (15). This intrinsic repressive potential of chromatin is essential for long-lasting regulatory decisions, such as terminal differentiation of cells (16). 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, such as an exposure to $1\alpha,25(OH)_2D_3$ (17). 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 class of co-regulatory proteins with either histone acetyltransferase, deacetylase methyltransferase or demethylase activity (18).

VDR-Retinoid X Receptor (RXR) Heterodimer Binding Sites

An essential prerequisite for a direct modulation of transcription *via* $1\alpha,25(OH)_2D_3$ is the location of activated VDR close to the basal transcriptional machinery. This is achieved through the specific binding of the VDR to $1\alpha,25(OH)_2D_3$ response elements (VDREs) in the regulatory regions of primary $1\alpha,25(OH)_2D_3$ target genes. VDREs are located up- and downstream of the transcription start site (TSS) of these primary target genes and *via* DNA looping can come in close vicinity to the basal transcriptional machinery on the TSS (12). The hexameric consensus sequence RGKTSA (R=A or G, K=G or T, S=C or G) is the direct recognition motif of the VDR DBD. However, since the affinity of monomeric VDR to a single binding motif is not sufficient for the formation of a stable protein-DNA complex, VDR forms homo- and heterodimeric complexes with a partner nuclear receptor in order to allow efficient DNA binding (19). The predominant partner of VDR is RXR. Steric constraints allow dimerization of the DBDs of VDR and RXR only on REs with properly spaced core binding motifs. The most efficient interface for VDR–RXR heterodimers is an asymmetric arrangement, *i.e.* head-to-tail, as a direct repeat with three intervening nucleotides, a so-called DR3-type RE (19, 20).

With internet-based software tools, such as TRANSFAC (21) and JASPAR (22), which use position weight matrices based on experimental data, such as a series of gel shift assays with a large number of natural binding sites (23, 24), one can screen genomic DNA for putative VDREs. Depending upon the individual position weight matrix description, this leads to a prediction of VDREs every 1 to 10 kb of genomic sequence. This high frequency of VDREs is certainly an overprediction, since the screening tools do not take the repressive nature of chromatin into account.

The Chromatin Immunoprecipitation Method

Due to the limitations of traditional experimental methods and *in silico* screening approaches, the chromatin immunoprecipitation (ChIP) method (25) became very popular for the investigation of gene regulatory regions. 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 the chromatin to 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 polymerase chain reaction (PCR) using gene-specific primers (regular ChIP), hybridized to a microarray of promoter sequences (ChIP-chip) or 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. Nuclear receptors have the additional advantage that their association with genomic DNA is often

triggered by ligand application. Regular ChIP has been performed, for example, for the VDR target genes vitamin D 24-hydroxylase (*CYP24A1*) (26), 25(OH)D_3-hydroxylase (*CYP27B1*) (27), cyclin C (*CCNC*) (28) and cyclin-dependent kinase inhibitor 1A (*CDKN1A* encoding for $p21^{WAF/CIP}$) (29, 30). In each of these studies, 7-10 kb upstream of the respective gene TSS was investigated by using primer sets for 20-25 overlapping genomic regions. This approach identified four VDR-binding sites for both the *CYP24A1* and the *CCNC* gene, three in the *CDKN1A* gene and two in the *CYP27B1* gene. Each of these VDR-binding sites seems to be functional as chromosome conformation capture assays showed that they are able to contact the basal transcriptional machinery (30, 31). This suggests a simultaneous communication of individual gene regulatory regions with RNA polymerase II molecules located on TSS regions (32).

The limitations of the ChIP-chip technology are that it is, like PCR, still based on DNA hybridization and the choice of the DNA probes, which are preferentially close to coding and TSS regions. However, Pike *et al*. (33) effectively used this method with custom probe sets for the analysis of VDR targets, such as the VDR gene itself (34), the *CYP24A1* gene (35), the intestinal calcium ion channel gene *TRPV6* (36), the *WNT* signaling co-regulator gene *LRP5* (37) and the tumor necrosis factor receptor ligand gene *TNFSF11* (38). For all these genes, a number of VDR-associated chromatin regions were identified, some of which were far upstream or downstream of the gene's TSS. This confirms that many, if not all, VDR target genes have multiple VDR-associated regulatory regions.

As of early November 2011, there are only two published ChIP-Seq studies for the VDR, which, interestingly, both were performed for cells belonging to the hematopoietic system. The investigations of Ramagopalan *et al*. (39) were carried out in lymphoblastoid cell lines (LCLs) obtained from two human individuals participating in the HapMap study, while we performed our own study (8) with the human monocytic leukemia cell line THP-1, which is able to differentiate into M1- and M2-type macrophages.

In contrast to regular ChIP and ChIP-chip, the ChIP-Seq method is not biased by the choice of primer pairs or hybridization probes. Instead, short (35 bp) but specific sequence tags of all chromatin fragments are obtained irrespective of their location in the genome (40). Typically a ChIP-Seq run results in 10-20 million sequence tags, which are aligned to the reference genome. When these tags significantly accumulate at the same genomic location, in comparison to the IgG control experiment, one refers to them as peaks due to their appearance in the typical style of visualization. As the chromatin fragments should only be 200-400 bp in size, the peaks should not spread over a sequence of more than 800 bp, but often far wider peaks were obtained. In most cases, one focuses on the sequence (±100 bp) below the peak summit, since it most likely contains the binding motif used by the transcription factor of choice.

Transcriptome-wide Analyses of $1\alpha,25(OH)_2D_3$ Signaling

The effects of $1\alpha,25(OH)_2D_3$ on mRNA expression, *i.e.* $1\alpha,25(OH)_2D_3$-induced changes of the transcriptome, have been assayed during the past eight years by multiple microarray experiments in many different cellular models (either established cell lines or primary cells) and in *in vivo* models (mostly rodents). The qualities of these datasets reflect the development of the microarray platforms during the last decade, where initially cDNA arrays with an incomplete number of genes were used. Therefore, the first studies reported rather short lists of VDR target genes, for example from colon (41), prostate (42-45), breast (46) and osteoblasts (47, 48). However, even in the early phase of microarray analysis, a few studies indicated many target genes, such as the 900 genes that were obtained in squamous cell carcinoma after a 12 h stimulation with $1\alpha,25(OH)_2D_3$ (49).

The VDR ChIP-Seq studies discussed in the next sections (8, 39) are associated with microarray analyses of the same cellular systems. In human LCLs, Ramagopalan *et al*. (39) found 229 differentially expressed genes 36 h after $1\alpha,25(OH)_2D_3$ treatment (226 genes up- and only 3 down-regulated), while in our own study (8) in human monocytes, we found 638 genes responding after a much shorter 4 h ligand treatment (408 genes up- and 230 down-regulated). The result from monocytes that the majority (64%) of the $1\alpha,25(OH)_2D_3$ target genes are up-regulated is typical for most VDR target tissues (50, 51), but the very low number of down-regulated genes (1.3%) in LCLs is an unusual result.

As expected from the chosen cell types, gene ontology analyses indicated that in both lists, genes with functions in the immune system were significantly enriched. In monocytes, the positive effect of $1\alpha,25(OH)_2D_3$ on IL1 production and secretion became apparent, which is in line with previous reports (52, 53). Since hardly any gene is down-regulated in LCLs after 36 h $1\alpha,25(OH)_2D_3$ treatment, we focused on the up-regulated genes in both cellular models and found that only 31 (7.6%) out of the 408 VDR target genes in monocytes overlap with those in LCLs (Figure 1). When comparing the gene list after 36 h-stimulated LCL with the 1680 genes of our unpublished microarray of 24 h stimulation in monocytes, the number of overlapping genes increased to 57, but due to the higher number of responding genes, their percentage decreased to 3.4%. Interestingly, in monocytes, 318 of the 408 early up-regulated $1\alpha,25(OH)_2D_3$ target genes (77.4%) are also found in the 24 h microarray

list. However, in our 24 h treatment of monocytes, 7.4-fold more up-regulated $1\alpha,25(OH)_2D_3$ target genes were identified than listed for the 36 h-treated LCLs, which is likely attributed to the larger sample-to-sample variation in the latter study due to the use of separate cell lines originating from different human individuals as biological replicates.

There is not only a large variation in the microarray platforms used for transcriptome studies with $1\alpha,25(OH)_2D_3$, but the experimental conditions, such as treatment time and ligand concentration, have also been rather divergent. Some studies focused on the identification of primary VDR target genes and used rather short incubations with the ligand (2 to 6 h), while others were more interested in the overall physiological effects of $1\alpha,25(OH)_2D_3$ and used far longer treatment times (24 to 72 h). Accordingly, the gene lists obtained by the latter studies contain many secondary or even tertiary $1\alpha,25(OH)_2D_3$ target genes. Furthermore, the results of many of the earlier microarray studies with $1\alpha,25(OH)_2D_3$ were not placed in public data repositories, such as the Gene Expression Omnibus (GEO) of NCBI (54), *i.e.* a direct comparison of the results is difficult.

Although the setups of all these microarray analyses were different in treatment times and probe sets, the overall impression remains that most VDR target genes respond to $1\alpha,25(OH)_2D_3$ in a very tissue-specific fashion and may manifest only a rather transient response. Although a number of these genes may not be primary $1\alpha,25(OH)_2D_3$ targets, they nevertheless contribute to the physiological effects of $1\alpha,25(OH)_2D_3$.

Genome-wide View of VDR Locations

The VDR ChIP-Seq in human LCLs reported 2776 genomic VDR-binding sites in cells treated for 36 h with $1\alpha,25(OH)_2D_3$ compared to resting cells (39), while in human monocytes, after 40 min ligand stimulation we observed 1820 VDR ChIP-Seq peaks, 1171 of which occur only in the presence of $1\alpha,25(OH)_2D_3$ (8). For comparison, in the absence of ligand in LCLs and monocytes, 623 and 520 genomic VDR sites were found, respectively. The numbers of both groups of VDR peaks are comparable as such. However, for the stimulated condition, only 708 (18.2%) binding locations were occupied by VDR in both cellular models. A partial explanation of this poor overlap may be the significant interindividual variation in chromatin organization and gene expression in cells of primary origin, such as those used by Ramagopalan *et al.*, in contrast to the cultured monocytic cell line that we used. However, the main reason is likely to be the very different duration of $1\alpha,25(OH)_2D_3$ treatment (36 h *versus* 40 min). This thus suggests that VDR utilizes considerably variant binding sites in different cells. Nevertheless, the ChIP-Seq studies confirmed a number of previously reported VDR-binding sites on known primary $1\alpha,25(OH)_2D_3$ targets, such as that of the genes *VDR* (55), *CCNC* (28) and arachidonate 5-lipoxygenase (*ALOX5*) (56). In addition, they found some extra sites for known $1\alpha,25(OH)_2D_3$ target genes and also indicated a large number of previously unknown targets of the nuclear hormone and its receptor.

The total number of genomic VDR sites in LCLs is around 3000 (Ramagopalan *et al.* did not report the exact overlap of their 623 non-stimulated and 2776 stimulated peaks), whereas it is 2340 in monocytes. ChIP-Seq studies with other nuclear receptors reported some 5000 to 10000 genome-wide binding sites (57, 58), *i.e.* the numbers for VDR are on the lower end. The other ChIP-Seq studies also indicated that the number of transcription factor binding sites exceeds the number of regulated genes, as detected by microarrays, by a factor of 10 or more. For LCLs, this observation is confirmed, while in monocytes, the number of VDR peaks is fewer than 5-fold that of $1\alpha,25(OH)_2D_3$ target genes. However, one has to take into account that both the genomic binding of VDR and the induction of its target genes are dynamic processes (30), *i.e.* numbers that were measured at one time point are only a subset of numbers that can be obtained when all time points are integrated. Moreover, since the binding of VDR precedes any modulation of the mRNA of its target genes by minutes to hours, it is not obvious which time points in ChIP-Seq and microarray experiments should be compared.

High-confidence VDR-binding site peaks were enriched in genomic regions up- and downstream of TSS regions compared to peaks with lower confidence, *i.e.* the probability of finding good VDR peaks declines symmetrically in both directions from the TSS (8). However, this analysis comprises the TSS regions of all genes and not only that of VDR target genes, and reflects primarily probably the higher density of accessible genomic DNA around TSS regions. Interestingly, the set of VDR peaks that are observed after ligand stimulation seems to take positions that are on average more distant from TSS regions than those that we found in the absence of ligand. This reflects the tendency that after ligand activation, VDR associates more specifically with its target genes and not in a seemingly random fashion with accessible genomic DNA as it does without the ligand. However, in the absence of ligand, VDR has also been shown to actively repress its target genes, likely *via* a mechanism involving an interaction with co-repressor proteins (14, 59).

DR3-type REs at Genomic VDR Locations

Typical to ChIP-Seq data analyses is the investigation of the sequences below the ChIP-Seq peaks (mostly ±100 bp of the peak summit) for any enriched sequence motif possibly reflecting a transcription factor binding site. In both VDR

ChIP-Seq studies (8, 39), such *de novo* binding site searches identified the classical DR3-type RE consensus sequence for VDR-RXR heterodimers as being the most highly enriched. This expected result increases the confidence in the quality of the ChIP-Seq datasets, but it also confirms that older, pre-genomic studies (19, 20), which highlighted DR3-type REs as the preferential VDR-binding sites, are still valid.

Interestingly, not all VDR peak summits contained a DR3-type RE. Taking the narrow window of ±100 bp, only 31.7% (742) of all 2340 VDR peak summits in monocytes include one or more DR3-type REs (8). Similar numbers apply for the dataset from LCLs. A plot of the similarity score of DR3-type REs to their consensus over their distance to the VDR peak summit is quite comparable for both ChIP-Seq datasets and is clearly distinguished from an unpublished formaldehyde-assisted identification of regulatory elements (FAIRE) dataset that serves as a reference set of accessible chromatin regions (Figure 2A). Moreover, when focusing only on $1\alpha,25(OH)_2D_3$ -dependent VDR peaks and plotting the percentage of DR3-type RE content over the quality of the VDR ChIP-Seq peak (Figure 2B), both ChIP-Seq datasets provide similar results: the higher the fold enrichment/value of a VDR peak, the higher is the chance that it contains a high-quality DR3-type RE. These curves show a saturation level of 90% for the DR3-type RE content in the monocyte data (8) and of 76% in the LCL data (39). In contrast, from the 520 genomic VDR-binding locations that occur in monocytes uniquely in the absence of ligand only 14% contain a DR3-type VDRE (8). 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, *i.e*. VDR becomes more specific in recognizing its genomic targets. Therefore, it is possible 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.

A *de novo* motif analysis for the 1598 VDR peaks without a DR3-type RE close to their summit provided indications for binding sites for the common transcription factors SP1 and SPI1/PU.1 in 538 and 274 of these peaks, respectively (8). However, there is no specific enrichment for such transcription factor binding sites in VDR-binding locations lacking a DR3-type VDRE. Nevertheless, this observation suggests that VDR may bind backpack to one of these transcription factors rather than directly contacting DNA. Searches for other VDRE types with different spacing or relative orientations of the core binding motifs did not provide any statistically significant enrichment for them within ±100 bp of the peak summit. Although it is still possible that a few individual regions carry such alternative VDRE types, in the present datasets, there is no genome-wide evidence for their widespread use.

VDR Peaks Related to Control of $1\alpha,25(OH)_2D_3$ Target Genes

Combining the microarray data of $1\alpha,25(OH)_2D_3$ treatments with VDR ChIP-Seq data allows the mechanisms of target gene regulation by the VDR to be explored. This was possible in particular for the study in monocytes (8), which used a $1\alpha,25(OH)_2D_3$ treatment of only 4 h for mRNA expression studies and a 40 min ligand stimulation for VDR location mapping. The short stimulation times allow it to be assumed that most of the 638 regulated genes are primary targets of $1\alpha,25(OH)_2D_3$, *i.e*. that their changes in mRNA expression are a direct consequence of VDR-binding in genomic regions sufficiently close to their TSS. For 93 out of the 408 up-regulated genes, the largest $1\alpha,25(OH)_2D_3$-stimulated VDR peak in the neighborhood is within 30 kb from their TSS. However, for another 201 genes, the most prominent VDR-binding site is between 30 and 400 kb away and often unique to the $1\alpha,25(OH)_2D_3$ treatment. It should be noted that in pre-genomic studies, a distance of 30 kb between a VDRE and the TSS was already considered large (12), while 400 kb was practically unimaginable. Plotting the positions of the $1\alpha,25(OH)_2D_3$-stimulated VDR ChIP-Seq peaks in relation to the TSS of the $1\alpha,25(OH)_2D_3$ target genes in monocytes and LCLs (Figure 3) provides comparable pictures for both datasets, with a clear peak at the TSS region and symmetrical decline towards both the upstream and downstream flanking regions. This suggests that VDR binds as likely upstream as downstream of the TSS of its target genes. This fits with insights of the ENCODE project (60) and indicates that the pre-genomic focus on the upstream region only addressed half of the regulatory regions of a gene.

Following these genomic insights, 294 (72.1%) of the up-regulated $1\alpha,25(OH)_2D_3$ target genes in monocytes have a $1\alpha,25(OH)_2D_3$-stimulated VDR peak within 400 kb of their TSS (8). In contrast, only 99 (43.0%) out of the 230 down-regulated genes have a $1\alpha,25(OH)_2D_3$-stimulated VDR peak in the ±400 kb region. Moreover, on average, the up-regulated genes had two VDR peaks in the vicinity of their TSS, while close to down-regulated genes, only one VDR site was found. The observations that the most dominant ligand-induced VDR-binding sites are highly enriched for DR3-type REs, and that these REs preferentially locate to the neighborhoods of up-regulated $1\alpha,25(OH)_2D_3$ target genes, suggest that DR3-type REs are primarily involved in gene activation. In contrast, the mechanisms of down-regulation of VDR target genes seem to be more complex and may require gene-specific investigations as demonstrated for *CYP27B1* (27). In that case, the repressive function of VDR is known to result from indirect interaction with the chromatin, *via* transcription factor 3, also known as VDR-interacting repressor (61).

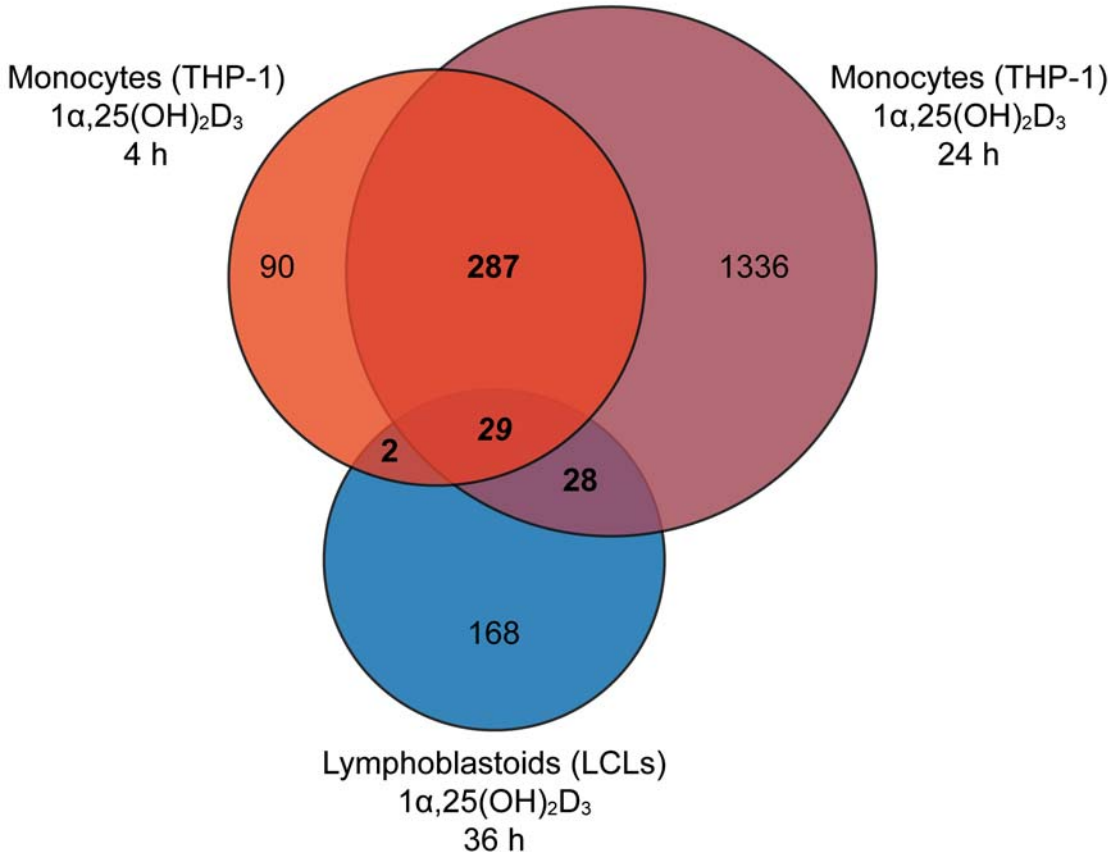

Figure 1. *1α,25(OH)$_2$D$_3$ target gene comparison. Up-regulated 1α,25(OH)$_2$D$_3$ target genes from three different microarray datasets were compared: THP-1 cells after short [4 h, red (8)] or long (24 h, purple, unpublished data) 1α,25(OH)$_2$D$_3$ treatment and in lymphoblastoid cell lines (LCLs) after long [36 h, blue (39)] 1α,25(OH)$_2$D$_3$ stimulation. Numbers of genes in each overlapping or unique gene set are given within the Venn diagram. Note that the circle sizes and degrees of overlap are only approximations.*

For some members of the nuclear receptor superfamily, such as the thyroid hormone receptor and the liver X receptor, a ligand-induced de-repression mechanism was described (62, 63). In monocytes, only 6 up-regulated genes meet the de-repression criteria that they have a VDR peak in the unstimulated sample and no peak in the 1α,25(OH)$_2$D$_3$-treated sample (8). 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 1α,25(OH)$_2$D$_3$ target genes, a de-repression mechanism may apply.

Nevertheless, for 26.5% of the up-regulated and 54.8% of the down-regulated 1α,25(OH)$_2$D$_3$ target genes in monocytes, the ChIP-Seq analyses did not suggest any VDR binding within the ±400 kb region neither in the 1α,25(OH)$_2$D$_3$-stimulated nor unstimulated state, *i.e.* for these genes there is no obvious explanation for their regulation by 1α,25(OH)$_2$D$_3$-activated VDR (8). However, gene regulation by VDR is a very dynamic process, with rapid changes of VDR-binding site occupancy (30, 64, 65), which our single, short time point at 40 min may have not fully captured. Therefore, 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.

VDR Location Scenarios

The regulatory scenario of most up-regulated 1α,25(OH)$_2$D$_3$ target genes in monocytes can be explained by one or multiple VDR-binding sites within 400 kb of their TSS (8). However, as already suggested for various VDR target genes, such as *CDKN1A* (29), *CYP24A1* (26, 35), *ALOX5* (56) and *CCNC* (28), there is quite a variation in the gene regulatory scenarios of up-regulated genes. Genome-wide, there are only about 20 genes that have, as in the case of the SP100 nuclear antigen (*SP100*) gene, a single VDR location close to the target gene TSS (8). However, more common than these rather simple scenarios are situations where one target gene has multiple VDR-binding sites in its regulatory region. This

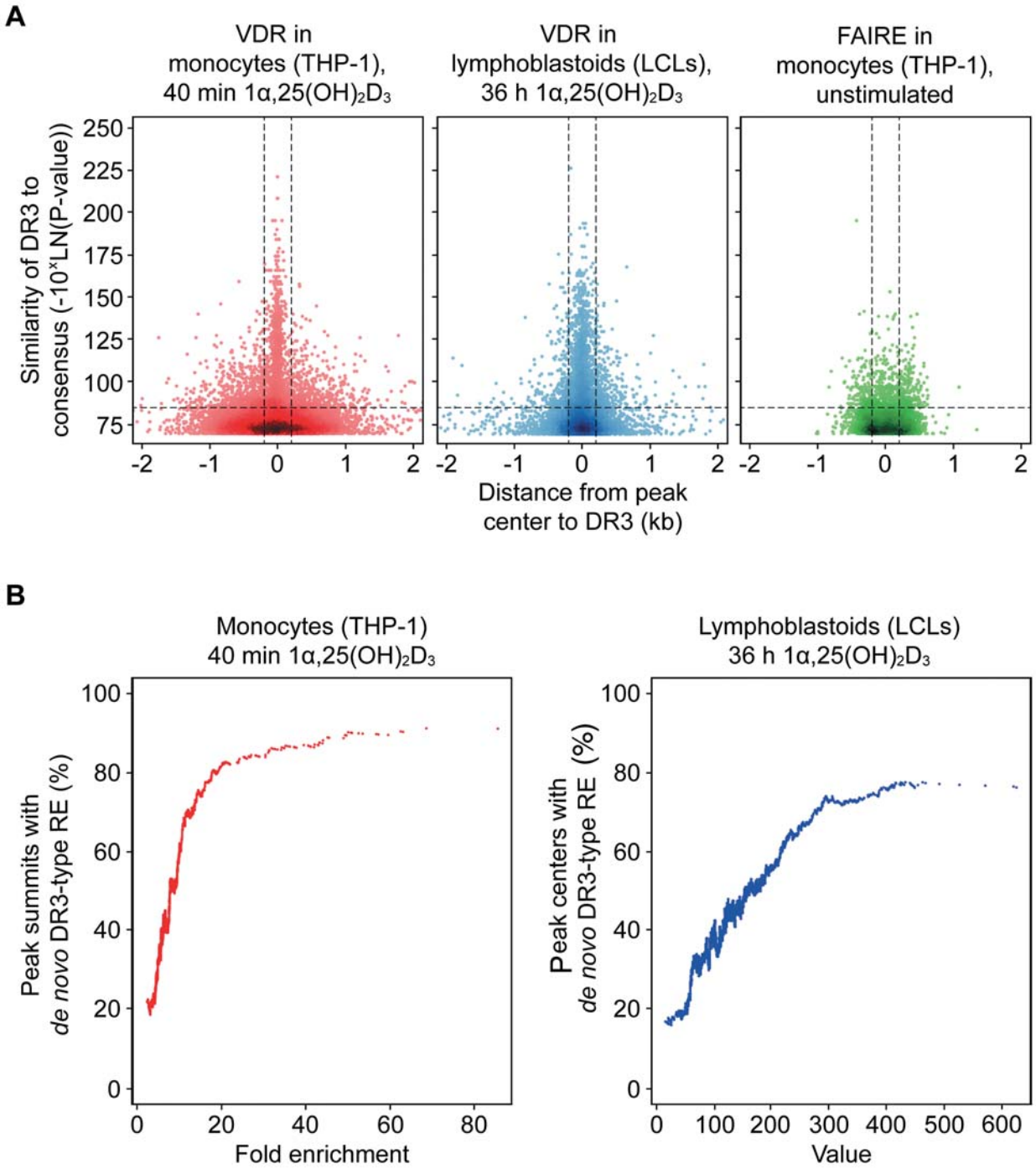

Figure 2. *Comparison of DR3-type response element (RE) usage in monocytes and lymphoblastoids. A: Distribution of DR3-type REs in vitamin D receptor (VDR) ChIP-Seq peaks in THP-1 cells after 40 min 1α,25(OH)$_2$D$_3$ treatment [left panel, 1820 peaks (8)], lymphoblastoid cell lines (LCLs) after 36 h 1α,25(OH)$_2$D$_3$ treatment [middle panel, 2773 peaks (39)] and, to represent random enhancers, 1894 biggest FAIRE-Seq peaks in unstimulated THP-1 cells (right panel, unpublished data). Dashed lines indicate the cut-offs reproducing our previous result that were uniformly used in B for DR3-type RE. P-value and distance from peak summit (THP-1 cells) or center (LCL). RSAT tools (67) were used to identify the respective DR3-type REs, which, for LCL, is based on a rGadem R-package de novo search of VDR ChIP-Seq peak centers. For the sake of visual clarity, the RSAT P-values for the similarity of DR3-type REs to the de novo consensus on the y-axis are represented as ten-fold negatives of their natural logarithms. B: DR3-type RE usage as a function of VDR-binding in THP-1 cells after 40 min 1α,25(OH)$_2$D$_3$ treatment (left panel) and LCLs after 36 h 1α,25(OH)$_2$D$_3$ treatment (right panel).*

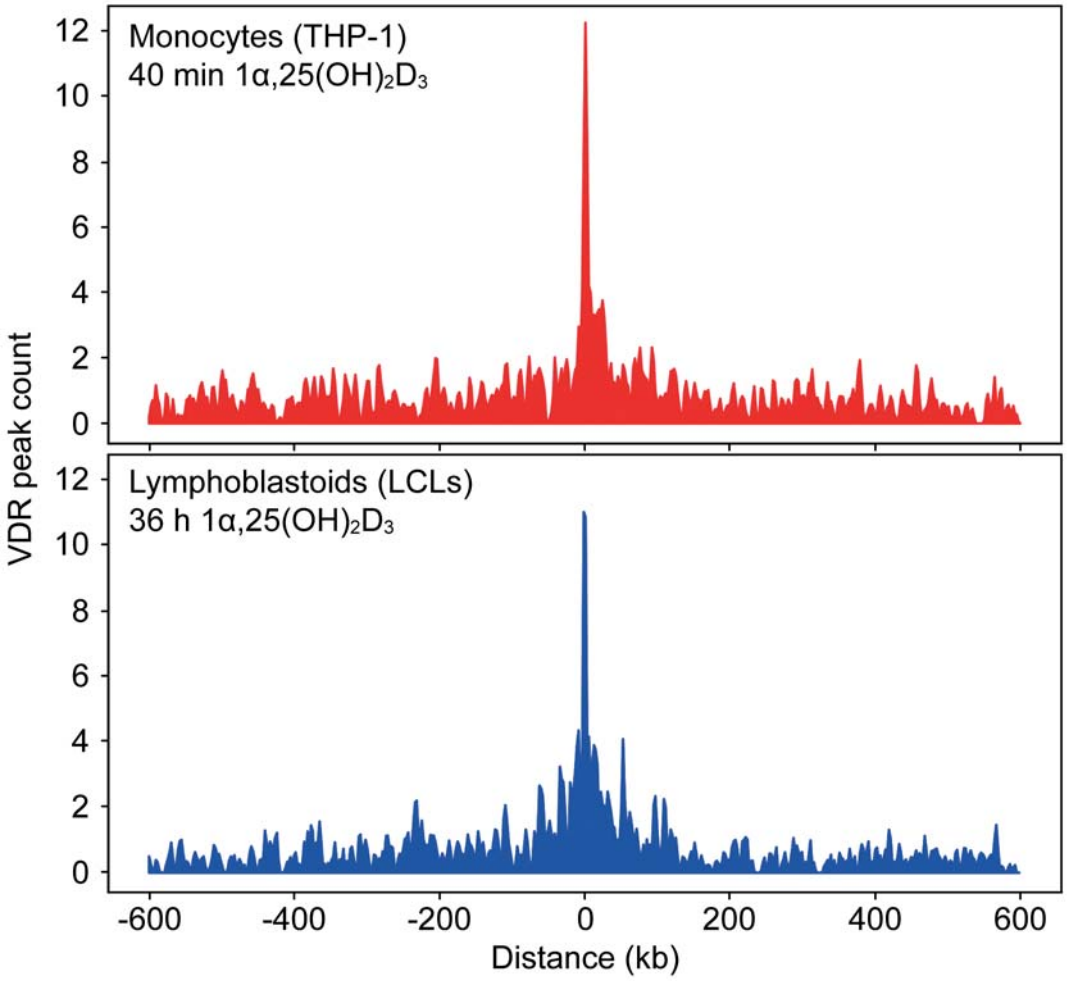

Figure 3. *Distribution of vitamin D receptor (VDR) ChIP-Seq peaks in the neighborhoods of 1α,25(OH)$_2$D$_3$ target genes. For the monocyte dataset [(8), top] and lymphoblastoid cell line (LCL) dataset [(39), bottom] the distribution of VDR ChIP-Seq peaks is plotted in relation to the transcription start site of the respective 1α,25(OH)$_2$D$_3$ target genes.*

has already been demonstrated for the above-mentioned VDR targets. Alternatively, there are constellations where a pair of closely located VDR target genes share one or more VDR-binding sites, as shown for the members of the insulin-like growth factor-binding protein (*IGFBP*) gene family (66). However, the most complex regulatory situations were found in the clusters around the VDR target genes thrombomodulin (*THBD*) and myosin IXB (*MYO9B*) which each contains five or six VDR-binding sites of different characteristics.

We assume that the strongest VDR-binding location within 400 kb of the TSS of a 1α,25(OH)$_2$D$_3$ target gene is its most likely regulatory site. Thus the VDR target genes can be divided into 36 sub-categories depending on whether i) the gene is up- or down-regulated; ii) the most likely regulatory site is located within the proximal (0 to ±30 kb of the TSS), distal (±30 to ±400 kb of the TSS), or 'desert' (more than 400 kb of the TSS) region; iii) the most likely regulatory site shows a high or low fold enrichment after ligand treatment; and iv) the gene has a high, medium or low fold change upon stimulation (8). In monocytes, 55 up-regulated VDR target genes have the most likely regulatory site with high fold enrichment in the proximal region, 120 in the distal region and 28 in the desert region. Out of these regulatory sites, 80-90% carry a DR3-type RE within their peak summit. Together, these sites represent fewer than 10% of all VDR peaks, but they seem to be the most relevant for the control of the up-regulated 1α,25(OH)$_2$D$_3$ target genes.

The poor overlap between monocytes and LCLs of 1α,25(OH)$_2$D$_3$ target gene sets on one hand and VDR peak locations on the other hand suggest that there are cell-specific modes of target gene regulation by VDR. Some such examples are given in Figure 4. First is the locus for *NINJ1*

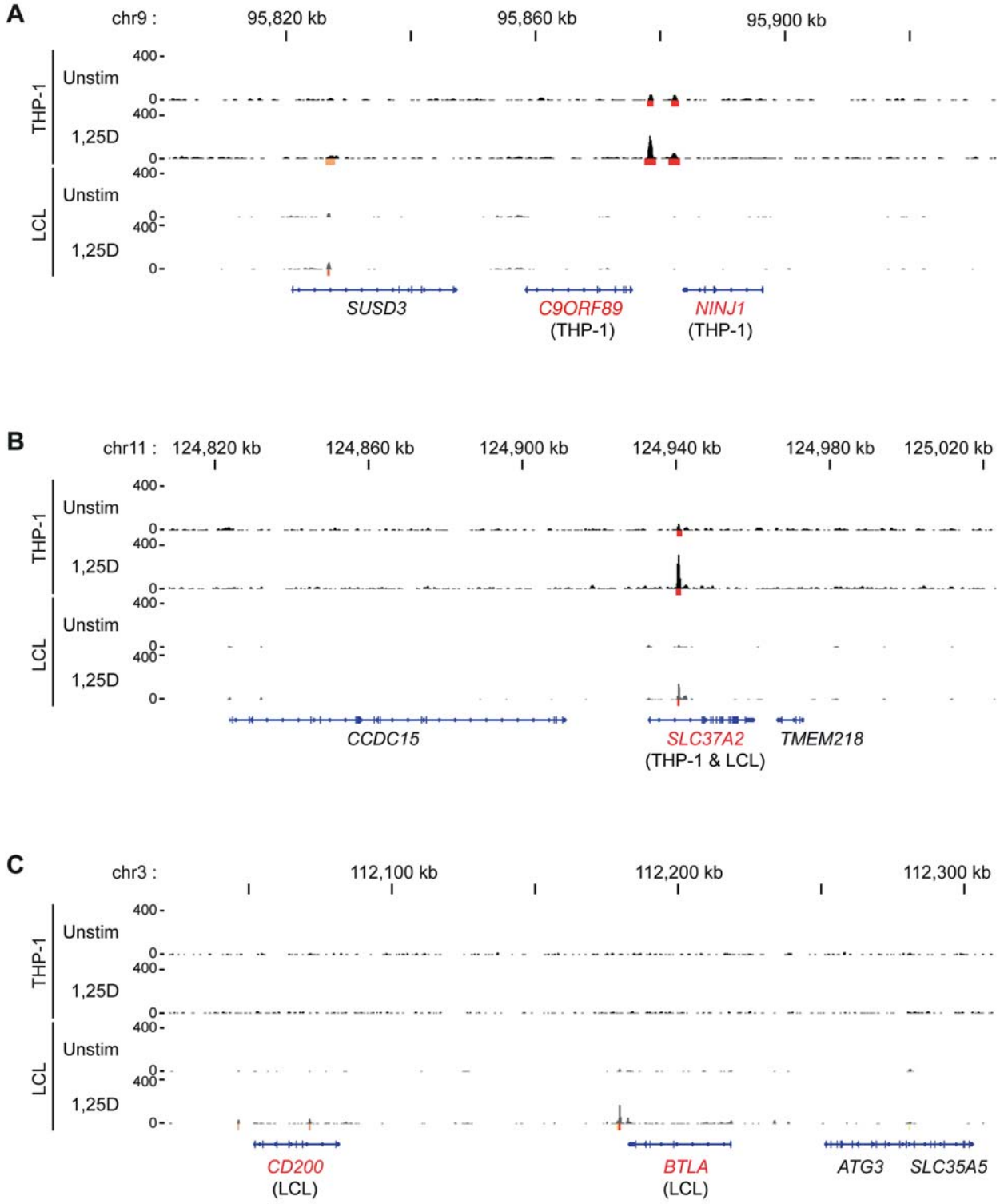

Figure 4. *Exemplary genomic views of VDR binding. ChIP-Seq analysis of genomic loci for A: NINJ1 and C9ORF89 genes uniquely up-regulated in monocytes; B: SLC37A2 up-regulated in both monocytes and lymphoblastoid cell lines (LCLs); and C: BTLA and CD200 uniquely up-regulated in LCLs. The peak tracks show the unstimulated and VDR peaks of 1α,25(OH)$_2$D$_3$-treated monocyte (8) and LCL (39) ChIP-Seq datasets. Summits of all the indicated VDR peaks contain a DR3-type RE either by the original de novo search criteria or that used for Figure 2B. The gene structures are shown in blue at the bottom. Gene names in red indicate up-regulated genes. Unstim, Unstimulated; 1,25D, 1α,25(OH)$_2$D$_3$-treated.*

and *C9ORF89*, where both genes are up-regulated by $1\alpha,25(OH)_2D_3$ and which carries two distinct VDR-binding locations between the two genes (Figure 4A). An example of a common target gene is *SLC37A2*, which is up-regulated and carries one distinct VDR-binding site in the first intron in both cellular models (Figure 4B). An LCL-specific example is the locus for two up-regulated, immune-related genes *BTLA* and *CD200*, which in monocytes lacks both VDR peaks and regulated genes (Figure 4C). An increasing knowledge base of VDR actions on chromatin and the transcriptome is thus already beginning to reveal cell-specific regulatory scenarios for VDR.

Conclusion

The molecular actions of $1\alpha,25(OH)_2D_3$ in many different tissues are becoming better understood based on large datasets obtained from genome- and transcriptome-wide investigations. These data can be used for follow-up studies in many directions. One of these is to associate the VDR-binding sites with single nucleotide polymorphisms causing diseases, such rs13385731 and rs947474 associated with the autoimmune diseases systemic lupus erythematosus and type 1 diabetes, respectively, and to investigate aspects of human evolution, such as an association of VDR-binding sites with genomic regions of positive selection, as already started by Ramagopalan *et al*. (39). Moreover, time emerges as a very critical parameter due to the dynamic response of cells and tissues, especially in the early phase of ligand treatment, suggesting time-course experiments for VDR ChIP-Seq and $1\alpha,25(OH)_2D_3$ microarrays. In addition, further VDR target tissues will be investigated in a similar way as presented here for monocytes and LCLs. All these studies will underline the broad physiological profile of the VDR and its ligands.

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