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# Epigenomic PU.1-VDR crosstalk modulates vitamin D signaling

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ABSTRACT

The ETS-domain transcription factor PU.1 acts as a pioneer factor for other transcription factors including nuclear receptors. In this study, we report that in THP-1 human monocytes the PU.1 cistrome comprises 122,319 genomic sites. Interestingly, at 6498 (5.3%) of these loci PU.1 binding was significantly modulated by the vitamin D receptor (VDR) ligand 1α,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$). In most cases $1,25(OH)_2D_3$ increased PU.1 association, which correlated strongly with VDR co-location and overlap ratios for canonical DR3-type VDR binding sites. Genome-wide 6488 sites associating both with PU.1 and VDR as well as 5649 non-VDR overlapping, $1,25(OH)_2D_3$-sensitive PU.1 loci represent the PU.1-VDR crosstalk and can be described by four gene regulatory scenarios, each. Chromatin accessibility was the major discriminator between these models. The location of the PU.1 binding loci in open chromatin coincided with a significantly smaller mean distance to the closest $1,25(OH)_2D_3$ target gene. PU.1 knockdown indicated that the pioneer factor is relevant for the transcriptional activation of $1,25(OH)_2D_3$ target genes but its impact differed in magnitude and orientation. In conclusion, PU.1 is an important modulator of VDR signaling in monocytes, including but also exceeding its role as a pioneer factor, but we found no evidence for a direct interaction of both proteins.



## 1. Introduction

The pioneer factor PU.1 (encoded by the gene *SPI1*) belongs to one of the largest transcription factor family, members of which bind via their ETS DNA-binding domain to GGAA/T sequence motifs [1,2]. In contrast to most other transcription factors, this short motif allows PU.1 to bind genomic DNA even in the co-presence of nucleosomes [3]. An additional winged helix domain mediates more specific DNA binding of PU.1 to a longer consensus sequence [4]. Furthermore, via a PEST domain PU.1 is involved in numerous protein-protein interactions and the properties of its partner proteins define whether PU.1 activates or represses transcription of a target gene [1]. For example, association of PU.1 with the histone acetyltransferases CBP and P300 causes local histone acetylation and a less dense packaging of nucleosomes at the respective genomic site, which is one of the main characteristics of a pioneer factor [5]. In contrast, PU.1 is also involved in the repression of transcription via direct interaction with the complex of histone deacetylase 1 with the co-repressor SIN3A [6]. PU.1 binding sites at promoter and enhancer regions are often of lower affinity and are flanked by binding regions for other transcription factors suggesting that combinatorial binding of PU.1 with its partner proteins may be necessary to create and maintain local regions of open chromatin. PU.1 is known best for its essential role in the development of several hematopoietic lineages and shows collaborative as well as antagonistic interactions with several lineage-specific transcription factors [7].

The transcription factor vitamin D receptor (VDR), the high-affinity nuclear receptor for the biologically most active vitamin D metabolite 1α,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) [8,9], and PU.1 are both regulators of the differentiation of monocytes and granulocytes and are tightly co-expressed in these lineages [10]. Vitamin D is best known for its role in the regulation of calcium and phosphorus homeostasis, but it also controls innate and adaptive immune responses [11–13]. According to the canonical nuclear receptor signaling model VDR binds as a heterodimer with the nuclear receptor retinoid X receptor (RXR) to DNA binding sites formed by a direct repeat of two hexameric sequence motifs spaced by three nucleotides (DR3) [14,15]. However, fewer than 15% of all known genomic VDR binding sites contain a DR3-type sequence [16], even though this number increases after ligand treatment [17]. Therefore, VDR has to use additional mechanisms to recognize its genomic targets [18]. In addition to PU.1 [10], VDR shows a close co-operation with other nuclear proteins, such as nuclear mediators of TGFβ signaling [19] and the transcription factors TCF4 [20], RUNX2 and CEBPβ [21].

In general, the genomic binding of nuclear receptors, such as androgen receptor (AR), glucocorticoid receptor (GR) and estrogen receptor (ER), is often facilitated by pioneer factors, such as FOXA or GATA

*Abbreviations:* $1,25(OH)_2D_3$ or 1,25D, 1α,25-dihydroxyvitamin $D_3$; ChIP, Chromatin immunoprecipitation; ChIP-seq, ChIP sequencing; CTCF, CCCTC-binding factor; DR3, Direct repeat spaced by 3 nucleotides; DsiRNA, Dicer substrate siRNA; FAIRE-seq, Formaldehyde-assisted isolation of regulatory elements sequencing; FC, Fold change; FE, Fold enrichment; KD, Knockdown; LD, Ligand-dependent; PU.1, Purine-rich box 1, Spi-1 proto-oncogene (official gene symbol: *SPI1*); RXR, Retinoid X receptor; TSS, Transcription start site; VDR, Vitamin D receptor.

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proteins, but there are also mechanisms, where the receptors act themselves as pioneer factors [3,22–26]. Thus, in certain gene regulatory scenarios also VDR may assume pioneer factor functions. In order to follow this hypothesis, we investigated in this study the crosstalk of PU.1 with genome-wide VDR signaling in THP-1 human monocytes. We found that in this cellular model the PU.1 cistrome is composed of 122,319 genomic sites, 5.3% of which were significantly modulated by $1,25(OH)_2D_3$ stimulation. Co-association of PU.1 and VDR preferentially occurred at open chromatin. We differentiate up to four gene regulatory scenarios of a direct PU.1-VDR crosstalk and another four for an indirect interaction between the two transcription factors providing novel insight into the mechanisms of $1,25(OH)_2D_3$-regulated gene expression.

## 2. Material and methods

### 2.1. Cell culture

The human acute monocytic leukemia cell line THP-1 [27] is a well responding and physiologically meaningful model system for the investigation of $1,25(OH)_2D_3$-triggered physiological processes, such as innate immunity and cellular growth [17,28–30]. The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin and were kept at 37 °C in a humidified 95% air/5% $CO_2$ incubator. Prior to mRNA or chromatin extraction, cells were first grown overnight in phenol red-free medium supplemented with charcoal-stripped FCS and then treated with vehicle (0.1% ethanol (EtOH)) or 100 nM $1,25(OH)_2D_3$ (Sigma-Aldrich). Three independent replicate experiments (biological repeats) were performed for obtaining the ChIP-seq data sets.

### 2.2. Dicer substrate siRNA (DsiRNA) silencing

THP-1 cells were transfected with either non-specific control DsiRNA oligomers (NC1) or specific DsiRNAs targeting PU.1/*SPI1* mRNA (Table S1). In addition, mock-transfected controls (no DsiRNA) were prepared. The cells were grown for 2 days in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. The transfection was performed in an Amaxa nucleofector I (Lonza) using the Cell Line Nucleofector Kit V according to the manufacturer's instructions with the following modifications. Per sample, $2 \times 10^6$ cells were harvested by centrifugation and resuspended in 100 μl Nucleofector Solution V. A total of 200 pmol of a mixture of three different DsiRNA oligonucleotides was added and the cell suspension was transferred to an electroporation cuvette. The Nucleofector T-12 program was applied. Immediately after the pulse, 500 μl pre-warmed RPMI 1640 medium supplemented with 5% charcoal-stripped FCS, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin was added and the cell suspension was transferred to pre-equilibrated 6-well plates containing 2.5 ml of medium per well. The extent of the knockdown was determined on mRNA level after an incubation for 48 h including vehicle and $1,25(OH)_2D_3$ treatment for 24 h. Transfection efficiency was determined after nucleofection of a fluorescently labeled DsiRNA oligonucleotide (IDT, Leuven, Belgium) by confocal microscopy to be ≥88%. Three independent experiments have been performed, each of these biological repeats consisting of three technical repeats.

### 2.3. RNA isolation, cDNA synthesis and qPCR

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. RNA quality was assessed by native agarose gel electrophoresis. cDNA synthesis and qPCR were performed as described previously [31]. qPCR reactions were performed with the LightCycler® 480 System (Roche) using 250 nM of reverse and forward primers, 2 μl cDNA and the LightCycler 480 SYBRGreen I Master mix (Roche). Primer-specific temperatures and sequences are listed in Table S2. Relative mRNA expression levels were determined using the formula $2^{-(\Delta Ct)}$, where ΔCt is $Ct_{(target\ gene)} - Ct_{(reference\ gene)}$. The genes *B2M*, *GAPDH* and *HPRT1* were used as references as described previously [30].

### 2.4. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described by Zhang et al. [32] with some modifications. After treatment of $20 \times 10^6$ THP-1 cells, nuclear proteins were cross-linked to genomic DNA by adding formaldehyde directly to the medium to a final concentration of 1% and incubating at room temperature for 10 min on a rocking platform. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and incubating at room temperature for 10 min on a rocking platform. The cells were collected by centrifugation and washed twice with ice cold phosphate-buffered saline (PBS). The cell pellets were subsequently resuspended twice in 10 ml cell lysis buffer (0.1% SDS, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors, 50 mM HEPES-KOH, pH 7.5) and once in 10 ml nuclear lysis buffer (1% SDS, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors, 50 mM HEPES-KOH, pH 7.5). After two washes with cell lysis buffer, the chromatin pellet was resuspended in 700 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris-HCl, pH 8.1) and the lysates were sonicated in a Bioruptor Plus (Diagenode) to result in DNA fragments of 200 to 500 bp. Cellular debris was removed by centrifugation. 340 μl aliquots of the lysate were diluted 1:5 in IP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, protease inhibitors, 20 mM Tris-HCl, pH 7.5). 4 μg anti-PU.1 antibody (Santa Cruz, sc-22805X), previously used by the ENCODE project [33] (data available at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) at GSE32465) and Gasper et al. [34] or 25 μl anti-VDR antibody (Cell Signaling Technology, #12550) were coated to 60 μl Dynabeads Protein G (Invitrogen) in an overnight incubation at 4 °C. The pre-formed bead-antibody complexes were then washed twice with beads wash buffer (0.1% Triton X-100, PBS, protease inhibitors) and added to the chromatin aliquots. The samples were incubated for overnight at 4 °C on a rotating wheel to form and collect the immuno-complexes. The beads were washed sequentially for 5 min on a rotating wheel with 1 ml of the following buffers, each: twice cell lysis buffer, once high salt buffer (0.1% SDS, 1% Triton X-100, 1 mM EDTA, 350 mM NaCl, 0.1% sodium deoxycholate, 50 mM HEPES-KOH, pH 7.5), once ChIP wash buffer (250 mM LiCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and twice 1 ml TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Then, the immune complexes were eluted using 250 μl ChIP elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5) at 37 °C for 30 min with rotation. The elution was repeated with a 10 min rotation and the supernatants were combined. The immune complexes were reverse cross-linked at 50 °C for 2 h in the presence of proteinase K (Fermentas) in a final concentration of 40 μg/ml. The genomic DNA was isolated with the ChIP DNA Clean&Concentrator Kit (Zymo Research).

### 2.5. ChIP-seq analysis

2–10 ng of each ChIP DNA template was used for library preparation with the NEBNext Ultra II kit (New England Biolabs) and libraries were sequenced at 50 bp read length on a HiSeq2000 system using standard Illumina protocols at the Gene Core of the EMBL (Heidelberg, Germany). ChIP-seq data was aligned with the human reference genome version hg19 using Bowtie software version 1.1.1 [35] with the following essential command line arguments: bowtie -n 1 -m 1 -k 1 -e 70 –best. The aligned input and PU.1 reads were converted to sorted BAM format using samtools [36] and, after merging the read sets per sample, converted to TDF format using igvtools, in order to allow efficient visualization in the Integrative Genomics Viewer (IGV) genome browser [37].