

A hierarchical regulatory network analysis of the vitamin D induced transcriptome reveals novel regulators and complete VDR dependency in monocytes

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Fig. S1

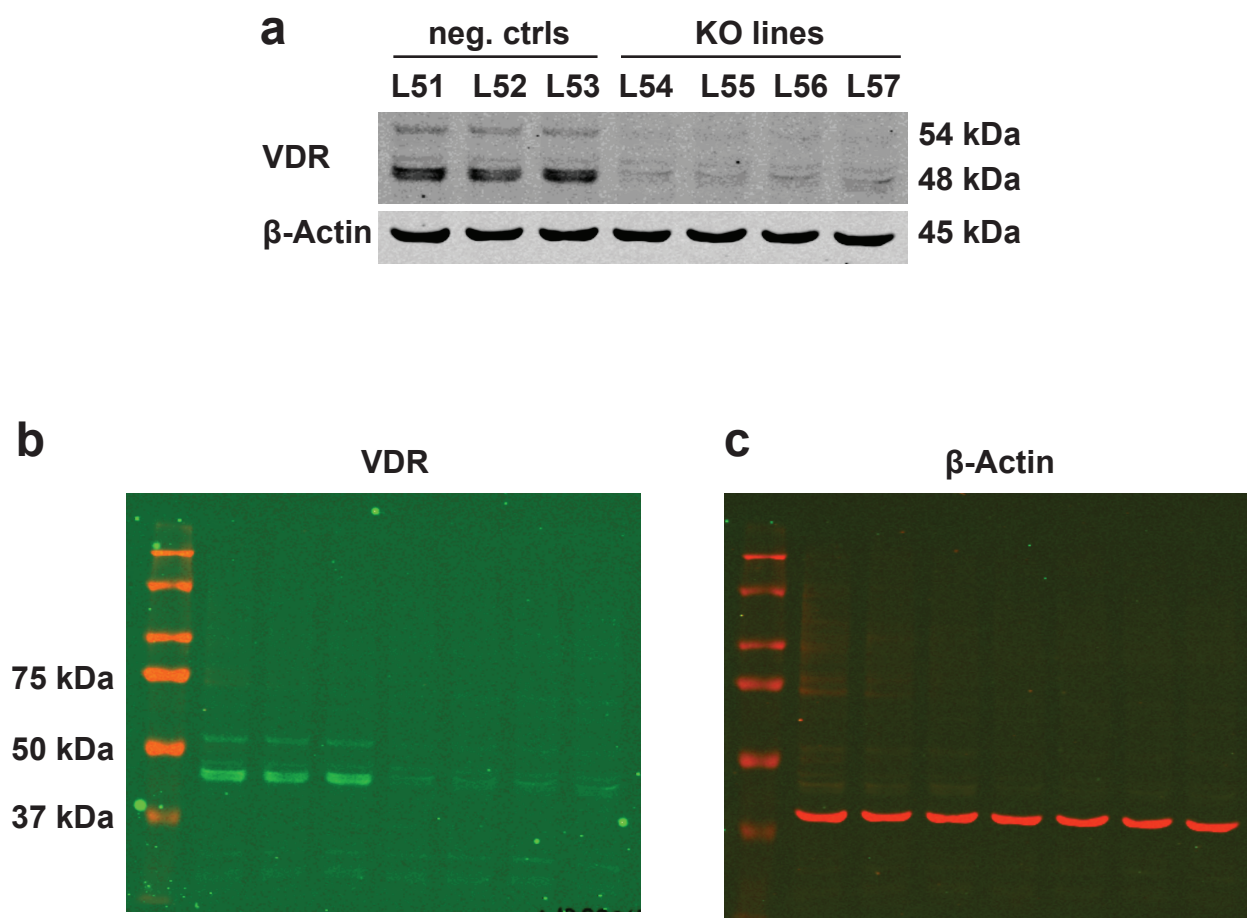


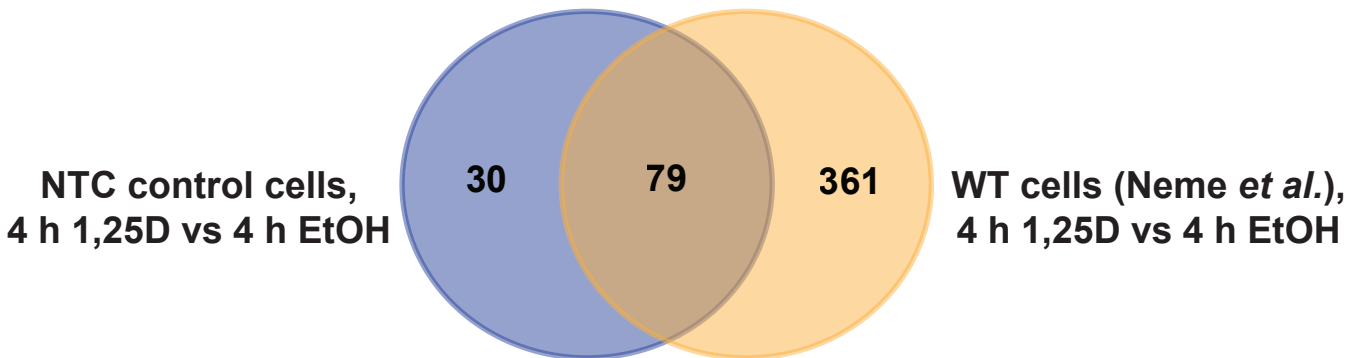
Figure S1: VDR knockout. The successful knockout of the VDR protein by CRISPR/Cas9 genome editing was verified by Western Blot in four different knockout lines (L54-L57), created by lentiviral transduction of four different gRNAs (Supplementary Table S1), compared to three different controls (L51 and L52 – non-targeting gRNAs NTC1 and NTC2, L53 – empty vector). The assembled bands converted to greyscale are shown in **a**, while the complete original scans of the blot are depicted in **b** (VDR) and **c** (β -Actin). The Precision Plus Protein All Blue Prestained Protein Standards (BioRad) were loaded as size marker. VDR knockout line L56 and control line L52 were used in the subsequent experiments.

Fig. S2

a



b



c



d DEGs by KO



Figure S2: Differential analysis of the 1,25(OH)₂D₃-regulated transcriptome in CRISPR/Cas9 control cells. THP-1 cells that have been transduced with a non-targeting gRNA (NTC) have been treated with 1,25(OH)₂D₃ or solvent (EtOH) for 4 or 24 h and four biological repeats of RNA-seq have been performed. The huge intersect between both time points (a) as well as the reasonably high overlap with the DEGs that had recently been identified by RNA-seq from THP-1 wild-type cells (b, c) indicate consistency with our previous studies. There was a strong increase in the number of genes differentially expressed by the VDR knockout in 24 h 1,25(OH)₂D₃ treated cells compared to 4 h stimulated cells and the majority of the early time point DEGs overlap with those of the late time point (d).

Fig. S3

a



b

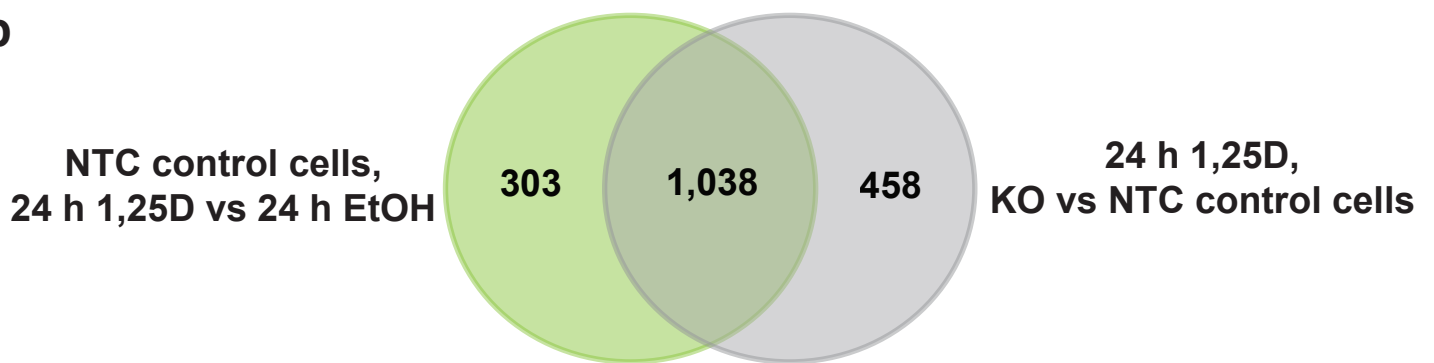
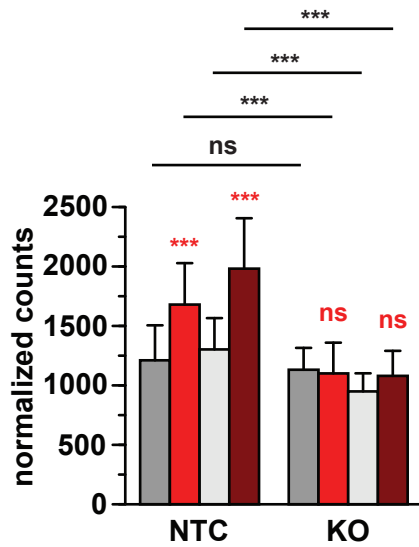


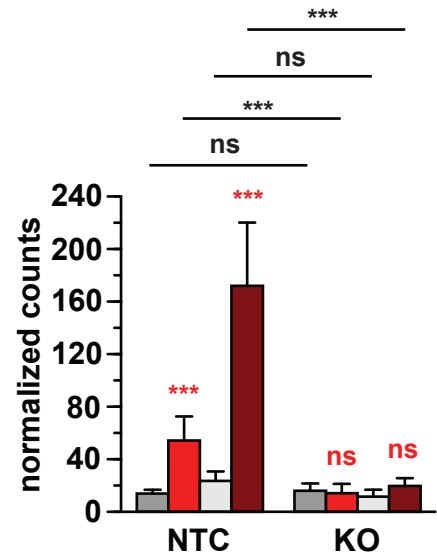
Figure S3: High overlap between 1,25(OH)₂D₃-regulated genes and genes with modulated expression by the absence of VDR. THP-1 cells that have been transduced with a VDR-specific (KO) or a non-targeting gRNA (NTC) have been treated with 1,25(OH)₂D₃ or solvent (EtOH) for 4 or 24 h and quadruplicate RNA-seq has been performed. Intersects between vitamin D target genes and genes differentially expressed by the VDR knockout for 4 h (a) and 24 h (b) VDR ligand treatment.

Fig. S4

a *ZFP36*: early primary



b *KCNF1*: early primary



c *IL8 (CXCL8)*: late primary

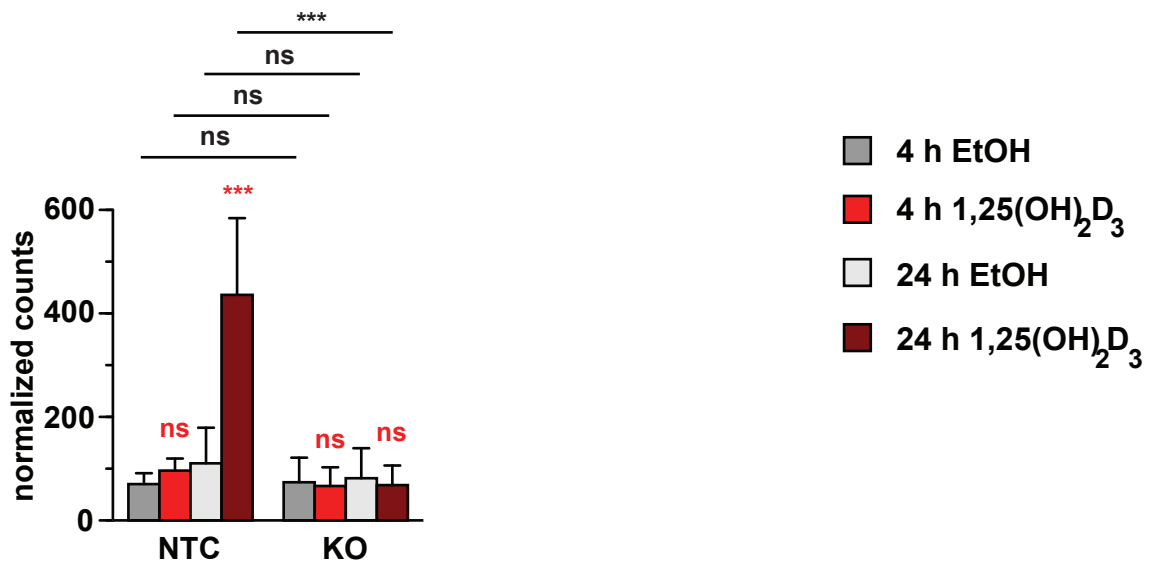
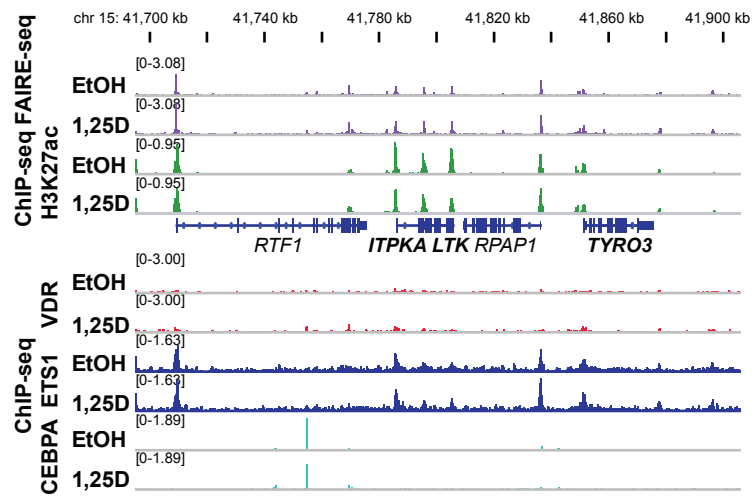


Figure S4: VDR knockout abolished transcriptomic vitamin D response. THP-1 cells that have been transduced with a VDR-specific (KO) or a non-targeting gRNA (NTC) were treated with 1,25(OH)₂D₃ or solvent (EtOH) for 4 or 24 h and quadruplicate RNA-seq was performed. The effects of the VDR knockout are displayed for two early primary (a, b) and one late primary (c) vitamin D target genes.

Fig. S5

a



b

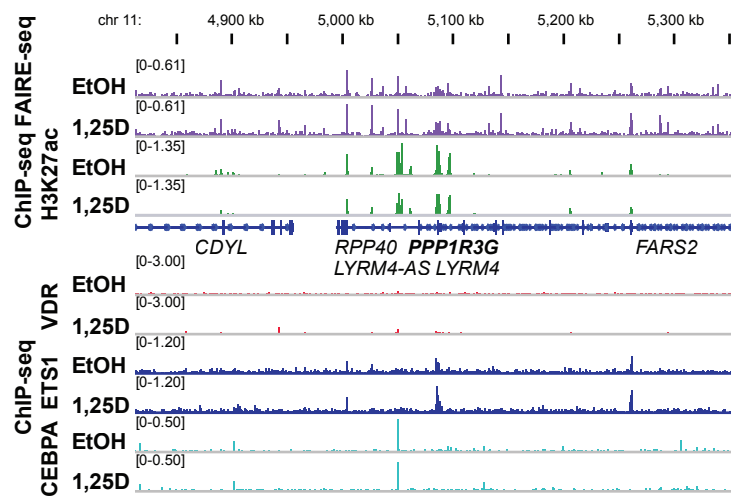
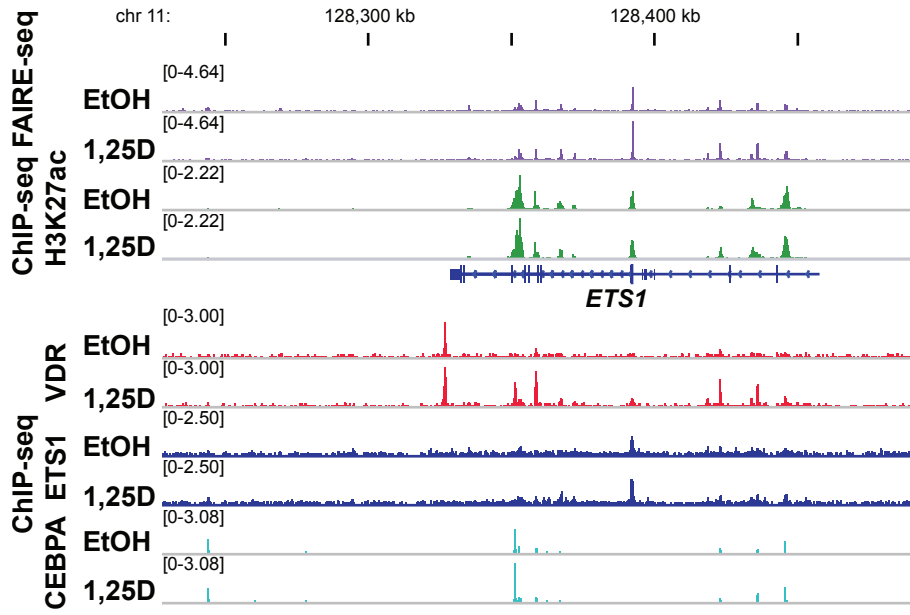


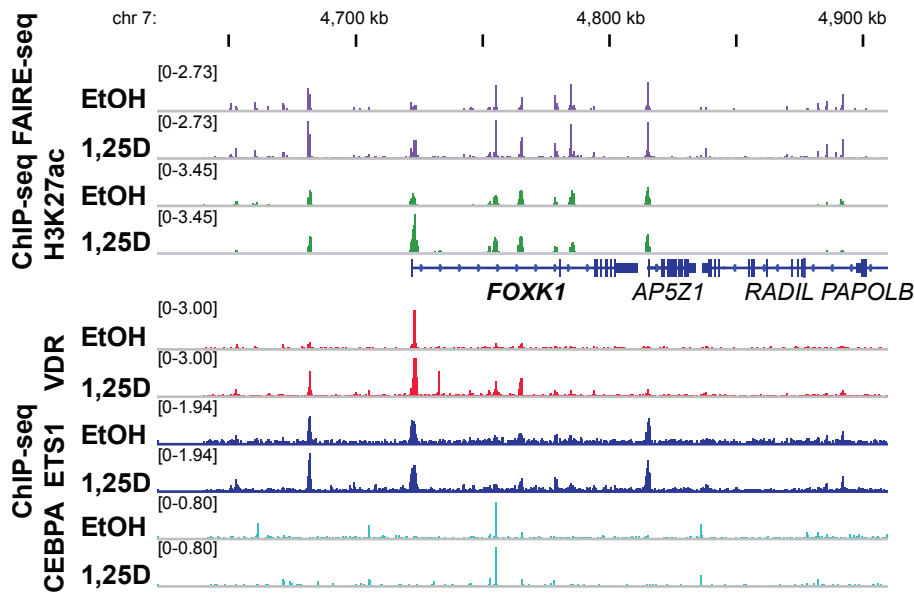
Figure S5: Transcription factor binding to the locus of secondary 1,25(OH)₂D₃-regulated genes. The predicted binding of CEBPA and ETS1 to the gene locus of the secondary VDR targets *LTK* (a) and of CEBPA to the *PPP1R3G* locus (b) was confirmed by ChIP-seq assays, while no significant VDR association could be seen. The binding occurred in open chromatin and co-located with the active enhancer mark H3K27ac. The gene names of all vitamin D target genes are shown in bold.

Fig. S6

a



b



c

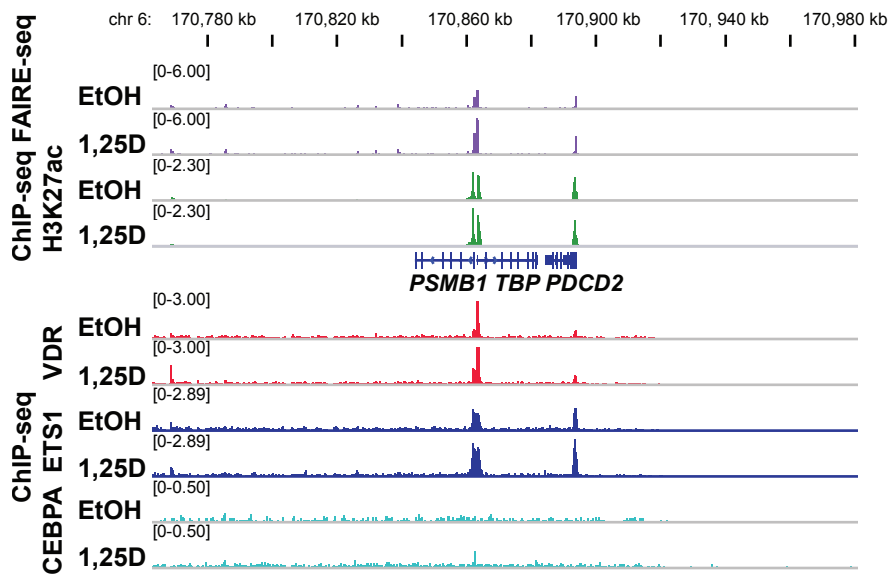


Figure S6: Transcription factor binding to loci of primary VDR target transcription factors. The predicted binding of VDR, ETS1 and CEBPA to the gene locus of the primary VDR target genes *ETS1* and *FOXK1* (a, b) and of VDR and ETS1 to *TBP* (c) was confirmed by ChIP-seq assays. The binding occurred in open chromatin and co-located with the active enhancer mark H3K27ac. Please note that for the VDR ChIP-seq tracks the scale has been chosen such as to be able to see also medium-sized peaks, meaning that the summits of the strongest peaks are cut. The gene names of all vitamin D target genes are shown in bold.

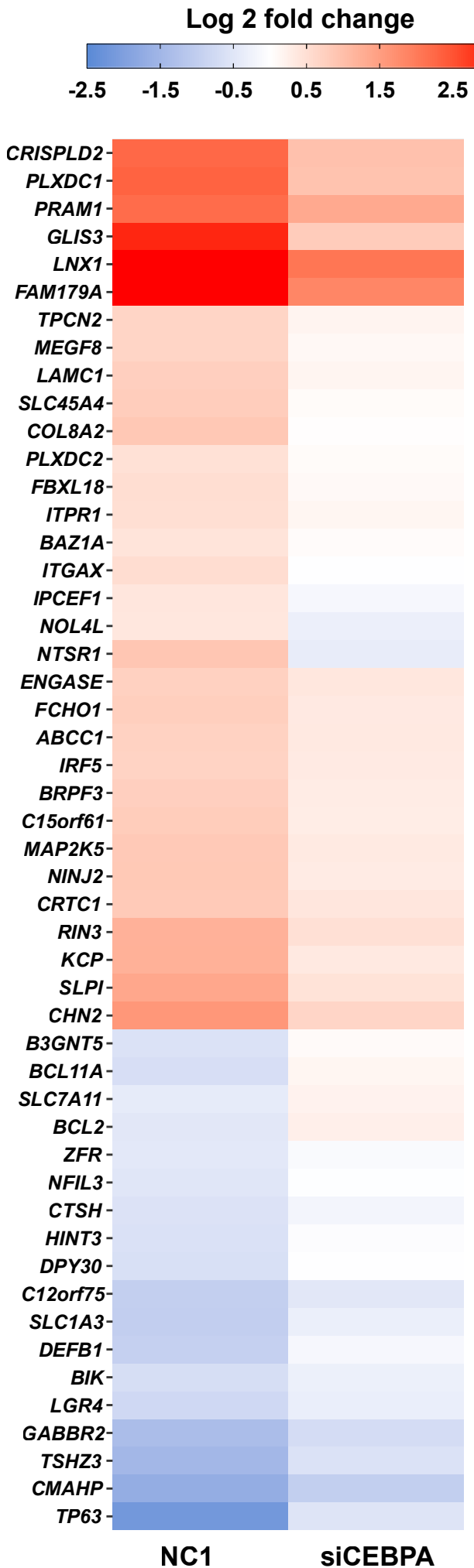
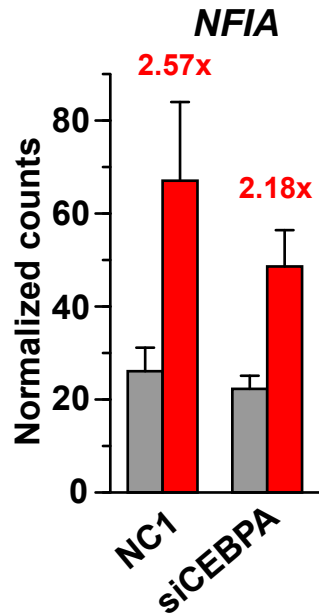
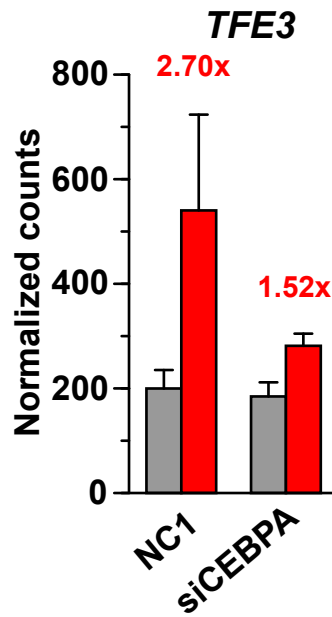
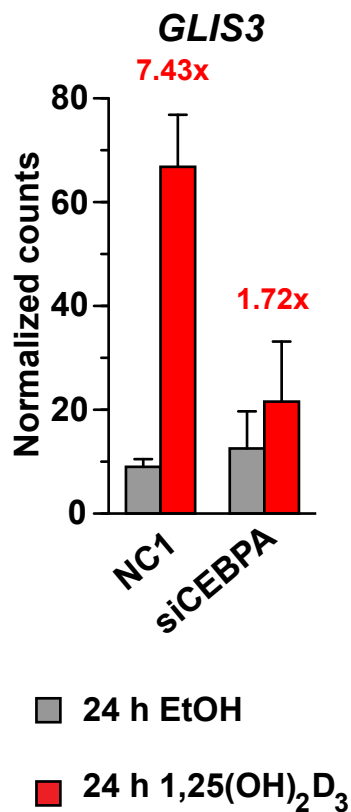
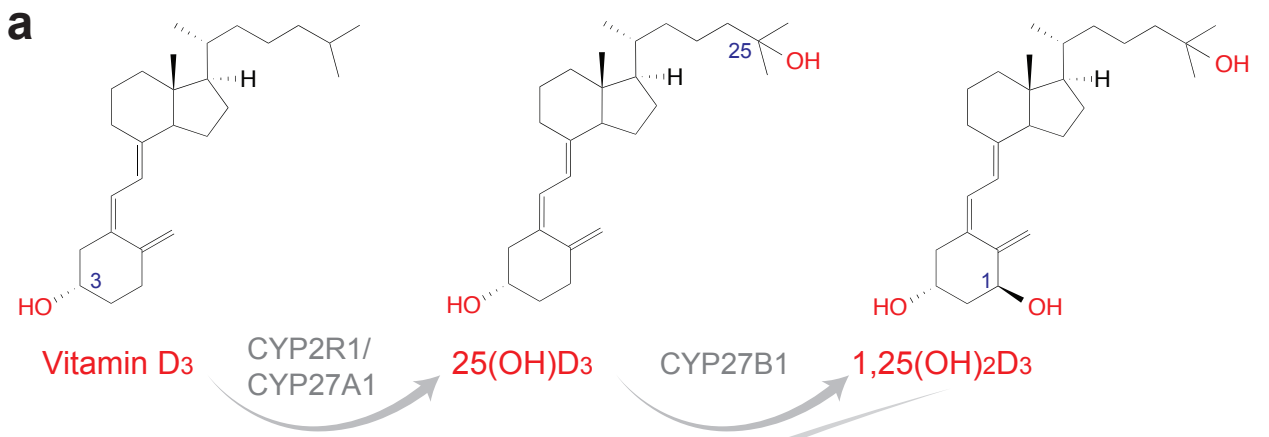
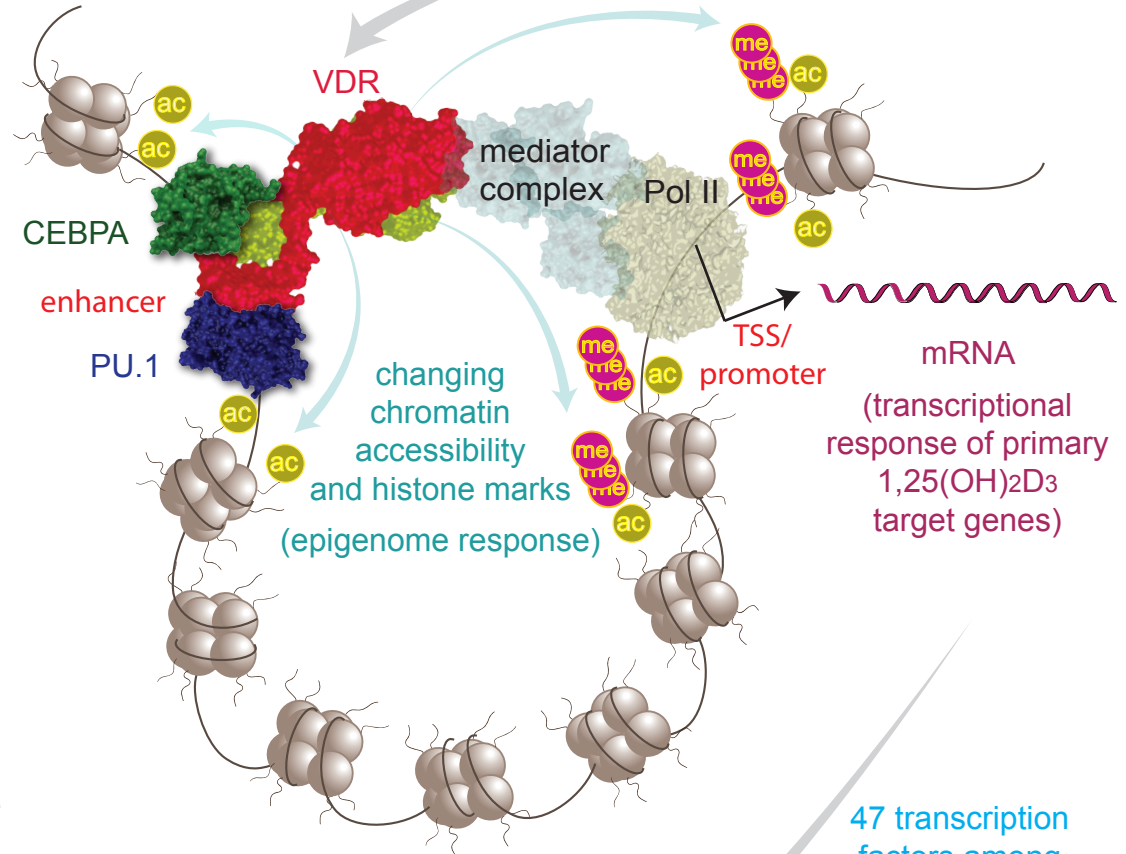
Fig. S7**a****b****c****d**

Figure S7: Effects of CEBPA knockdown on vitamin D target genes, to which CEBPA was predicted to bind. From the 1,25(OH)₂D₃ regulated genes identified from a previously published RNA-seq dataset in THP-1 cells with CEBPA knockdown that were predicted to bind CEBPA we selected those that do not gain or lose their response to the VDR ligand by the CEBPA knockdown (Figure 5). The log₂ fold changes in gene expression of the top 50 most significant of these DEGs in both siCEBPA and NC1 control cells demonstrated the almost complete loss of 1,25(OH)₂D₃ response (a). The genes *NFIA*, *TFE3* and *GLIS3* are shown to further illustrate this effect (b).

Fig. S8

b Primary response



c Secondary response

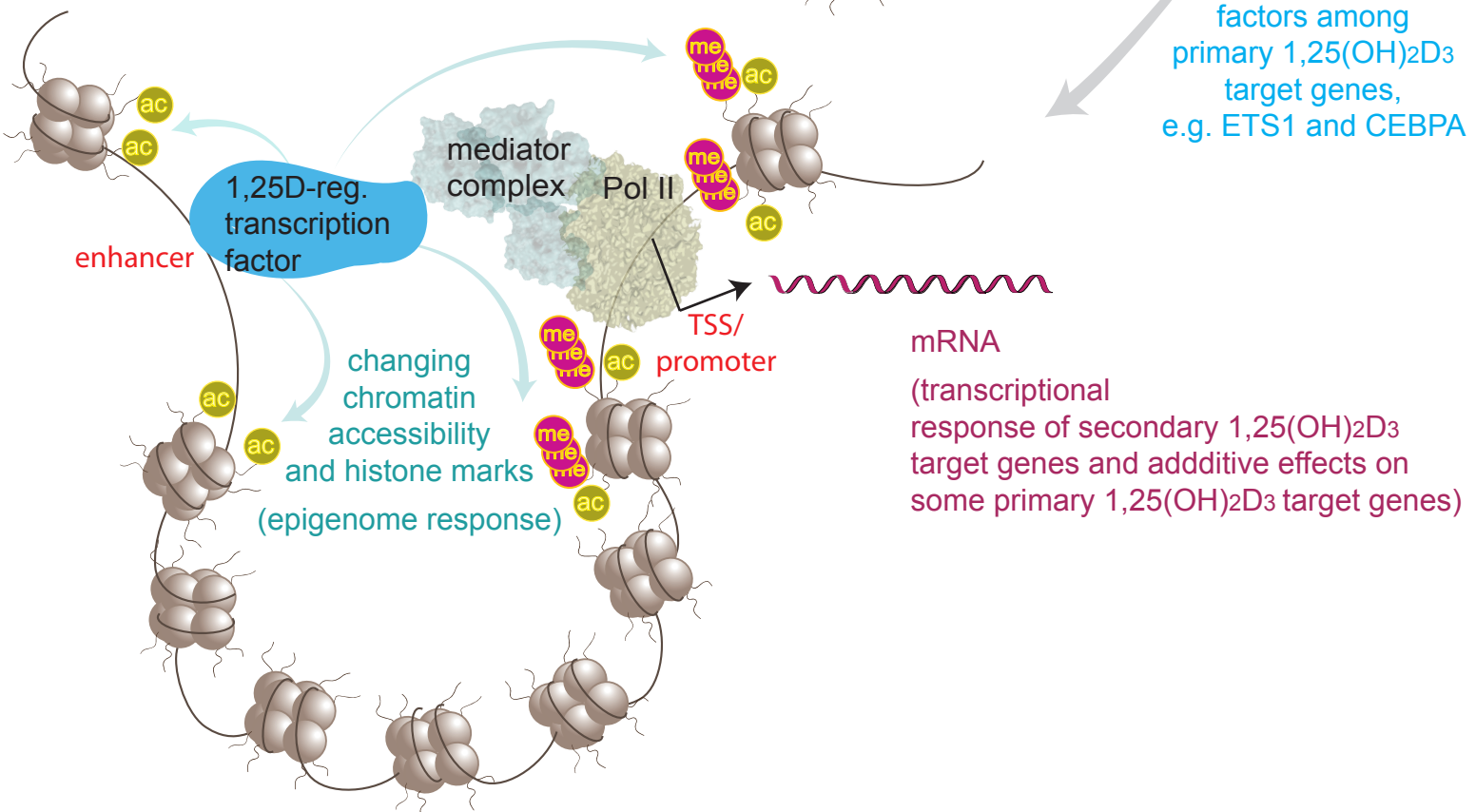


Figure S8: Model of vitamin D signaling. Vitamin D₃ is hydroxylated to 25-hydroxyvitamin D₃ (25(OH)D₃) and the high affinity VDR ligand 1,25(OH)₂D₃ (a). Pioneer transcription factors, such as PU.1 (dark blue) and CEBPA (green), increase chromatin accessibility and facilitate binding of VDR to enhancer sites and histone acetylation (H3K27ac) (b). Interaction of VDR-bound enhancers with mediator complexes at TSS regions induces changes of histone marks at promoters (H3K4me3 and H3K27ac) and activates RNA polymerase II. Subsequently, the respective primary vitamin D target genes are transcribed. Among these primary vitamin D targets are 47 transcription factor genes. When these transcription factors are translated, they associate to their binding sites at enhancer regions to regulate genes which are secondary vitamin D target genes or primary target genes that are regulated by the other transcription factor as well (c).

SUPPLEMENTARY DATA

SUPPLEMENTARY TABLES

Table S1: Oligonucleotides for gRNA cloning into lentiGuide-Puro (Addgene #52963).

Gene	Position in chr12 (hg19)	Oligonucleotide sequences (5'-3')	KO line
<i>VDR</i>	47,857,227	CACCGCATCATTACACGAACTGG AAACCCAGTTCGTGTGAATGATGC	L57
<i>VDR</i>	47,857,532	CACCGGGGTCGTAGGTCTTATGGT AAACACCATAAAGACCTACGACCCC	L56
<i>VDR</i>	47,865,123	CACCGGCGGCAGTCCCCGTTGAAG AAACCTTCAACGGGGACTGCCGCC	L55
<i>VDR</i>	47,879,056	CACCGCGTTCGGTCAAAGTCTCC AAACGGAGACTTTGACCGGAACGC	L54
NTC1	non-targeting control	CACCGTTCGGGCTAACAAGTCCT AAACAGGACTTGTTAGCCCGGAAC	L51
NTC2	non-targeting control	CACCGAATATCAACGCGAGCAAGG AAACCCCTGCTCGCGTTGATATTC	L52

Table S2: Vitamin D-triggered transcriptome of VDR knockout and control cells. The VDR was knocked out by CRISPR/Cas9 genome editing in THP-1 cells. VDR knockout (KO) cells and control (NTC) cells were treated with 1,25(OH)₂D₃ (V) or solvent (EtOH; E) for 4 or 24 h and four biological repeats (N1-N4) of RNA-seq have been performed. The raw data were analyzed using STAR for genomic alignment and DESeq2 for differential expression for different pairwise comparisons between the conditions.

Table S3: Prediction of primary VDR target transcription factors to mediate secondary vitamin D responses. The DYNAMITE analysis pipeline was used to identify primary VDR targeted transcription factors (TFs) with importance for the regulation of secondary vitamin D target genes. Normalized absolute classifier coefficients are listed alongside the rank in the entire DYNAMITE analysis for primary VDR targeted transcription factors.

Table S4: Pathway enrichment analysis for the 1,25(OH)₂D₃-regulated genes targeted by CEBPA or ETS1. ReactomePA was used to identify pathways that are enriched in the DEG subsets that have been shown to be bound by CEBPA or ETS1 within +/-25 kb of their TSS by ChIP-seq. BgRatio, background ratio; GeneRatio, number of genes of interest annotated to the pathway, out of the total size of the geneset of interest.