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Switches in histone modifications epigenetically control vitamin D3-dependent transcriptional upregulation of the CYP24A1 gene in osteoblastic cells

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Abstract

In bone cells vitamin D dependent regulation of gene expression principally occurs through modulation of gene transcription. Binding of the active vitamin D metabolite $1,25(OH)_2D_3$ to the vitamin D receptor (VDR) induces conformational changes in its C-terminal domain enabling competency for interaction with physiologically relevant co-activators, including SRC-1. Consequently, regulatory complexes can be assembled that support intrinsic enzymatic activities with competency to post-translationally modify chromatin histones at target genomic sequences to epigenetically alter transcription. Here we examine specific transitions in representation and/or enrichment of epigenetic histone marks during $1,25(OH)_2D_3$ mediated upregulation of CYP24A1 gene expression in osteoblastic cells. This gene encodes the 24-Hydroxylase enzyme, essential for biological control of vitamin D levels. We demonstrate that as the CYP24A1 gene promoter remains transcriptionally silent, there is enrichment of H4R3me2s together with its "writing" enzyme PRMT5 and decreased abundance of the H3ac/H4ac, H3R17me2a, and H4R3me2a marks as well as of their corresponding "writers". Exposure of osteoblastic cells to $1,25(OH)_2D_3$ stimulates the recruitment of a VDR/SRC-1 containing complex to the CYP24A1 promoter to mediate increased H3/H4 acetylation. VDR/SRC-1 binding occurs concomitant with the release of PRMT5 and the recruitment of the arginine methyltransferases CARM1 and PRMT1 to catalyze the deposition of the H3R17me2a and H4R3me2a marks, respectively. Our results indicate that these dynamic transitions of histone marks at the CYP24A1 promoter, provide a "chromatin

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Author contributions

D.M. and M. M. conceived the study and wrote most parts of the manuscript. D. M. performed most of the experimental work. P.M. provided methodological support in the study design and experimental work. J. B. L., G. S. S. and J.L.S. contributed to experimental design, data analysis and critically revised the manuscript text.

Conflict of interests

The authors declare that they have no conflict of interests.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

context" that is transcriptionally competent for activation of the CYP24A1 gene in osteoblastic cells in response to $1,25(OH)_2D_3$.

Keywords

Vitamin D; Vitamin D3 mediated transcription; Epigenetic control of osteoblast gene transcription

Introduction

Vitamin D is a principal mediator in biological control, including regulation of cell proliferation and differentiation during development, and has an obligatory role for postnatal remodeling and skeletal homeostasis throughout life (Pike & Christakos, 2017). It is well documented that Vitamin D is a critical component of the regulatory processes that mediate bone formation and resorption, by modulating the expression of a cohort of relevant bone-related genes (Carlberg, 2019b; Pike & Meyer, 2012). The active form of Vitamin D, 1,25-dihydroxy vitamin D3 ($1,25(OH)_2D_3$), binds to the vitamin D receptor (VDR) to induce its preferential localization in the cell nucleus and support interactions with specific DNA motifs at target regulatory genomic sequences to control gene transcription (Montecino et al., 2007; Pike et al., 2017).

VDR is a member of the superfamily of nuclear receptors that form ligand-dependent high molecular weight complexes that include transcription factors and coregulators (Montecino et al., 2007; Pike et al., 2017). Tissue specific transcription factors (Christakos et al., 2007; Paredes et al., 2004) and epigenetic regulators (Meyer & Pike, 2013; Seth-Vollenweider et al., 2014; Sierra et al., 2003; Zella et al., 2006) together modify the epigenetic landscape at specific genomic domains, change local chromatin structure and modulate $1,25(OH)_2D_3$-responsive transcriptional activity (Pike et al., 2015).

Work from our team and others has shown that increased histone acetylation accompanies $1,25(OH)_2D_3$ dependent enhancement of gene transcription in osteoblastic cells (Carvallo et al., 2008; Kim et al., 2006; Shen et al., 2003; Shen et al., 2002; Zella et al., 2006). This enrichment in histone H3 and H4 acetylation (H3ac and H4ac, respectively) are mediated, at least in part, by the recruitment of histone acetyl transferases (HATs) that include p300/CBP and the SRC family of nuclear receptor coactivators to the promoter regions of target genes (Carvallo et al., 2007; Dhawan et al., 2005; Kim et al., 2005; Sierra et al., 2003; Yamamoto et al., 2003). Analysis of histone modifications suggests that $1,25(OH)_2D_3$ promotes VDR binding at genomic sites that are marked by H3K9ac and H4K5ac, and that the interaction of VDR-containing complexes further enhances the enrichment in these epigenetic marks. Moreover, it was reported that these three modifications are increasingly enriched at sites of VDR binding and function (Meyer et al., 2014; St. John et al., 2014).

The CYP24A1 mammalian gene provides a paradigm for studying mechanisms associated with $1,25(OH)_2D_3$ dependent upregulation of transcription (Zierold et al., 1995). This gene encodes an enzyme that catabolizes $1,25(OH_2D_3$ by transforming it into a functionally inactive compound ($1,25,24(OH_2D_3$) in all tissues (Pike & Christakos, 2017). The expression of this gene is upregulated in response to the presence of $1,25(OH)_2D_3$ due to the

interaction of VDR and associated cofactors to regulatory sequences located proximally and distally from the transcription start site (TSS) (Meyer et al., 2010). More importantly, it was shown that during osteogenic differentiation VDR increasingly occupies several of these VDREs in a $1,25(OH)_2D_3$ dependent manner (Meyer et al., 2014; Meyer et al., 2015). This interaction of the VDR complex with the CYP24A1 locus results in significant changes in the enrichment of several epigenetic marks that accompany CYP24A1 mRNA expression (Pike et al., 2015).

It has been demonstrated that prior to this VDR dependent transcriptional activation of the CYP24A1 gene, a repressive complex including the arginine methyltransferase PRMT5 binds to the CYP24A1 promoter (Seth-Vollenweider et al., 2014). PRMT5 mediated gene repression at this locus is maintained through elevated levels of the histone epigenetic marks symmetric di-methylation of histone H3 arginine 8 (H3R8me2s) and H4R3me2s, that have been previously reported to be strongly associated with gene silencing (Blanc & Richard, 2017).

Based on these important findings, there is a compelling requirement to understand whether coordinated mechanisms that mediate “writing” and “erasing” of the different epigenetic marks are operating at the CYP24A1 promoter in response to $1,25(OH)_2D_3$. We address the contribution to transcriptional activity of enzymes that catalyze the deposit (“writing”) of epigenetic marks at the CYP24A1 gene before and after exposure of osteoblastic cells to $1,25(OH)_2D_3$. We specifically address whether hierarchical roles among these modifications are operating during $1,25(OH)_2D_3$ dependent epigenetic upregulation of CYP24A1 transcription.

Methods

Cell culture and vitamin D3 treatment

ROS 17/2.8 rat osteosarcoma cells were cultured in F12 (Invitrogen) media as previously described (Majeska et al., 1980). The media was supplemented with 5% bovine fetal calf serum (Hyclone). For the experiments with 1α,25-dihydroxy vitamin D3 ($1,25(OH)_2D_3$), cells were pre-incubated in F12 media with Charcoal/Dextran-treated fetal serum (Hyclone) for 24 h. Cell cultures were then treated with 10^{-8} M $1,25(OH)_2D_3$ or vehicle, for different periods of time (see below each figure) to define, under our experimental conditions, the profile of transcriptional responses of osseous and non-osseous genes.

Chromatin immunoprecipitation (ChIP) analyses

ChIP studies were performed as described earlier (Paredes et al., 2004), with modifications. ROS 17/2.8 cell cultures (100-mm-diameter plates), previously treated with 10^{-8} M $1,25(OH)_2D_3$ or vehicle, were incubated for 10 min with 1% formaldehyde and gentle agitation. Crosslinking was stopped by addition of 0,125 M glycine. The cells were then washed three times with 10 ml of PBS. Cells were scrapped off in 5 ml PBS and collected by centrifugation at 1,000g for 5 min. The cell pellet was resuspended in 1 ml of lysis buffer (50 mM Hepes pH 7.8, 20 mM KCl, 3 mM MgCl2, 0.1% NP-40, and a cocktail of proteinase inhibitors) and incubated for 10 min on ice. The cell extract was then collected by

centrifugation at 1,000g for 5 min, resuspended in 0.3 ml of sonication buffer (50 mM Hepes pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate acid, 0.1% SDS, and a cocktail of proteinase inhibitors). Chromatin was sheared in a water bath sonicator Bioruptor (Diagenode, NJ, USA) to obtain fragments of 200–500 bp. Extracts were sonicated at high power for four pulses of 5 min each, 30 sec on, 30 sec off, and centrifuged at 16,000g for 15 min at 4 °C. Supernatant was collected, aliquoted, frozen in liquid nitrogen, and stored at −80 °C; one aliquot was used for A260 measurements to determine concentration and chromatin size was confirmed by electrophoretic analysis. Cross linked extracts (500 A260 units) were resuspended in sonication buffer to a final volume of 500 μL. Samples were pre-cleared by incubating with 2–4 μg of normal IgG and 50 μL of protein A/G-agarose beads (Santa Cruz Biotechnology, CA, USA) for 2 h, at 4 °C with agitation. Chromatin was centrifuged at 4000g for 5 min, the supernatant was collected and immunoprecipitated with either anti CARM1 (sc-33176, Santa Cruz Biotechnology, TX), anti PRMT1 (sc-13392, Santa Cruz Biotechnology, TX), anti PRMT5 (611539, BD. B Pharmingen), anti SRC-1 (sc-8995, Santa Cruz Biotechnology, TX), anti H3Ac (06–599, Millipore), anti H4Ac (06–866, Millipore), anti H3R17me2a (ab8284, Abcam), anti H4R3me2a (39705, Active Motif) and anti H4R3me2s (ab5823, Abcam) for 12–16 h at 4 °C. The immunocomplexes were recovered with addition of 50 μL of protein A or G-agarose beads, followed by incubation for 1 h, at 4 °C with gentle agitation. Immunoprecipitated complexes were washed once with sonication buffer, twice with LiCl buffer (100 mM Tris-HCl pH 8.0, 500 mM LiCl, 1.0% NP-40, and 1.0% deoxycholic acid), and once with Tris–EDTA (TE) buffer pH 8.0 (2 mM EDTA and 50 mM Tris–HCl, pH 8.0), each time for 5 min at 4 °C; this was followed by centrifugation at 4000g for 5 min. Protein–DNA complexes were eluted by incubation with 100 μL of elution buffer (50 mM NaHCO3 and 1% SDS) for 15 min at 65 °C. Extracts were centrifuged at 16,000g for 30 sec, and the supernatant was collected and incubated for 12–16 h at 65 °C, to revert the cross-linking. Proteins were digested with 25 μg of proteinase K (Merck Millipore) for 2 h at 50 °C, and the DNA was recovered by phenol/chloroform extraction and ethanol precipitation using glycogen (20 μg/mL) as a precipitation carrier. The PCR primers used to evaluate the rat CYP24A1 gene promoter (−423/−199) were: 5’-TATTGGAAGGCGGACACTCT-3’ (forward) and 5’-GACTCCACCCCGGAGATAAC-3’ (reverse).

Silencing of SRC-1 gene expression

SRC-1 expression was downregulated in ROS 17/2.8 cells by specific SRC-1 small interfering RNAs (siRNA; SRC-1 Silencer Select predesigned siRNAs s162900, Ambion) that were transfected using Oligofectamine (Invitrogen) for 4 h, following the manufacturer’s instructions. Control cells were transfected with an unrelated random siRNA mix (Silencer Select negative control 1 siRNA; catalog number 4390844; Ambion). After treatment, 3 volumes of fresh media (F12 with 15% FBS) were added to the cells and then incubated for 48 h.

Lentivirus production and infection of ROS 17/2.8 cells

Downregulation of CARM1, PRMT1 and PRMT5 expression in ROS 17/2.8 cells was carried out by infecting with Lentivirus particles carrying sequences coding for specific shRNA molecules against the corresponding rat mRNAs. Viral particles were produced in

HEK293FT cells (Life Technologies) by transfecting with the plasmids pCMVVSVg, pCMV-dR8.91 and pLKO.1-shRNA (at a 1:2:3 ratio, respectively, with a maximum total DNA concentration of 10 μg per 60 mm plate). pLKO.1 empty vector was used as a control. All short hairpin-containing plasmids were acquired at Open Biosystems (GE Healthcare Dharmacon, UK). After 16–18 h, the culture medium was replaced and cells were maintained at 32 °C for 48 h. Supernatants containing pseudo typed particles were collected, filtered through a PVDF filter (0.45 μm pore size) and stored at −80 ºC. Titration and quantitation were performed by using the Lenti-X qRT-PCR Titration kit (Clontech) and the Lenti-X Provirus Quantitation kit (Clontech). ROS 17/2.8 cells were plated in 6-well culture plates and infected for 48 h with 300 μl lentiviral particles coding for shRNA-CARM1 (TRCN0000039118), shRNA-PRMT1 (TRCN0000018491), shRNA-PRMT5 (TRCN0000182229) or empty vector.

Nuclear extracts and protein expression analyses

Nuclear extracts were prepared as reported previously (Paredes et al., 2004). Protein levels were quantified by the Bradford assay (Bio-Rad Protein Assay Reagent, Bio-Rad, CA, USA) using bovine serum albumin as a standard. For western blot analyses, 10 μg of total protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose. Immunoblotting was performed with secondary antibodies conjugated to horseradish peroxide (Santa Cruz Biotechnology) and enhanced chemiluminescence solutions (Thermo Scientific, IL, USA). Primary antibodies used to detect proteins were as follows: anti SRC-1 (05–522, Upstate Biotechnology), anti TFIIB C-18 (sc-225, Santa Cruz Biotechnology), anti RNA-PoIII N-20 (sc-899, Santa Cruz Biotechnology), anti PRMT5/JBP1 (611539, BD Biosciences), anti PRMT1 (sc-13392, Santa Cruz Biotechnology), and anti CARM1/PRMT4 (sc-33176, Santa Cruz Biotechnology).

Reverse transcriptase and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted with TRIzol (Life Technologies), according to the manufacturer's protocol. An equal amount of each sample (2 μg) was used for reverse transcription. qPCR was performed using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, CA, USA). Data were normalized to GAPDH mRNA levels. Expression of mRNAs coding for CYP24A1, SRC-1, CARM1, PRMT1, PRMT5 and GAPDH, was determined using the following primers; rat CYP24A1: 5’- GCATGGATGAGCTGTGCGA-3’ (forward) and 5’-AATGGTGTCCCAAGCCAGC-3’ (reverse); rat SRC-1: 5’-GCAAGCTATCTTGAACCAGTTTGCAG-3’ (forward) and 5’-GCTCTTTGCTGCTGGATAATTTGCC-3’ (reverse); rat CARM1: 5’-AACAATCTGACAGACCGCATC-3’ (forward) and 5’-TTCAGGTACTTTTTGGCATGG-3’ (reverse); rat PRMT1: 5’-CTGTGGCCAAGCAGAAAGTAG-3’ (forward) and 5’-GAGATGCCGATTGTGAAACAT-3’ (reverse); rat PRMT5: 5’-GCTGTGGTGACGCTAGAGAAC-3’ (forward) and 5’-AGCCCAGAAGTTCACTGACAA-3’ (reverse); and rat GAPDH: 5’-CATGGCCTTCCGTGTTCCTA-3’ (forward) and 5’-CCTGCTTCACCACCTTCTTGAT-3’ (reverse).

Statistical analyses

For ChIP assays, we used a one-way ANOVA analysis followed by the Dunnett post-test to compare significant changes with respect to control. For mRNA expression analysis, we used the Student's t-test. In all figures, error bars represent the mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Results

SRC-1 mediates CYP24A1 transcriptional activation and chromatin hyperacetylation at the CYP24A1 promoter

It has been shown in several cell types that the CYP24A1 gene responds to the presence of $1,25(OH)_2D_3$ with a rapid increase in transcription (Kim et al., 2005). Using rat-derived osteosarcoma ROS 17/2.8 cells we determined that treatments with $1,25(OH)_2D_3$ (10^{-8} M) for 1, 2, 3, and 4 h (or longer, but not shown) produces a time-dependent accumulation of CYP24A1 mRNA (Fig 1A). This increase in CYP24A1 mRNA expression is accompanied by enrichment of the transcriptional co-activator SRC-1 at the CYP24A1 proximal promoter (−423 to −199) (Fig 1B). This co-activator has been shown to interact with VDR in a ligand-dependent manner and to be recruited to $1,25(OH)_2D_3$ target genes forming a complex with VDR and other regulatory proteins (Carvallo et al., 2007; Kim et al., 2005). This complex possesses histone acetyl transferase (HAT) activity and therefore mediates an increase in histone acetylation at target genomic sequences (Glass & Rosenfeld, 2000; Spencer et al., 1997). As shown in Fig 1C and 1D, following 3 h of $1,25(OH)_2D_3$ treatment, the CYP24A1 promoter exhibits significant enrichments in both histone H3 (Fig 1C) and histone H4 (Figure 1D) acetylation.

To determine whether expression of SRC-1 is critical for CYP24A1 responsiveness to $1,25(OH)_2D_3$ in these osteoblastic cells, we performed shRNA driven knockdown of SRC-1 expression (Fig 2A). The SRC-1 depletion was found to significantly inhibit CYP24A1 mRNA increase in response to $1,25(OH)_2D_3$ treatment (Fig 2B). Importantly, this inhibition of CYP24A1 transcription was accompanied by a reduced enrichment of H3ac (Fig 2C) and H4ac (Fig 2D) at the CYP24A1 promoter. Together, these results indicate that induction of CYP24A1 gene expression in osteoblastic cells in response to $1,25(OH)_2D_3$ requires the transcriptional co-activator SRC-1, which binds to the CYP24A1 promoter and mediates chromatin hyperacetylation.

Methylation of histone H3 arginine 17 accompanies $1,25(OH)_2D_3$-mediated CYP24A1 transcription

Growing evidence demonstrates a linkage between arginine methylation at chromosomal histones and epigenetic control of transcription (Blanc & Richard, 2017). To assess the contribution of this histone modification during transcriptional induction of the CYP24A1 gene in osteoblasts, we systematically evaluated critical methylations at histone H3 and H4 arginine residues and genomic occupancy of the enzymes that have been reported to mediate these modifications in mammalian cells.

We first determined that the class I arginine methyltransferase CARM1/PRMT4 exhibits increased occupancy at the CYP24A1 gene proximal promoter in osteoblastic cells exposed to 1,25$(OH)_2D_3$ for 3 h (Fig 3A). Importantly, the mark asymmetric di-methylation of histone H3 arginine 17 (H3R17me2a), a product of CARM1 activity is found significantly enriched at the CYP24A1 promoter in response to 1,25$(OH)_2D_3$ and nearly absent from this regulatory region in the absence of the ligand (Fig 3B). This result is in agreement with previous reports indicating that the H3R17me2a mark accompanies transcriptional activation in mammalian cells (Franek et al., 2015; Wu & Xu, 2012).

To demonstrate that CARM1 is necessary for the enrichment of H3R17me2a at the CYP24A1 gene promoter, a shRNA-mediated knockdown of this methyltransferase was performed in ROS 17/2.8 osteoblastic cells (Fig 3C). As shown in Fig 3D, CARM1 knockdown prevents the 1,25$(OH)_2D_3$ dependent enrichment of H3R17me2a at the CYP24A1 promoter. Importantly, the absence of CARM1 in osteoblastic cells also inhibits ligand dependent activation of CYP24A1 transcription (Fig 3E). Taken together these results indicate that CARM1 binding and CARM1-mediated increase in the H3R17me2a mark at the CYP24A1 promoter are required for a full 1,25$(OH)_2D_3$-dependent induction of this gene in osteoblastic cells.

The stimulatory role of CARM1 binding at the CYP24A1 promoter appears to operate independent of histone acetylation as CARM1 knockdown does not affect enrichment of H4ac (and of H3ac, but not shown) at this promoter (Fig S1A). It is significant that SRC-1 knockdown in these cells results in a strong inhibition of H3R17me2a enrichment at the CYP24A1 promoter (Fig S1B). Together these results reinforce a critical role of SRC-1 dependent histone acetylation at the CYP24A1 gene promoter during 1,25$(OH)_2D_3$ mediated transcriptional enhancement of this gene. Moreover, these data suggest a hierarchical relationship among these two histone modifications that accompany activation of CYP24A1 gene transcription, where H3R17me2a enrichment by CARM1 requires SRC-1 mediated increase in histone acetylation.

Switches in histone modifications epigenetically control 1,25$(OH)_2D_3$ dependent transcriptional upregulation of the CYP24A1 gene in osteoblastic cells

In a recent report Christakos and coworkers showed that a reduction in the H4R3me2s mark at the CYP24A1 gene promoter accompanies 1,25$(OH)_2D_3$ dependent upregulation of this gene in both osteoblastic and non-osteoblastic cells (Seth-Vollenweider et al., 2014). Moreover, their results suggested that this modification, catalyzed by the methyltransferase PRMT5 contributes to maintainance of CYP24A1 gene repression in the absence of ligand. In agreement with these findings we demonstrate that in untreated ROS 17/2.8 osteoblasts the CYP24A1 proximal promoter is enriched in both PRMT5 and H4R3me2s (Fig 4A and 4B, respectively). These enrichments are significantly reduced in osteoblastic cells treated with 1,25$(OH)_2D_3$ for 3 h (Fig 4A and 4B), further indicating that ligand dependent increase of CYP24A1 transcription requires a decrease in the H4R3me2s repressive mark. Importantly, it was determined that PRMT5 mediates the H4R3me2s enrichment at the CYP24A1 gene promoter; PRMT5 knockdown using a specific shRNA against the PRMT5

mRNA (Fig 4C) significantly reduces the H4R3me2s mark at the CYP24A1 promoter (Fig 4D).

In striking contrast, it was found that PRMT5 knockdown and concomitant H4R3me2s reduction do not significantly affect the transcriptionally silent status of the CYP24A1 gene in ROS 17/2.8 osteoblastic cells in the absence of $1,25(OH)_2D_3$ (Fig 4E). This PRMT5 knockdown, however, resulted in a significantly higher CYP24A1 mRNA expression in cells exposed to $1,25(OH)_2D_3$ for 3 h (Fig 4E). Taken together, these results indicate that PRMT5-dependent enrichment of the H4R3me2s mark can contribute to epigenetic mechanisms that sustain transcriptional repression of the CYP24A1 gene in the absence of $1,25(OH)_2D_3$. However, a reduction in this modification is not sufficient to epigenetically reprogram the silent chromatin conformation of this promoter and activate transcription in the absence of hormone. These data also indicate that PRMT5 departure from the CYP24A1 promoter and the concomitant decrease in H4R3me2s are molecular events that together significantly contribute to a more effective transcriptional response to $1,25(OH)_2D_3$.

In contrast to H4R3me2s, asymmetric di-methylation of the H4R3 residue (H4R3me2a) has been identified as an epigenetic mark associated with genes that are actively transcribing (Tikhanovich et al., 2017; Yang et al., 2014). As shown in Fig 5A, PRMT1, the arginine methyltransferase that can catalyze the deposition of the H4R3me2a mark, is bound at the CYP24A1 gene promoter in ROS 17/2.8 osteoblastic cells growing in the absence of $1,25(OH)_2D_3$ (Fig 5A). Importantly, we find that enrichment of both PRMT1 and H4R3me2a occurs in response to treatment with $1,25(OH)_2D_3$ for 3h (Fig 5A and 5B, respectively), concomitant with the decrease in the H4R3me2s mark (see Fig 4B). This ligand dependent H4R3me2a enrichment requires PRMT1, as the knockdown of PRMT1 expression in these osteoblastic cells using specific shRNAs (Fig 5C) prevents the increase of the H4R3me2a mark at the CYP24A1 gene promoter (Fig 5D). It is important to note that PRMT1 downregulation does not significantly affect the $1,25(OH)_2D_3$ dependent activation of CYP24A1 gene expression (Fig 5E).

These results indicate that while PRMT1 dependent enrichment of H4R3me2a at the CYP24A1 promoter accompanies ligand induced transcription of this gene, a decrease in this mark does not prevent expression of this gene in response to $1,25(OH)_2D_3$. In agreement with these findings, PRMT1 knockdown does not significantly affect $1,25(OH)_2D_3$ dependent H4ac at the CYP24A1 gene promoter (Fig S2A). Also, the H4R3me2a enrichment following $1,25(OH)_2D_3$ stimulation in ROS 17/2.8 osteoblastic cells occurs independent of SRC-1 expression (Fig S2B) and, in turn, independent from both SRC-1 mediated CYP24A1 gene transcription (Fig 2B) and SRC-1 mediated increase in histone acetylation at the CYP24A1 promoter (Fig 2C and 2D).

Taken together, these results suggest that binding of the regulatory complexes that include PRMT1 and SRC-1 to the CYP24A1 promoter occurs through mutually independent mechanisms. Additionally, our results further confirm that acetylation of histone H3 and histone H4 at the CYP24A1 gene promoter likely represents an epigenetic hallmark of CYP24A1 transcription in response to $1,25(OH)_2D_3$.

Discussion

In this study we have addressed how switches in the epigenetic profile of histone posttranslational modifications accompany and/or regulate transcriptional induction of the CYP24A1 gene in ROS 17/2.8 osteoblastic cells exposed to $1,25(OH)_2D_3$. We report that SRC-1 mediated histone acetylation plays a hierarchically dominant role during active transcription of the CYP24A1 gene. SRC-1 binds, in a ligand dependent manner, to the VDR and this complex is then recruited to the CYP24A1 gene promoter to activate transcription (Kim et al., 2005). We and others have previously shown that the intrinsic HAT activity of SRC-1 is required for both activating transcription and mediating histone hyperacetylation (Carvallo et al., 2007; Glass & Rosenfeld, 2000; Spencer et al., 1997). Moreover, our findings are supported by reports indicating that SRC-1 can directly interact with the HAT containing coactivator p300, hence forming a multi subunit regulatory complex with VDR that is capable of bringing strong hyperacetylating power to target gene promoters (Li et al., 2000; Spencer et al., 1997).

Whether SRC-1 can also form complexes with the histone arginine methyltransferases that we find enriched at the CYP24A1 promoter following $1,25(OH)_2D_3$ treatment remains to be formally established. An increase of the H3R17me2a mark at this promoter is a process mediated by CARM1 that accompanies CYP24A1 gene upregulation in response to 1,25(OH)2D3. Interestingly, we show that this process requires SRC-1 expression and binding to the CYP24A1 promoter. Whereas these results argue in favor of the potential formation of a complex including VDR/SRC-1/CARM1 at the CYP24A1 promoter, other results do not necessarily support this possibility; SRC-1 mediated histone acetylation at the CYP24A1 promoter is independent of CARM1 expression and H3R17me2a enrichment, although both molecular events are required for full transcriptional stimulation of the CYP24A1 gene in response to $1,25(OH)_2D_3$.

Christakos and colleagues have shown that an enrichment in the H4R3me2s mark represents an important component of the transcriptionally silent CYP24A1 gene promoter (Seth-Vollenweider et al., 2014). This team also showed that the H4R3me2s mark is erased from the promoter as CYP24A1 gene transcription is activated by exposure of the cells to $1,25(OH)_2D_3$. Here we demonstrate that this H4R3me2s enrichment at the CYP24A1 promoter requires PRMT5 and that PRMT5 knockdown together with reduction of H4R3me2s enrichment, does not modify the silent status of the CYP24A1 gene in the absence of $1,25(OH)_2D_3$. Our results support a model in which additional epigenetic mechanisms are operating at the transcriptionally silent CYP24A1 gene to prevent its activation prior to exposure to $1,25(OH)_2D_3$.

Additionally, we find that asymmetrically di-methylated H4R3 (H4R3me2a) is located at the CYP24A1 promoter of osteoblastic cells in a PRMT1 and $1,25(OH)_2D_3$ dependent manner. These results indicate that a fine epigenetic tuning, represented by a specific switch in alternative di-methylation at the H4R3 residue occurs on this promoter during transcriptional upregulation; H4R3me2s is erased and replaced by H4R3me2a. To our knowledge, this is the first time that such a switch in these two opposite epigenetic marks is reported during steroid hormone regulation of transcription.

Strikingly, H4R3me2a enrichment does not seem to represent a critical component for $1,25(OH)_2D_3$ dependent upregulation of CYP24A1 mRNA expression; PRMT1 knockdown affects neither CYP24A1 upregulation nor histone acetylation at the CYP24A1 promoter in cells exposed to the ligand. We propose that H4R3me2a enrichment occurs as this H4R3 residue becomes “available” following H4R3me2s erasure and as PRMT1 is recruited to the CYP24A1 promoter. This possibility is currently under investigation. The specific mechanisms mediating PRMT5 release from the CYP24A1 promoter and PRMT1 binding to this sequence remain to be defined. Preliminary results indicate that recruitment of PRMT1 is independent of VDR/SRC-1 binding to the CYP24A1 proximal promoter (data not shown) and hence does not require SRC-1 mediated hyperacetylation.

Taken together, these results support a model (see Fig 6) in which specific transitions in enrichment of epigenetic histone marks accompany transcriptional upregulation of the CYP24A1 gene in osteoblastic cells exposed to $1,25(OH)_2D_3$. First, in the absence of the ligand this promoter remains transcriptionally silent, a status that is reflected by elevated levels of H4R3me2s (and of its “writing” enzyme PRMT5) and lower abundance of the H3ac/H4ac, H3R17me2a, and H4R3me2a marks and their corresponding “writers”. Second, exposure of the cells to $1,25(OH)_2D_3$ results in the recruitment of the VDR/SRC-1 complex to the CYP24A1 promoter, which in turn mediates H3/H4 acetylation. VDR/SRC-binding occurs prior to or together with the release of PRMT5 and the recruitment of the arginine methylases CARM1 and PRMT1 that catalyze the deposition of H3R17me2a and H4R3me2a marks, respectively. Our results indicate that these histone mark transitions, in addition to other components of the epigenetic landscape that are known to occur at transcriptionally active genes (Carlberg, 2019a; Kouzarides, 2007; Pike et al., 2016), provide the “appropriate epigenetic context” for transcriptional induction of the CYP24A1 gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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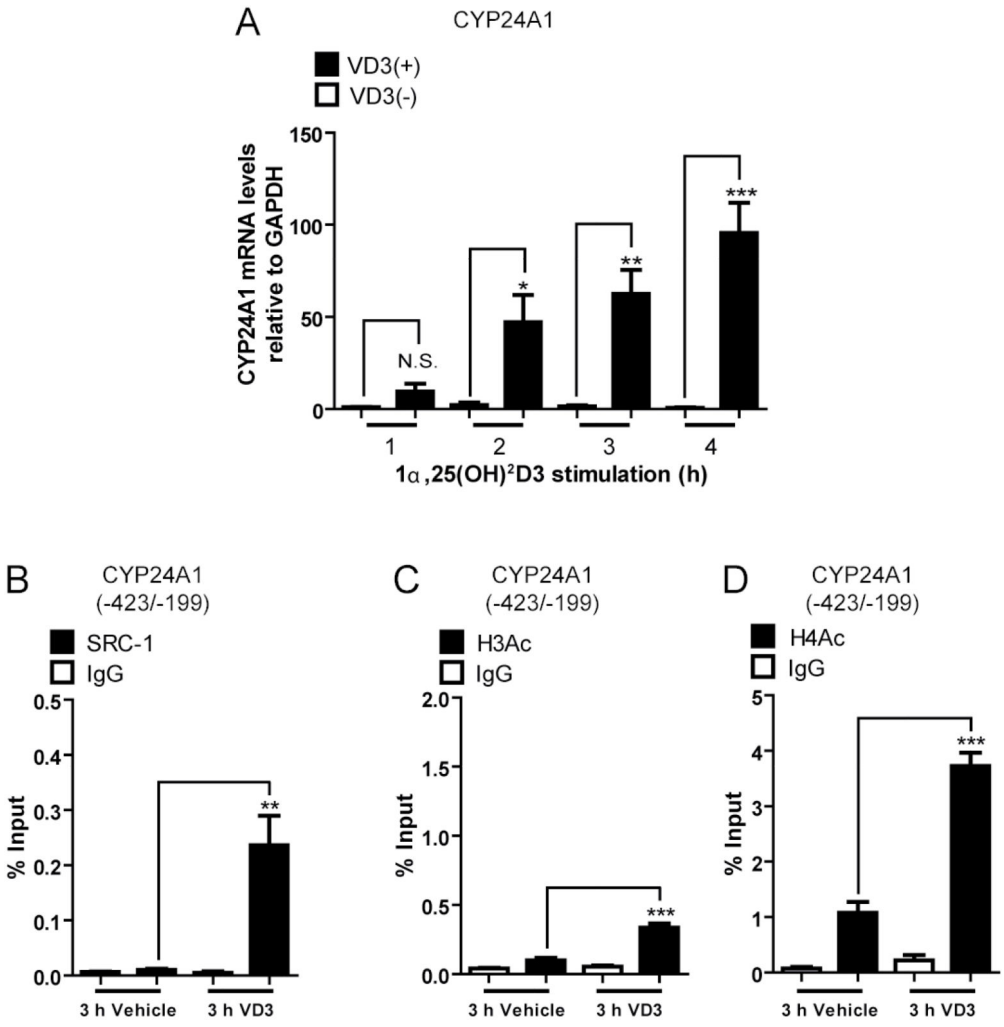

Figure 1.

1,25(OH)2D3 dependent activation of the CYP24A1 gene is mediated by SRC-1 binding and H3Ac and H4Ac enrichment. (A) ROS17/2.8 cells were treated with 10–8 M 1,25(OH)2D3 or vehicle for 1 to 4 h periods. CYP24A1 mRNA levels were determined by qRT-PCR using specific primers. Values were normalized against GAPDH mRNA. (B–D) Recruitment of SRC-1 and enrichment of H3Ac and H4Ac marks at the CYP24A1 promoter in ROS17/2.8 cells exposed to 10–8 M 1,25(OH)2D3 or vehicle for 3 h. ChIP assays were performed using antibodies against SRC-1 protein (B) or against the H3Ac (C) and H4Ac (D) histone marks. ChIP values are expressed as % input ± SEM. Normal IgG was used as specificity control. Statistical analyses were carried out with respect to vehicle. *, p 0.05; **, p 0.01***, p 0.001; N.S. Statistically not significant differences.

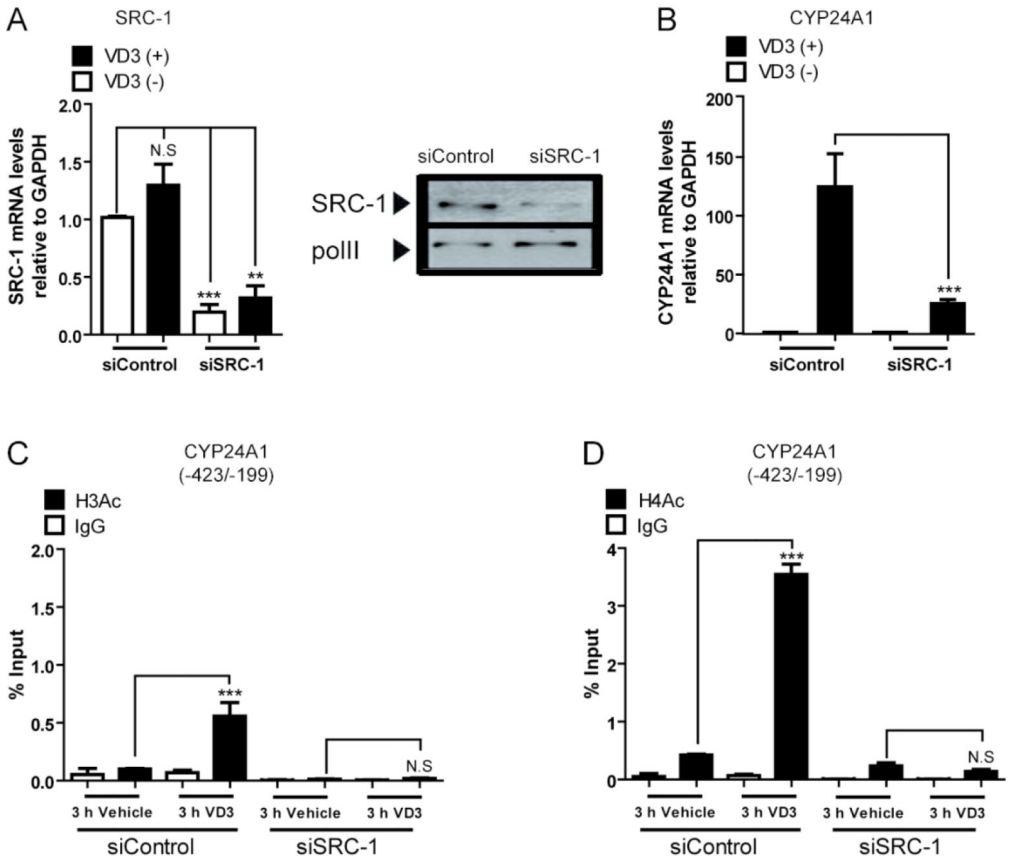

Figure 2.

Knockdown of SRC-1 decreases 1,25(OH)2D3 dependent CYP24A1 gene expression and reduces H3Ac and H4Ac enrichment at the CYP24A1 promoter. ROS17/2.8 cells treated with siRNAs against SRC-1 or siRNA control for 48 h were exposed to 10–8 M 1,25(OH)2D3 or vehicle for 3 h. (A) SRC-1 knockdown was confirmed by measuring SRC-1 mRNA (left) and protein (right) levels by qRT-PCR and western blot analyses, respectively. GAPDH mRNA was determined as mRNA expression control. Detection of RNA polymerase II (pol II) was used to control for equal protein loading. (B) Decrease in CYP24A1 mRNA levels were measured by qRT-PCR; Data were normalized against GAPDH mRNA levels. ChIP assays in cells with reduced expression of SRC-1 showed decreased H3Ac (C) and H4Ac (D) enrichments at the CYP24A1 promoter. ChIP values are expressed as % input ± SEM. Normal IgG was used 624 as specificity control. Statistical analyses were performed with respect to vehicle. **, p 0.01***, p 0.001; N.S. Statistically not significant differences.

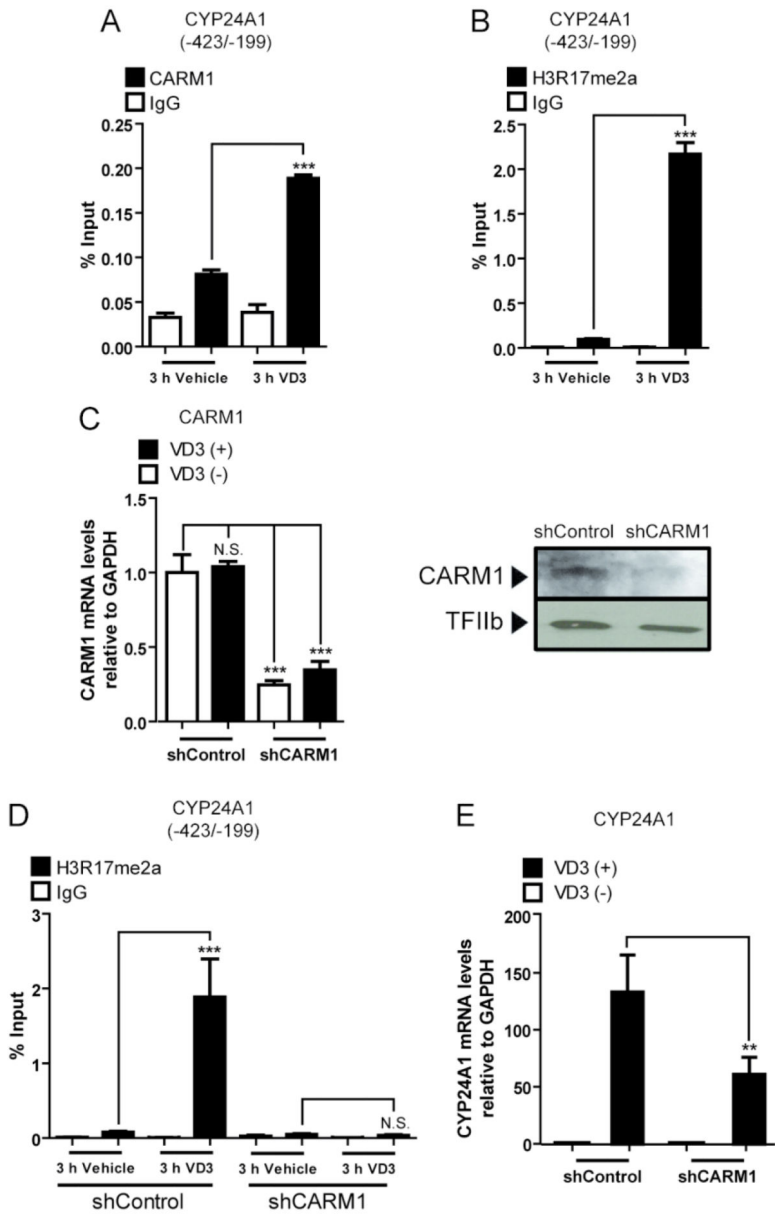

Figure 3.

1,25(OH)2D3 dependent binding of CARM1 to the CYP24A1 promoter is required for enrichment of the H3R17me2a mark and transcriptional enhancement. ROS17/2.8 osteoblastic cells were incubated with 10–8 M 1,25(OH)2D3 or vehicle for 3 h. ChIP assays using antibodies against CARM1 protein (A) protein and the H3R17me2a histone mark (B). (C-D) ROS17/2.8 cells infected with shRNAs against CARM1 or shRNA control for 48 h were exposed to 10–8 M 1,25(OH)2D3 or vehicle for 3 h. (C) CARM1 knockdown was confirmed at mRNA (left) and protein (right) expression levels as described in figure legend 2. GAPDH mRNA is shown as mRNA expression control. Detection of TFIIb was used to control for equal protein loading. (D) Effect of CARM1 knockdown on H3R17me2a enrichment at the CYP24A1 promoter. (E) Knockdown of CARM1 decreases 1,25(OH)2D3 dependent CYP24A1 expression. ChIP values are expressed as % input ± SEM. Normal IgG

was used as specificity control. Statistical analyses were performed with respect to vehicle. **, p 0.01***; p 0.001; N.S. Statistically not significant differences.

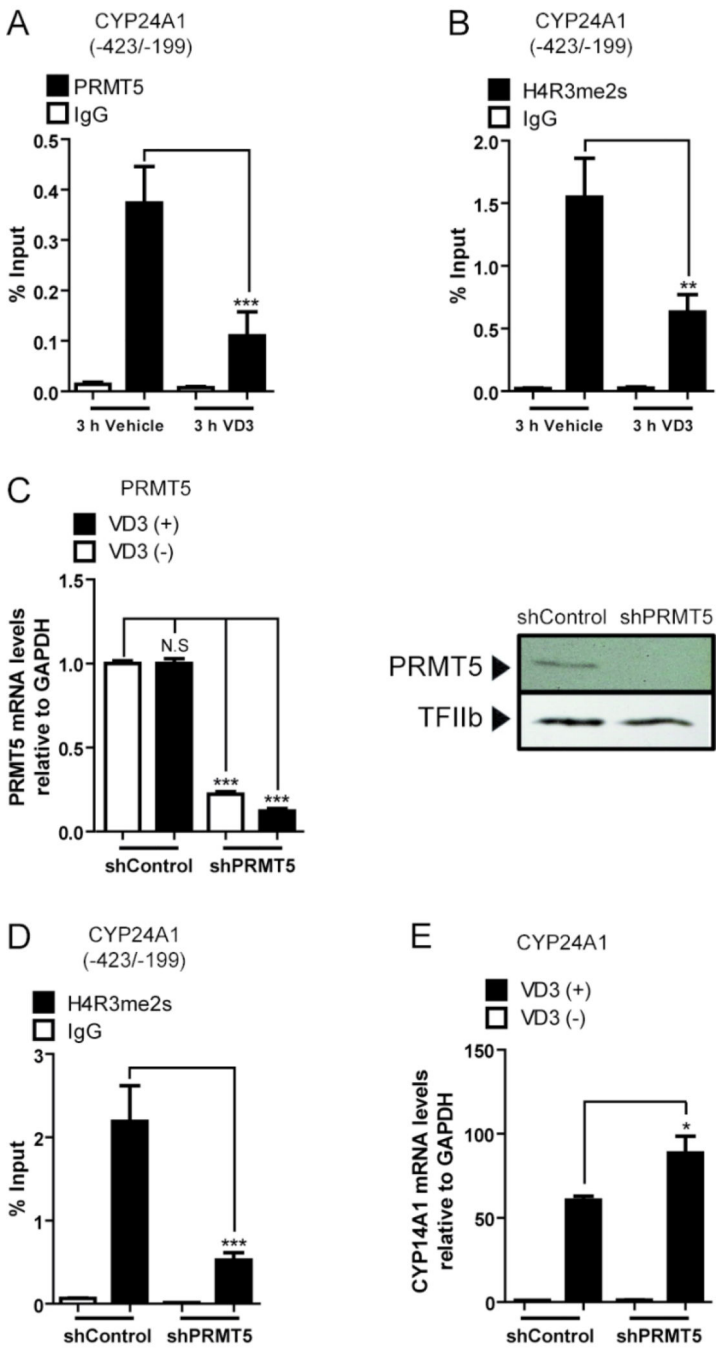

Figure 4.

Binding of PRMT5 to the CYP24A1 gene promoter and PRMT5 dependent enrichment of H4R3me2s are inhibited by treatment with 1,25(OH)2D3. Binding of PRMT5 (A) and enrichment of the H4R3me2s mark (B) at the CYP24A1 promoter were measured by ChIP in ROS17/2.8 cells incubated with 10–8 M 1,25(OH)2D3 or vehicle for 3 h. (C) Knockdown with shRNAs against PRMT5 (48 h) was confirmed by evaluating mRNA (left) and protein (right) levels. (D) The effect of PRMT5 knockdown on H3R17me2s enrichment at the CYP24A1 promoter was determined by ChIP in samples from ROS17/2.8 cells treated with

10–8 M 1,25(OH)2D3 or vehicle for 3 h. (E) knockdown of PRMT5 increases CYP24A1 gene responsiveness to 1,25(OH)2D3. CYP24A1 mRNA 650 levels were measured by qRT-PCR, as described in figure legend 1. ChIP values are expressed as % input ± SEM. Normal IgG was used as specificity control. Statistical analyses were performed with respect to vehicle. *, p 0.05; **, p 0.01***; p 0.001; N.S. Statistically not significant differences.

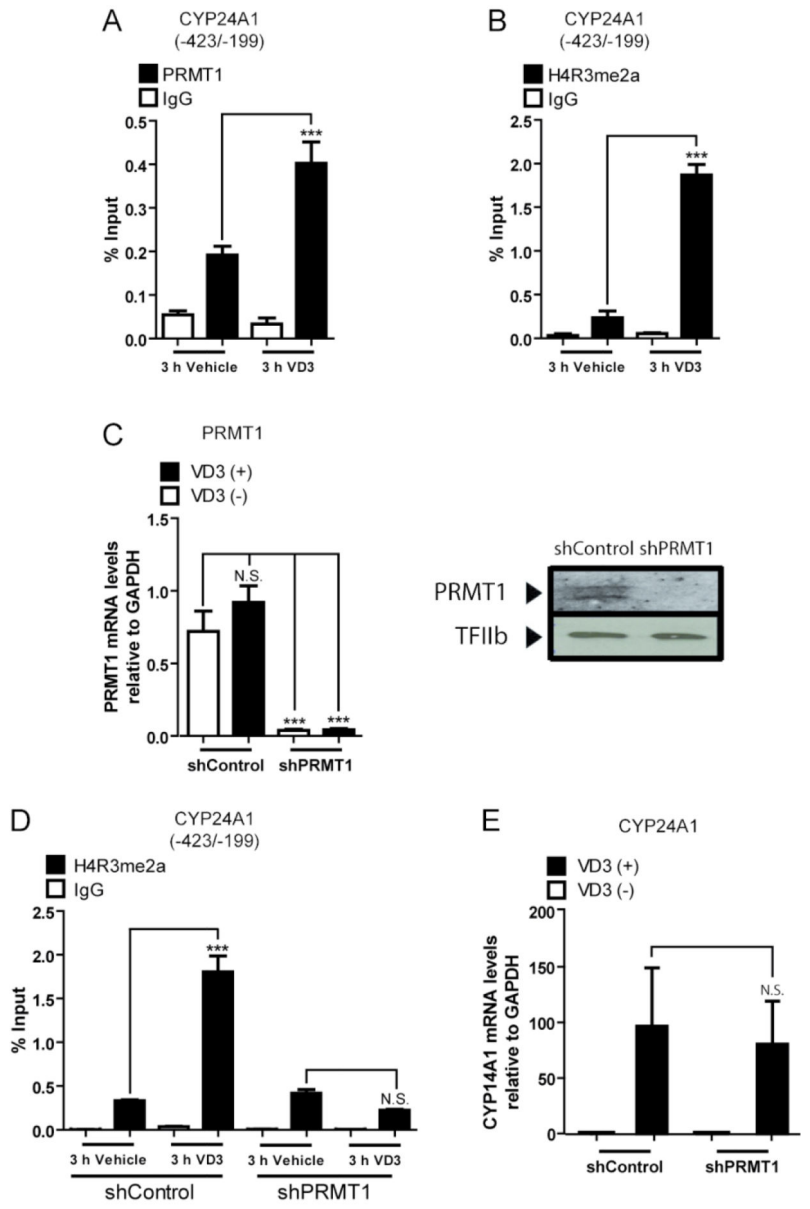

Figure 5.

(A,B) 1,25(OH)2D3 dependent binding of PRMT1 to the CYP24A1 gene promoter is accompanied by enrichment of the H4R3me2a histone mark. Recruitment of PRMT1 and enrichment of H4R3me2a histone modifications at the CYP24A1 promoter were measured by ChIP in samples from ROS17/2.8 cells incubated with 10–8 M 1,25(OH)2D3 or vehicle for 3 h. (C-D) Cells were infected with lentiviruses coding for shRNAs against PRMT1 or shRNA control for 48 h, and then incubated with 10–8 M 1,25(OH)2D3 or vehicle for 3 h. (C) PRMT1 knockdown was confirmed at mRNA (left) and protein (right) levels. (D) Effect of PRMT1 knockdown on H4R3me2a enrichment measured by ChIP. (E) CYP24A1 mRNA levels in cells with downregulated expression of PRMT1. ChIP values are expressed as % input ± SEM. Normal IgG was used as specificity control. Statistical analyses were performed with respect to vehicle. *, p 0.001; N.S. Statistically not significant differences.

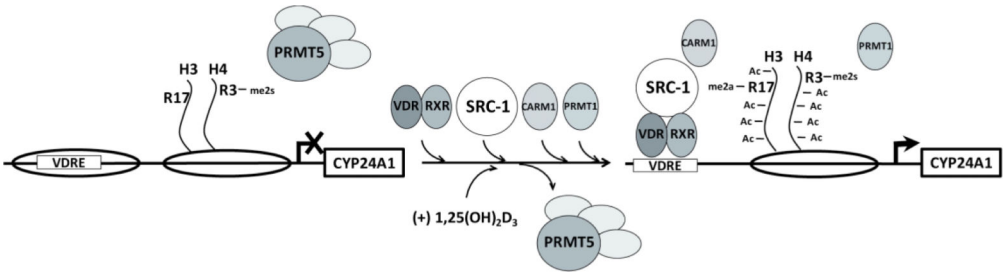

Figure 6.

Proposed model describing switches in epigenetic control at the CYP24A1 gene in osteoblastic cells. Schematic representation of the CYP24A1 gene promoter including changes in H3Ac, H4Ac, H3R17me2a, H4R3me2s and H4R3me2a histone marks as well as binding of different chromatin modifiers to the proximal CYP24A1 promoter under control and 1,25(OH)2D3 enhanced conditions. The arrowhead represents the transcription start site. The open ellipses reflect nucleosomes localized at the CYP24A1 promoter. The levels of enrichment of histone marks as well as their enzymatic writers are represented by the sizes of each component. Figure S1. H3R17me2a enrichment during 1,25(OH)2D3 dependent transcriptional activation of the CYP24A1 gene requires SRC-1 mediated histone acetylation. (S1A) Knockdown of SRC-1 prevents the enrichment of H4Ac associated with 1,25(OH)2D3 dependent activation of the CYP24A1 gene promoter. ChIP against H4Ac were performed in chromatin samples of ROS17/2.8 cells transduced with shRNAs against CARM1 or shRNA control for 48 h and then treated with 10–8 M 1,25(OH)2D3 or vehicle for 3 h. (S1B) Downregulation of SRC-1 prevents the enrichment of H3R17me2a at the CYP24A1 gene promoter in cells exposed to 1,25(OH)2D3. ChIP assays to detect H3R17me2a were performed in samples from cells treated with siRNAs against SRC-1 or siRNA control for 48 h, and then incubated with 10–8 M 1,25(OH)2D3 or vehicle for 3 h. ChIP values are expressed as % input ± SEM. Normal IgG was used as specificity control. Statistical analyses were performed with respect to vehicle. *, p 0.001; N.S. Statistically not significant differences. Figure S2. 1,25(OH)2D3 mediated enrichment of the H4Ac and H4R3me2a marks at the CYP24A1 gene promoter are mutually independent molecular events. (S2A) Knockdown of PRMT1 does not affect the increased enrichment of H4Ac at the CYP24A1 promoter in cells exposed to 1,25(OH)2D3. ChIP assays against H4Ac were performed in ROS17/2.8 cells transduced with shRNAs against PRMT1 or shRNA control for 48 h, and then incubated with 10–8 M 1,25(OH)2D3 or vehicle for 3 h. (S2B) Downregulation of SRC-1 expression does not prevent the 1,25(OH)2D3 dependent increase in enrichment of H4R3me2a at the CYP24A1 promoter. ChIP assays to detect H4R3me2a were performed with chromatin samples from ROS17/2.8 cells treated with siRNAs against SRC-1 or siRNA control for 48 h, and then incubated with 10–8 M 1,25(OH)2D3 or vehicle for 3 h. ChIP values are expressed as % input ± SEM. Normal IgG was used as specificity control. Statistical analyses were performed with respect to vehicle. *, 702 p 0.001; N.S. Statistically not significant differences.