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PU.1 and Epigenetic Signals Modulate 1,25-Dihydroxyvitamin D₃ and C/EBP α Regulation of the Human Cathelicidin Antimicrobial Peptide Gene in Lung Epithelial Cells

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Abstract

LL-37, the only known human cathelicidin which is encoded by the human antimicrobial peptide (CAMP) gene, plays a critical role in protection against bacterial infection. We previously demonstrated that cathelicidin is induced by 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in human airway epithelial cells with a resultant increase in bactericidal activity. In this study we identify key factors that cooperate with 1,25(OH)₂D₃ in the regulation of CAMP. Our results show for the first time that PU.1, the myeloid transcription factor (which has also been identified in lung epithelial cells), cooperates with the vitamin D receptor and C/EBP α to enhance the induction of CAMP in lung epithelial cells. Our findings also indicate that enhancement of 1,25(OH)₂D₃ regulation of CAMP by histone deacetylase inhibitors involves cooperation between acetylation and chromatin remodeling through BRG1 (a component of the SWI/SNF complex). BRG1 can be an activator or repressor depending on BRG1 associated factors. PRMT5, a methyltransferase which interacts with BRG1, represses 1,25(OH)₂D₃ induced CAMP in part through dimethylation of H4R3. Our findings identify key mediators involved in the regulation of CAMP gene in lung epithelial cells and suggest new approaches for therapeutic manipulation of gene expression in order to increase the antibacterial capability of the airway.

Keywords

1,25-dihydroxyvitamin D₃; cathelicidin; PU.1; Brahma-related gene 1; histone acetylation; protein arginine methyltransferase 5

INTRODUCTION

As important elements of innate immunity, antimicrobial peptides are known to provide protection against bacterial infection. Defensins and cathelicidins are the two principle families of antimicrobial peptides in mammals. With the ability to disrupt the integrity of

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microbe membranes, both families of antimicrobial peptides have a broad spectrum of antimicrobial activity (Arranz-Trullen et al., 2017; Cederlund et al., 2011; Tecle et al., 2010). The human cationic antimicrobial protein 18 (hCAP18) is the only known human cathelicidin and is encoded by the human cathelicidin antimicrobial peptide (CAMP) gene (Cederlund et al., 2011). hCAP18 is the inactive storage form which is cleaved to generate the mature, 37 amino acid active peptide LL-37 (Sorensen et al., 2001). LL-37 is produced in neutrophils as well as in other immune cells including B and T cells, monocytes and natural killer cells (Agerberth et al., 2000; Cederlund et al., 2011; Cowland et al., 1995). Epithelial cells of the skin, intestine and respiratory tract also produce LL-37 (Bals et al., 1998; Cederlund et al., 2011; Diamond et al., 1991; Dorschner et al., 2001; Tecle et al., 2010; Tollin et al., 2003; Vandamme et al., 2012; Yim et al., 2007). The respiratory tract is constantly exposed to inspired pathogens. The airway epithelium is the initial line of defense against lung infection by contributing barrier function as well as by secreting antimicrobial peptides (Tecle et al., 2010). Mice deficient in the murine homologue of human LL-37 (mCRAMP) have impaired host defense against *P. aeruginosa* lung infection (Beaumont et al., 2014). Delivery of exogenous LL-37 was shown to enhance clearance of the lung infection providing *in vivo* evidence for the role of LL-37 in antibacterial defense in the lung (Beaumont et al., 2014). In light of the increased prevalence of antibiotic-resistant pathogens and since bacterial resistance to antimicrobial peptides is uncommon, enhancement of the endogenous expression of these peptides may represent a novel therapeutic approach for treatment of infection caused by multi-resistant bacteria. Understanding the specific factors that regulate antimicrobial peptide expression may be the key to the development of modulators of innate immune responses for adjunct therapy. We previously demonstrated that physiological levels of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the biologically active form of vitamin D, induce expression of CAMP mRNA and hCAP18 in human airway epithelial cells with a resultant increase in bactericidal activity (Yim et al., 2007). Subsequent studies by others using human bronchial or tracheobronchial epithelial cells also noted increased expression of cathelicidin in response to 1,25(OH)₂D₃ (Hansdottir et al., 2008; Steinmann et al., 2009). These findings suggest that vitamin D by inducing cathelicidin could be useful as a potential addition to antibiotic therapy for the treatment of airway infections.

Although the importance of vitamin D for maintaining calcium homeostasis is well known, recent studies have shown that 1,25(OH)₂D₃ is also a key regulator of the immune system. The actions of 1,25(OH)₂D₃, similar to other steroid hormones, are mediated by a nuclear receptor (vitamin D receptor, VDR) which heterodimerizes with the retinoid X receptor and interacts with specific DNA sequences (vitamin D response elements, VDREs) in target genes (Christakos et al., 2016; Pike and Meyer, 2010). The ligand bound VDR further recruits a coregulatory complex that initiates transcriptional activity. Although progress has been made with regard to mechanisms involved in the regulation of adaptive immunity by 1,25(OH)₂D₃ (Christakos et al., 2016; Wei and Christakos, 2015), little is known about the molecular level changes that are involved in the effects of 1,25(OH)₂D₃ on innate immunity. The identification of a functional VDRE in the human CAMP gene provided evidence that CAMP is a direct transcriptional target of 1,25(OH)₂D₃ in myeloid cells and keratinocytes (Gombart et al., 2005; Weber et al., 2005). We previously demonstrated that C/EBPα is a

potent enhancer of CAMP transcription and that C/EBP α cooperates with 1,25(OH) $_2$ D $_3$ to induce CAMP transcription and mRNA in lung epithelial cells (Dhawan et al., 2015). In addition to the VDRE and C/EBP sites, sequence analysis indicated putative PU.1 binding sites in the CAMP promoter (Gombart et al., 2005). PU.1 is a key transcription factor that regulates myeloid and B cell lineage development and has been reported to regulate critical components of the innate immune system (Singh, 2008). PU.1 and C/EBP family members have been reported to cooperate in the regulation of many genes involved in immunity and hematopoiesis including granulocyte macrophage colony stimulating factor receptor and IL-1 β (Hohaus et al., 1995; Yang et al., 2000). Only more recently has PU.1 been described in non-hematopoietic cells including lung epithelial cells (Joshi et al., 2006; Rigo et al., 2012). Our findings provide evidence for a cooperative mechanism involving PU.1, VDR and C/EBP α for the regulation of CAMP gene expression in lung epithelial cells. Histone acetylation has also been reported to enhance 1,25(OH) $_2$ D $_3$ regulation of CAMP and the use of histone deacetylase inhibitors (HDACi) to enhance expression of CAMP has been suggested as a novel approach of strengthening innate immunity to treat bacterial infections (Steinmann et al., 2009). However, the underlying mechanisms responsible for the effects of HDACi to enhance CAMP expression remain unclear. In addition to covalent modification of histones, transcription is also facilitated by ATP dependent chromatin remodeling. Our findings suggest that one mechanism of enhancement of 1,25(OH) $_2$ D $_3$ regulation of CAMP by HDACi is enhanced cooperation between acetylation and chromatin remodeling through BRG-1, a component of the SWI/SNF chromatin remodeling complex which interacts with C/EBP α and is recruited by 1,25(OH) $_2$ D $_3$ to the C/EBP site in the CAMP promoter. BRG-1 can be an activator or a repressor depending on differential recruitment of BRG1 associated factors (Trotter and Archer, 2008). We found that protein arginine methyltransferase 5 (PRMT5) a type II methyltransferase, which has been associated with transcriptional repression through repressive histone marks (Karkhanis et al., 2011), interacts with BRG1 and represses 1,25(OH) $_2$ D $_3$ induction of CAMP mRNA and transcription. These results collectively identify novel networks involved in vitamin D mediated regulation of cathelicidin in lung epithelial cells and suggest new approaches of targeted therapy to treat respiratory tract infection and to combat infection caused by multi-resistant bacteria.

MATERIALS AND METHODS

Materials

Polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad Laboratories, Inc. PRMT5 antiserum (C-20, sc-22132), BRG-1 antiserum (H88, sc-10768), PU.1 antiserum (T-21, sc-352) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-acetyl histone H4 antibody (06-866) was purchased from Millipore. Anti-Histone H4 (symmetric dimethyl R3; H4(me2s)R3) antibody (ab5823) was obtained from Abcam. Anti-goat (sc-2020), anti-mouse (sc-2005) and anti-rabbit (sc-2004) HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology. 1,25(OH) $_2$ D $_3$ was purchased from Cayman Chemical Company (Ann Arbor, Michigan). The luciferase assay system (E1501) was purchased from Promega (Madison, WI). RiboZol RNA extraction reagent was purchased from Amresco (Solon, OH). Prestained protein markers were obtained from Bio-Rad. The

specific small molecule inhibitor of PRMT5, CMP5, was developed and characterized as previously described (Alinari et al., 2015).

Cell Culture

Cell culture medium DMEM was purchased from Mediatech, Inc. (a Corning subsidiary, Manassas, VA). BEGM^{TM} bronchial epithelial cell growth medium was from Lonza Group (Walkersville, MD). Fetal bovine serum (FBS) and charcoal-stripped fetal bovine serum (CS-FBS) were from Gemini Biological Products (Calabasas, CA). Penicillin, streptomycin and neomycin mixture (PSN) was purchased from ThermoFisher Scientific, Inc. Beas-2B human bronchial epithelial cells and A549 adenocarcinomic human alveolar basal epithelial cells were obtained from American Type Culture Collection (Manassas, VA). Normal human bronchial epithelial (NHBE) cells were obtained from Lonza, Inc. and from the UNC CF Center Tissue Procurement and Cell Culture Core (Dr. Scott Randell, director). Beas-2B cells and A549 cells were cultured in DMEM medium supplemented with 10% FBS and 1% PSN. NHBE cells were cultured in BEGM^{TM} (bronchial epithelial cell growth medium) supplemented with 1% PSN. HEK293 cells (a PU.1, C/EBP α and VDR negative cell line) were cultured in DMEM supplemented with 5% FBS and 1% PSN. Cells were grown in a humidified atmosphere of 95% air, 5% CO₂ at 37°C. Cells were seeded at 60-70% confluence prior to treatment. Cells were treated with vehicle or the compounds noted, at the concentrations and time points indicated, in medium supplemented with 2% CS-FBS.

Plasmids, Transfections and Assay of Luciferase Activity

Human CAMP promoter (−693 to +17) luciferase reporter construct (pXP2-CAMP-Luc) was generated as previously described (from Drs. Gombart and Koeffler; Gombart et al., 2005). The PU.1 site at −195/−185 was mutated by site directed mutagenesis using QuikChange II site-directed mutagenesis kit from Agilent (Santa Clara, CA). The oligonucleotides used to generate the mutated PU.1 site (PU.1 site is shown in bold, the mutated nucleotides are underlined) were as follows: 5'–

TCCTTGTGTGCAAGATG**ACCAAGTGGTTTCTTCATCTCTGA**-3' and 5'-
TCAGAGATGAAGAA**CCACTTGGT**TCATCTTGCACACAAGGA-3'.

pAV-hVDR was from J. Wesley Pike (University of Wisconsin, Madison). C/EBP α expression vector was a gift from Simon Williams, Texas Tech University, Lubbock, Tx. The BRG1-DN expression vector (pBJ5-BRG-DN) was obtained from James DiRenzo (Geisel School of Medicine at Dartmouth, Hanover, NH) and Myles Brown (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA). PRMT5 expression vector (pBabe/Fl-PRMT5) was from Said Sif (Qatar University, Doha, Qatar). PU.1 and PU.1-DN (lacking transactivation domain amino acids from 33 to 74) were kindly provided by Pavan Reddy (University of Michigan Comprehensive Cancer Center, Department of Internal Medicine). Cells were seeded in a 24-well culture plate 24 hours prior transfection at 50-60% confluence. Cells in each well were transfected using BioT reagent purchased from Morganville Scientific, (Morganville, NJ) or Lipofectamine 2000 (ThermoFisher Scientific, Inc.) according to the user manuals. Empty vectors were used to keep the total concentration of DNA the same. NHBE cells were not used for studies examining transcriptional regulation due to difficulty of transfection of primary cells (transfection efficiency was not

consistently high). Treatment using vehicle or the compounds indicated in the Results section was performed 16 h post-transfection in media supplemented with 2% CS-FBS for 24 h. After cells were harvested using 1× passive lysis buffer from Promega (Madison, WI), luciferase activity of cell lysate was measured according to the standard protocol of Promega luciferase assay system, normalized to total protein and expressed as fold induction compared to basal.

qRT-PCR Analysis and Western Blotting

Total RNA from was isolated from cells grown in 5 cm plates using RiboZol extraction reagent. Cells were treated with 1,25(OH)₂D₃ and/or transfected with the indicated expression vectors using Bio T reagent or Lipfectamine 2000. A 10 fold greater concentration of expression vectors was used for studies related to RNA analysis compared to luciferase assay due to the use of 5 cm plates for preparation of RNA compared to 24 well plates for luciferase assay. 2µg of total RNA was reverse transcribed using Superscript III Reverse Transcriptase (ThermoFisher Scientific, Inc.). Relative quantification of target gene expression was performed using real time quantitative PCR (Taqman analyses). Taqman Gene Expression Probes (Applied Biosystems) used for qRT-PCR are the following: CAMP (Hs00189038_m1), GAPDH (Hs04420697_g1). For each sample, qPCR reactions were performed in triplicate and normalized to GAPDH. The 2^{-Ct} method was used to calculate relative gene expression. For Western blot, cell lysate was prepared using a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1.0% NP-40 and protease inhibitors. The protein concentration was measured using the RC DC Protein Assay (detergent compatible; Bio-rad, Hercules, CA). Denatured protein was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes for Western blot analysis using an enhanced chemiluminescent detection system (Denville Scientific). GAPDH immunoblotting was used as a loading control.

Co-Immunoprecipitation Assay

Cells were lysed by five freeze-thaw cycles. 1mg of protein extract was used for immunoprecipitation with the addition of 4 µg BRG1 or PRMT5 antibody and incubation overnight at 4°C. 30µl protein A-Sepharose 4 Fast Flow beads (Rockland Immunochemical Inc., Gilbertsville, PA) was added to each sample followed by incubation by rotating at 4°C for 24h. Immunoprecipitates were collected by centrifugation and the complex was separated by 7.5% SDS-PAGE and analyzed by Western blot using BRG-1 or PRMT 5 antibody.

Chromatin Immunoprecipitation (ChIP) assay

ChIP was performed to Beas-2B or NHBE cells as previously described (Dhawan et al., 2015). Qiagen QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) was used to purify DNA fragments that were subjected to qPCR analysis. Primers were designed to amplify fragments in human CAMP promoter containing the C/EBP site (at -627/-619) (forward, 5' - GTT ACC CAG GCT GGA GTG C - 3'; reverse, 5' - ACG GTC TGC ACG CCT ATA AT - 3') and the PU.1 binding site (at -195/-185) (forward, 5'-GCC ACC GTG CCC TGC CTC ATT CAT CAA TTC-3'; reverse, GGG TGT GGG CTG GGG TTT GCT TTA-3'). PCR analysis was performed in the linear range of DNA amplification. 2% agarose

gel with ethidium bromide staining is used to visualize PCR products. 5% DNA acquired prior to immunoprecipitation was used as input for PCR evaluation. In re-chromatin immunoprecipitation (re-ChIP) experiments, two sequential chromatin immunoprecipitation with different antibodies were performed. On the second day of re-ChIP experiments after pull-down with first antibody, complexes were eluted by incubation in 60 μ l of elution buffer containing 10 mM dithiothreitol at 37°C for 30 min. The eluted samples were further diluted 50 times with ChIP dilution buffer and subjected to the ChIP procedure with specific second antibody.

Statistical Analysis

Results are expressed as mean $\pm$ SE. Student's t test or ANOVA were used to determine significance of two-group comparison or multiple-group comparison, respectively.

RESULTS

Functional cooperation between PU.1, C/EBP α and VDR in the regulation of CAMP

Although CAMP, which plays a multifunctional role in host defense, is induced by 1,25(OH) $_2$ D $_3$ in lung epithelial cells (Fig. 1A, Yim et al., 2007), little is known about the mechanisms that regulate CAMP. In order to address a possible crosstalk between PU.1 and C/EBP α in the 1,25(OH) $_2$ D $_3$ regulation of CAMP transcription, initial studies were done in HEK293 cells that lack endogenous PU.1, VDR and C/EBP α . 1,25(OH) $_2$ D $_3$ (10 nM) or C/EBP α stimulated CAMP transcription 1.5 – 3 fold and C/EBP α combined with 1,25(OH) $_2$ D $_3$ treatment resulted in a significant enhancement of CAMP transcription compared to 1,25(OH) $_2$ D $_3$ alone (Fig. 1B). PU.1 (6.25 ng) alone did not affect basal promoter activity and did not enhance 1,25(OH) $_2$ D $_3$ induced CAMP transcription (Fig. 1B). Similar results were observed using increasing concentrations of PU.1 (12.5 – 50 ng; not shown). However cotransfection of C/EBP α and PU.1 resulted in a significant enhancement of CAMP transcription compared to C/EBP α alone which was further enhanced by 1,25(OH) $_2$ D $_3$ (Fig. 1B), suggesting functional cooperation between VDR, C/EBP α and PU.1 in the regulation of CAMP. In Beas-2B human bronchial epithelial cells that contain PU.1 endogenously (as well as VDR and C/EBP α), the induction by 1,25(OH) $_2$ D $_3$ of CAMP transcription as well as the enhancement by C/EBP of the inductive action of 1,25(OH) $_2$ D $_3$ was inhibited by PU.1 DN (which blocks PU.1 mediated transcriptional activation; (Sun et al., 2013) (Fig. 1C), further indicating cooperation between VDR, PU.1 and C/EBP α in the regulation of CAMP. In addition qRT-PCR analysis indicated that CAMP gene expression was induced by 1,25(OH) $_2$ D $_3$, C/EBP α , the combination of C/EBP α and 1,25(OH) $_2$ D $_3$ as well as by the combination of C/EBP α and PU.1 or C/EBP α , PU.1 and 1,25(OH) $_2$ D $_3$ in NHBE cells (Fig. 1D). qRT PCR was also performed to assess the effect of PU.1 DN on endogenous 1,25(OH) $_2$ D $_3$ or C/EBP α induced CAMP mRNA. Expression of PU.1 DN resulted in a significant decrease in 1,25(OH) $_2$ D $_3$ or C/EBP α induction of CAMP mRNA. (Fig. 1E; upper and lower panel respectively). PU.1 expression in Beas-2B cells is noted in Fig. 1F and, similar to our findings in NHBE cells (Rigo et al. 2012), is unaffected by 1,25(OH) $_2$ D $_3$ treatment. HEK cells were used as a negative control.

Identification of a PU.1 binding site in the CAMP promoter

By sequence analysis a putative PU.1 site was identified in the CAMP promoter between -195/-185. Mutation of this site resulted in a loss of the enhancement of C/EBP α induced CAMP transcription in the presence of PU.1 and a marked decrease in the cooperative regulation of CAMP transcription by 1,25(OH) $_2$ D $_3$, PU.1 and C/EBP α (Fig. 2 A). The enhanced CAMP transcription observed in the presence of PU.1, C/EBP α + D treatment using the mutated promoter may be due to cooperative effects between VDR and C/EBP α at the intact VDR and C/EBP sites. Binding of PU.1 to the putative PU.1 site was assessed by ChIP assay using primers to amplify fragments of the CAMP promoter containing the PU.1 site (-195/-185). Compared to vehicle control, Beas-2B cells and NHBE cells treated with 1,25(OH) $_2$ D $_3$ showed enhanced PU.1 recruitment to that site, indicating the involvement of endogenous PU.1 in 1,25(OH) $_2$ D $_3$ induced CAMP transcription in lung epithelial cells (Fig. 2 B, C).

BRG1, a component of the SWI/SNF chromatin remodeling complex, VDR, and C/EBP α cooperate in the regulation of CAMP transcription

In addition to binding of transcription factors to cis acting regulatory elements, epigenetic modulation also plays an important role in directing gene expression. We previously demonstrated that BRG1 [a component of the SWI/SNF complex which uses the energy of ATP hydrolysis to alter histone DNA interaction; (Bultman et al., 2000; King et al., 2012)], via association with C/EBP, plays a fundamental role in the induction by 1,25(OH) $_2$ D $_3$ of CYP24A1 transcription (Seth-Vollenweider et al., 2014). We thus examined a possible role of BRG1 in 1,25(OH) $_2$ D $_3$ and C/EBP α regulation of CAMP transcription. In the presence of BRG1 DN (K798R; which has a mutation in the ATPase domain and functions as a DN) there is a dose dependent inhibition of the stimulating effect of 1,25(OH) $_2$ D $_3$ and 1,25(OH) $_2$ D $_3$ + C/EBP α on CAMP transcription (Fig. 3A). Brm is another component of the SWI/SNF complex with ATPase activity (Bultman et al., 2000; King et al., 2012). Brm-DN also inhibits 1,25(OH) $_2$ D $_3$ and 1,25(OH) $_2$ D $_3$ + C/EBP α induced CAMP transcription (Dhawan et al., 2015). However, we found that CAMP transcription is more sensitive to inhibition by BRG1 DN (by approximately two fold; data not shown). Expression of CAMP mRNA induced by 1,25(OH) $_2$ D $_3$ or C/EBP α was also suppressed by BRG1 DN (Fig. 3B; upper and lower panel respectively). These findings suggest a role for BRG1 in the cooperative regulation of CAMP by VDR and C/EBP α .

It has been reported that the remodeling activity of SWI/SNF can act cooperatively with histone acetylation to regulate transcription (DiRenzo et al., 2000; Hassan et al., 2001; Narlikar et al., 2002). Although previous studies showed that histone deacetylase inhibitors (HDACi) can induce CAMP expression (Steinmann et al., 2009), the molecular mechanisms involved in the effect of HDACi on the expression of CAMP are not fully understood. We next examined possible cooperativity between chromatin remodeling and histone acetylation in the regulation of CAMP. Treatment of Beas-2B cells with the HDACi butyrate results in a dose dependent induction of CAMP mRNA (Fig. 4 A). Enhancement by butyrate of VDR mRNA was not observed (data not shown). Butyrate alone also induced CAMP transcription in Beas 2B cells and this induction was further enhanced by 1,25(OH) $_2$ D $_3$ treatment (Fig. 4B). There was no effect of butyrate or butyrate + 1,25(OH) $_2$ D $_3$ on empty vector (pXP2;

Fig. 4B). Treatment with butyrate [or TSA (trichostatinA), not shown] relieved the inhibition by BRG1 DN of 1,25(OH)₂D₃ and 1,25(OH)₂D₃ + C/EBPα induction of CAMP transcription (Fig. 4 C). The BRG1 DN mediated suppression of 1,25(OH)₂D₃ induction of CAMP mRNA was partially relieved by butyrate treatment (Fig. 4D, upper panel). Increasing concentrations of butyrate relieved the inhibition by BRG1 DN of C/EBPα induction of CAMP mRNA and resulted in a significant enhancement of CAMP mRNA expression (Fig. 4 D, lower panel). Previous studies have shown that C/EBPα can interact with both Brm and BRG1 (Inayoshi et al., 2006) and that C/EBPα and Brm or BRG1, as indicated by ChIP/re-ChIP experiments, can bind simultaneously to the CAMP promoter in lung epithelial cells [(Dhawan et al., 2015); and data not shown]. ChIP assays were performed using NHBE cells (Fig. 4 E) or Beas-2B cells (not shown) and primers designed to amplify the fragments in the CAMP promoter containing the C/EBP site (627/–619). ChIP assays showed recruitment of BRG1 to the CAMP promoter in response to 1,25(OH)₂D₃. ChIP re-ChIP analysis also indicated an increase in acetylated histone 4 in association with BRG1 in response to 1,25(OH)₂D₃ at this site (Fig. 4 E). These findings indicate cooperativity between chromatin remodeling and histone acetylation and suggest that one mechanism of enhancement of 1,25(OH)₂D₃ regulation of CAMP by HDACi is enhanced cooperation between acetylation and chromatin remodeling (though BRG1).

PRMT5 Inhibits CAMP Transcription Induced by 1,25D₃ and C/EBPα

In addition to histone acetylation, histone methylation also plays an important role in transcriptional regulation. Protein arginine methyltransferase 5 (PRMT5) is a type II PRMT that targets H4R3 and H3R8 and has been reported to be involved in transcriptional repression (Karkhanis et al., 2011). Since PRMT5 has been shown to be associated with BRG1-based SWI/SNF complexes (Pal et al., 2004; Pal et al., 2003), we examined a possible role of PRMT5 in the cross talk between VDR, C/EBPα and BRG1 in the regulation of CAMP. Co-immunoprecipitation experiments in Beas-2B human bronchial epithelial cells indicated an endogenous association between PRMT5 and BRG1 (Fig. 5A). To determine whether CAMP is regulated by PRMT5, transcription assays and qRT-PCR analyses were done. We found that PRMT5 inhibited 1,25(OH)₂D₃ or C/EBPα + 1,25(OH)₂D₃ induction of CAMP transcription (Fig. 5B). PRMT5 also suppressed CAMP mRNA induced by 1,25(OH)₂D₃ or C/EBPα (Fig. 5C; upper and lower panel respectively). Inhibition of PRMT5 using a small molecule inhibitor specific for PRMT5, CMP5 (small molecule compound 5), that selectively blocks symmetrical dimethylation of H4R3 (H4R3Me2s) (Alinari et al., 2015), resulted in enhanced 1,25(OH)₂D₃ induction of CAMP mRNA (Fig. 5D), suggesting that PRMT5 mediated H4R3 methylation mediates, at least in part, repression of VDR mediated CAMP gene expression. Since BRG1 is recruited to the C/EBP site on the CAMP promoter and PRMT5 interacts with BRG1, ChIP assays were performed using Beas 2B cells and primers containing the C/EBP site on the CAMP promoter. In cells treated with 1,25(OH)₂D₃ after 6h there was increased symmetrical dimethylation of H4R3 (Fig. 5E). Enhanced PRMT5 binding after 1,25(OH)₂D₃ treatment of cells for 6h (but not at earlier times after treatment) was also observed (not shown). Western blot analysis showed an increase in H4R3Me2s at later times after 1,25(OH)₂D₃ treatment in Beas-2B cells (Fig. 5F), further suggesting that at later times [and not at early times when there is enhanced histone acetylation (Fig. 4E)] repressive epigenetic mechanisms are involved in the cyclical

transcriptional process. These results further indicate a role for epigenetic events mediated by PRMT5 in the modulation of $1,25(\text{OH})_2\text{D}_3$ regulation of CAMP.

A model for the induction of CAMP in lung epithelial cells is shown in Fig. 6. Induction of CAMP in lung epithelial cells involves cooperation among VDR, C/EBP α , PU.1, histone acetylation and chromatin remodeling. PRMT5 can repress induction of CAMP transcription at least in part via symmetrical dimethylation of H4R3 as described above.

DISCUSSION

In this study we define novel mechanisms and key mediators involved in the regulation by $1,25(\text{OH})_2\text{D}_3$ of CAMP in lung epithelial cells. Our results show for the first time that PU.1, the myeloid transcription factor, which has also been identified in lung epithelial cells, cooperates with VDR and C/EBP α to enhance CAMP transcription and mRNA expression in lung epithelial cells. Our findings also provide evidence indicating that one mechanism of enhancement of $1,25(\text{OH})_2\text{D}_3$ induction of CAMP by HDACi is enhanced cooperation between acetylation and chromatin remodeling (through BRG1) allowing for efficient initiation of transcription. PRMT5, a type II methyltransferase which interacts with BRG1, was found to repress CAMP transcription, at least in part, through symmetrical dimethylation of H4R3. Therefore, BRG1 may act as a scaffold that allows recruitment to the promoter of BRG1 interacting proteins in response to activating or repressing signals. Our findings identify key mediators and epigenetic events that regulate CAMP expression in lung epithelial cells and thus affect the regulation of the antibacterial capability of the airway.

Although PU.1 was thought to be restricted to hematopoietic cells, more recently PU.1 has been described in non-hematopoietic cells including lung epithelial cells (Harendza et al., 2000; Joshi et al., 2006; Rigo et al., 2012; Tissieres et al., 2009). We are only now beginning to understand the functional significance of PU.1 in non-myeloid cells. With regard to regulation of CAMP, in previous studies PU.1 knock down was shown to result in a reduction of $1,25(\text{OH})_2\text{D}_3$ mediated induction of CAMP in THP.1 cells (Seuter et al., 2017). Our findings related to CAMP regulation suggest that the PU.1 signaling pathway may be a key defense pathway which is not myeloid specific. Although functionality was not addressed, non-myeloid regulation of CAMP by PU.1 was also suggested in studies by Termen et al who noted PU.1 recruitment to the CAMP promoter by $1,25(\text{OH})_2\text{D}_3$ in colon epithelial cells (Termen et al., 2008). In addition to CAMP, PU.1 has been shown to control the expression of other genes involved in immune function in non-myeloid cells. Studies from the Guidot lab provided evidence for GM-CSF-PU.1 signaling in pulmonary epithelial cells and suggested that GM-CSF-PU.1 signaling is a critical regulator and a common defense pathway in both alveolar macrophages and epithelial cells (Joshi et al., 2006). Subsequent studies showed that PU.1 is a critical regulator of MD-2, a type II acute phase protein of innate immunity, in human hepatocytes as well as in myeloid cells (Tissieres et al., 2009). In addition, in glomerular mesangial cells PU.1 has been reported to repress gelatinase A, a matrix metalloproteinase which plays a key role in renal inflammatory processes (Harendza et al., 2000). These findings further suggest that the role of PU.1 as central regulator of immune function may not be myeloid specific.

We have demonstrated that PU.1 cooperates with C/EBP α and VDR to activate CAMP transcription and mRNA expression in lung epithelial cells. Combinatorial transcriptional activation by PU.1, C/EBP and VDR had not been previously demonstrated. It is well recognized that many myeloid genes including granulocyte macrophage colony stimulating factor (GM-CSF) receptor α , macrophage colony stimulating factor (M-CSF) receptor and neutrophil elastase (NE) utilize PU.1 and C/EBP to direct their expression (Hohaus et al., 1995; Oelgeschlager et al., 1996; Petrovick et al., 1998). Cooperation between PU.1 and C/EBP β was also noted in the differentiation associated induction of the macrophage-specific chitotriosidase (CHIT1) gene and in the induction of MD-2 production by IL-6 in hepatocytes as well as in myeloid cells (Pham et al., 2007; Tissieres et al., 2009). In addition, C/EBP β and PU.1 functionally cooperate to regulate the expression of IL-1 β , an inflammatory cytokine expressed by activated monocytes/macrophages (Yang et al., 2000). For regulation of IL-1 β , C/EBP β alone had a minimal effect, PU.1 alone induced transcription 20 fold and a synergistic effect was observed in the presence of both C/EBP β and PU.1 (Yang et al., 2000). In our studies, unlike the regulation of IL-1 β , C/EBP alone but not PU.1 alone can activate CAMP transcription. However, PU.1 enhanced transcription induced by C/EBP α alone or C/EBP α + 1,25(OH) $_2$ D $_3$. In monocytes, although PU.1 knock down was also shown to repress 1,25(OH) $_2$ D $_3$ induced CAMP mRNA, the authors suggested that PU.1 may act together with other transcription factors to affect VDR signaling (Seuter et al., 2017). Our findings suggest that C/EBP α is one factor that can act together with PU.1 to enhance VDR mediated regulation of CAMP. It is of interest that, in studies using genome-wide analysis to determine NF κ B target selection in THP.1 cells, PU.1 was shown not to be sufficient to establish an active chromatin mark (Jin et al., 2011). However, sites bound by both PU.1 and C/EBP α were most likely to be bound by p65 after TNF treatment, suggesting that C/EBP α may function with PU.1 to affect NF- κ B target gene selection (Jin et al., 2011). In the differentiation associated induction of CHIT1 by PU.1 and C/EBP β it was suggested that C/EBP β is responsible for the activation of the CHIT1 gene by facilitating remodeling through its capacity to recruit SWI/SNF, thus allowing PU.1 to target its sites (Pham et al., 2007). Further studies involving genome scale definition will be required to determine whether similar mechanisms are involved in the cooperative regulation of CAMP by PU.1, C/EBP α and VDR. It will also be of interest in future studies to determine whether cooperative activation between C/EBP α , PU.1 and VDR could be a general mechanism involved in the induction by 1,25(OH) $_2$ D $_3$ of innate immune responses in myeloid cells as well as in non-myeloid cells.

In addition to sequence specific transcription factors, chromatin modifying complexes also play an important role in the regulation of gene expression. We previously showed that BRG1, a component of the SWI/SNF chromatin remodeling complex plays a fundamental role via association with C/EBP, in the induction by 1,25(OH) $_2$ D $_3$ of CYP24A1 transcription in kidney and osteoblastic cells (Seth-Vollenweider et al., 2014). C/EBP dependent recruitment of BRG1 has also been shown to be important for the transcriptional activity of the osteocalcin gene in osteoblastic cells and for the regulation of CYP27b1, the gene which encodes 25-hydroxyvitamin D3 1 α -hydroxylase, the enzyme that catalyzes the conversion of 25(OH)D $_3$ to 1,25(OH) $_2$ D $_3$ (Villagra et al., 2006; Zhong et al., 2009). It has also been reported that C/EBP β and C/EBP α can form a complex with SWI/SNF and activate myeloid

and adipocyte specific genes (Kowenz-Leutz and Leutz, 1999; Pedersen et al., 2001). In addition, SWI/SNF regulates liver specific albumin gene transcription in cooperation with C/EBP α (Inayoshi et al., 2006). Our findings indicate that cooperation between SWI/SNF and C/EBP is also an important component for VDR mediated transcriptional activation of CAMP. In addition to C/EBPs, multiple mechanisms have been reported for the recruitment of SWI/SNF to target genes. With regard to steroid receptor mediated transcriptional activation, it has been suggested that interaction with ER α and GR, which relies on BRG1 associated factors (BAFs) or steroid receptor coactivators as a bridge to BRG1, is responsible for recruitment of SWI/SNF (Jeong et al., 2009; King et al., 2012). Whether additional mechanisms are involved in the functional relationship between the SWI/SNF complex and VDR mediated gene regulation remains to be determined.

Previous studies have suggested that combination therapy with HDACi and vitamin D could result in bolstering innate immune defense (Steinmann et al., 2009). However, the mechanisms involved have not been clearly defined. Our findings indicated an increase in acetylated histone 4 in association with BRG1 in response to 1,25(OH) $_2$ D $_3$ and that butyrate relieves the suppression by BRG1 DN of 1,25(OH) $_2$ D $_3$ and C/EBP α + 1,25(OH) $_2$ D $_3$ induction of transcription. These findings suggest that one mechanism of enhancement of 1,25(OH) $_2$ D $_3$ regulation of CAMP by HDACi, involves enhanced cooperation between acetylation and chromatin remodeling (through BRG1) allowing for efficient initiation of transcription. Histone acetylation and recruitment of SWI/SNF have previously been shown to cooperate in mediating changes in chromatin structure that lead to efficient transcriptional activation. (DiRenzo et al., 2000; Narlikar et al., 2002). The effect of butyrate on relief of the suppression of CAMP transcription may involve, in part, the histone acetyltransferase (HAT) p300 since p300 has been reported to act as a transcriptional coactivator for C/EBP α and VDR, additional VDR coactivators with HAT activity or inhibition of NCOR and SMRT, co-repressors of VDR/RXR mediated transactivation which recruit deacetylases (Erickson et al., 2001; Pike and Meyer, 2010; Sanchez-Martinez et al., 2008). Cell type specific mechanisms may also be involved in the regulation of CAMP expression by HDACi alone and in combination with 1,25(OH) $_2$ D $_3$. In keratinocytes siRNA silencing of the VDR coactivator SRC3 (steroid receptor coactivator 3) which has intrinsic histone acetyltransferase activity, resulted in inhibition of 1,25(OH) $_2$ D $_3$ induction of CAMP gene expression which could not be reversed by butyrate, indicating that SRC3 mediated VDR transcription is required before VDR mediated CAMP transcription can be enhanced by HDACi (Schauber et al., 2008). In lung epithelial cells other mechanisms that have been reported to be involved in the enhancement of CAMP by HDACi include MAP kinase signaling, VDR (siRNA silencing of VDR reduced butyrate induced CAMP expression) and histone acetylation resulting in enhanced AP1 binding (Kida et al., 2006; Kulkarni et al., 2015; Steinmann et al., 2009). Together these findings suggest that the control of CAMP expression by HDACi and 1,25(OH) $_2$ D $_3$ in lung epithelial cells can be influenced by transcriptional and post transcriptional mechanisms.

Phenylbutyrate has been reported to augment vitamin D mediated CAMP dependent M. tuberculosis growth restriction in human macrophages (Coussens et al., 2015). In addition, using cultured supernatants from the bronchial epithelial cell line VA10 co-treated with phenylbutyrate and 1,25(OH) $_2$ D $_3$, a reduction in bacterial growth of *P. aeruginosa*, a

common pathogen in patients with compromised host defense mechanisms, was observed (Kulkarni et al., 2015). Thus, our findings and those of others suggest the possibility that combination therapy with HDACi and vitamin D (or low dose 1,25(OH)₂D₃) could be effective for the treatment of bacterial infection, at least in part, by enhancing CAMP expression. Since CAMP regulation is cell type specific and since HDACi have diverse physiological functions (and thus treatment with HDACi may not always be clinically beneficial), novel drug design approaches that lead to development of cell type selective HDACi that can target individual biological functions are needed.

BRG1 can act as a repressor of gene expression as well as a transcriptional activator depending on its association with interacting proteins, which mediate different functions (Trotter and Archer, 2008). BRG1-associated PRMT5 was reported to be involved with repression of several tumor suppressor genes such as ST7, NM23, and retinoblastoma-like protein 2 (Pal et al., 2003; Wang et al., 2008). More recently, our lab found that PRMT5 is a negative regulator of 1,25(OH)₂D₃-induced *Cyp24a1* transcription in kidney and osteoblastic cells, and this inhibition is also associated with PRMT5 interaction with BRG1 (Seth-Vollenweider et al., 2014). In this study we found that PRMT5 is associated with BRG1 in lung epithelial cells and that PRMT5 can also negatively regulate 1,25(OH)₂D₃ induced CAMP expression. Inhibition of PRMT5 using a small molecule inhibitor specific for PRMT5, that selectively blocks symmetrical dimethylation of H4R3 (Alinari et al., 2015), resulted in an enhancement of 1,25(OH)₂D₃ induction of CAMP gene expression. Thus, our findings suggest that PRMT5 silencing of CAMP expression involves repressive histone marks including H4R3me2s. In addition to histone methylation it is also possible that PRMT5 mediated arginine methylation of non-histone proteins, which has previously been reported, may be involved in epigenetic repression of CAMP (Wei et al., 2014; Stopa et al., 2015). Further studies are needed to determine additional mechanisms involved in repression of 1,25(OH)₂D₃ target genes by PRMT5. Although the role of corepressors such as NCoR and SMART in the control of steroid receptor transcriptional activity has been extensively investigated, the role of repressors in controlling VDR mediated transcription has been controversial. In the classical model, in the absence of ligand, corepressors bind to the nuclear receptor. In order to recruit coactivators, corepressors are released after ligand binding (Nagy et al., 1999). Studies by Sanchez-Martinez et al. showed that, unlike other nuclear receptors, there is very weak binding of SMRT and NCoR to unoccupied VDR (Sanchez-Martinez et al., 2008). However, SMRT and NCoR (as well as coactivators) are recruited to VDR/RXR upon 1,25(OH)₂D₃ binding (Sanchez-Martinez et al., 2008). siRNA silencing of SMRT and NCoR resulted in enrichment of acetylated histone H4 to the *Cyp24a1* promoter (Sanchez-Martinez et al., 2008). The authors suggested that ligand dependent target gene repression may be part of the cyclic transcriptional process and that corepressors are involved in deacetylation. A similar mechanism may be involved in the repression of VDR mediated transcription by PRMT5. In future studies it will be of interest to determine, using cell specific inhibitors, whether inhibition of PRMT5 will have a therapeutic role in enhancing 1,25(OH)₂D₃ mediated immune responses.

In summary, our findings provide new insight into cellular mechanisms and key mediators involved in the effects of 1,25(OH)₂D₃ on innate immunity in lung epithelial cells. It is indeed possible that the mechanisms and cofactors we have identified in the regulation of

CAMP may reflect more general mechanisms involved in innate host defense mediated by $1,25(\text{OH})_2\text{D}_3$ and that our findings will suggest potential candidates for development of modulators of innate immune responses for adjunct therapies in the treatment of bacterial infection. A future challenge will involve the examination of long range genomic interactions of the key mediators that have been identified and how functional relationships are altered after bacterial infection in the presence or absence of $1,25(\text{OH})_2\text{D}_3$.

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References

- Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jornvall H, Wigzell H, Gudmundsson GH. 2000 The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* 96(9):3086–3093. [PubMed: 11049988]
- Alinari L, Mahasenan KV, Yan F, Karkhanis V, Chung JH, Smith EM, Quinion C, Smith PL, Kim L, Patton JT, Lapalombella R, Yu B, Wu Y, Roy S, De Leo A, Pileri S, Agostinelli C, Ayers L, Bradner JE, Chen-Kiang S, Elemento O, Motiwala T, Majumder S, Byrd JC, Jacob S, Sif S, Li C, Baiocchi RA. 2015 Selective inhibition of protein arginine methyltransferase 5 blocks initiation and maintenance of B-cell transformation. *Blood* 125(16):2530–2543. [PubMed: 25742700]
- Arranz-Trullen J, Lu L, Pulido D, Bhakta S, Boix E. 2017 Host Antimicrobial Peptides: The Promise of New Treatment Strategies against Tuberculosis. *Front Immunol* 8:1499. [PubMed: 29163551]
- Bals R, Wang X, Zasloff M, Wilson JM. 1998 The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc Natl Acad Sci U S A* 95(16):9541–9546. [PubMed: 9689116]
- Beaumont PE, McHugh B, Gwyer Findlay E, Mackellar A, Mackenzie KJ, Gallo RL, Govan JR, Simpson AJ, Davidson DJ. 2014 Cathelicidin host defence peptide augments clearance of pulmonary *Pseudomonas aeruginosa* infection by its influence on neutrophil function in vivo. *PLoS One* 9(6):e99029. [PubMed: 24887410]
- Bultman S, Gebuhr T, Yee D, La Mantia C, Nicholson J, Gilliam A, Randazzo F, Metzger D, Chambon P, Crabtree G, Magnuson T. 2000 A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. *Mol Cell* 6(6):1287–1295. [PubMed: 11163203]
- Cederlund A, Gudmundsson GH, Agerberth B. 2011 Antimicrobial peptides important in innate immunity. *FEBS J* 278(20):3942–3951. [PubMed: 21848912]
- Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. 2016 Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev* 96(1):365–408. [PubMed: 26681795]
- Coussens AK, Wilkinson RJ, Martineau AR. 2015 Phenylbutyrate Is Bacteriostatic against *Mycobacterium tuberculosis* and Regulates the Macrophage Response to Infection, Synergistically with 25-Hydroxy-Vitamin D3. *PLoS Pathog* 11(7):e1005007. [PubMed: 26133770]
- Cowland JB, Johnsen AH, Borregaard N. 1995 hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. *FEBS Lett* 368(1):173–176. [PubMed: 7615076]
- Dhawan P, Wei R, Sun C, Gombart AF, Koeffler HP, Diamond G, Christakos S. 2015 C/EBPalpha and the Vitamin D Receptor Cooperate in the Regulation of Cathelicidin in Lung Epithelial Cells. *J Cell Physiol* 230(2):464–472. [PubMed: 25078430]
- Diamond G, Zasloff M, Eck H, Brasseur M, Maloy WL, Bevins CL. 1991 Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. *Proc Natl Acad Sci U S A* 88(9):3952–3956. [PubMed: 2023943]

- DiRenzo J, Shang Y, Phelan M, Sif S, Myers M, Kingston R, Brown M. 2000 BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation. *Mol Cell Biol* 20(20):7541–7549. [PubMed: 11003650]
- Dorschner RA, Pestonjamasp VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, Agerberth B, Gudmundsson GH, Gallo RL. 2001 Cutaneous injury induces the release of cathelicidin antimicrobial peptides active against group A *Streptococcus*. *J Invest Dermatol* 117(1):91–97. [PubMed: 11442754]
- Erickson RL, Hemati N, Ross SE, MacDougald OA. 2001 p300 coactivates the adipogenic transcription factor CCAAT/enhancer-binding protein alpha. *J Biol Chem* 276(19):16348–16355. [PubMed: 11340085]
- Gombart AF, Borregaard N, Koeffler HP. 2005 Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J* 19(9):1067–1077. [PubMed: 15985530]
- Hansdottir S, Monick MM, Hinde SL, Lovan N, Look DC, Hunninghake GW. 2008 Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *J Immunol* 181 (10): 7090–7099 [PubMed: 18981129]
- Harendza S, Lovett DH, Stahl RA. 2000 The hematopoietic transcription factor PU.1 represses gelatinase A transcription in glomerular mesangial cells. *J Biol Chem* 275(26):19552–19559. [PubMed: 10867017]
- Hassan AH, Neely KE, Workman JL. 2001 Histone acetyltransferase complexes stabilize swi/snf binding to promoter nucleosomes. *Cell* 104(6):817–827. [PubMed: 11290320]
- Hohaus S, Petrovick MS, Voso MT, Sun Z, Zhang DE, Tenen DG. 1995 PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Mol Cell Biol* 15(10):5830–5845. [PubMed: 7565736]
- Inayoshi Y, Miyake K, Machida Y, Kaneoka H, Terajima M, Dohda T, Takahashi M, Iijima S. 2006 Mammalian chromatin remodeling complex SWI/SNF is essential for enhanced expression of the albumin gene during liver development. *J Biochem* 139(2):177–188. [PubMed: 16452305]
- Jeong KW, Lee YH, Stallcup MR. 2009 Recruitment of the SWI/SNF chromatin remodeling complex to steroid hormone-regulated promoters by nuclear receptor coactivator flightless-I. *J Biol Chem* 284(43):29298–29309. [PubMed: 19720835]
- Jin F, Li Y, Ren B, Natarajan R. 2011 PU.1 and C/EBP(alpha) synergistically program distinct response to NF-kappaB activation through establishing monocyte specific enhancers. *Proc Natl Acad Sci U S A* 108(13):5290–5295. [PubMed: 21402921]
- Joshi PC, Applewhite L, Mitchell PO, Fernainy K, Roman J, Eaton DC, Guidot DM. 2006 GM-CSF receptor expression and signaling is decreased in lungs of ethanol-fed rats. *Am J Physiol Lung Cell Mol Physiol* 291(6):L1150–L1158. [PubMed: 16877635]
- Karkhanis V, Hu YJ, Baiocchi RA, Imbalzano AN, Sif S. 2011 Versatility of PRMT5-induced methylation in growth control and development. *Trends Biochem Sci* 36(12):633–641. [PubMed: 21975038]
- Kida Y, Shimizu T, Kuwano K. 2006 Sodium butyrate up-regulates cathelicidin gene expression via activator protein-1 and histone acetylation at the promoter region in a human lung epithelial cell line, EBC-1. *Mol Immunol* 43(12):1972–1981. [PubMed: 16423398]
- King HA, Trotter KW, Archer TK. 2012 Chromatin remodeling during glucocorticoid receptor regulated transactivation. *Biochim Biophys Acta* 1819(7):716–726. [PubMed: 22425674]
- Kowenz-Leutz E, Leutz A. 1999 A C/EBP beta isoform recruits the SWI/SNF complex to activate myeloid genes. *Mol Cell* 4(5):735–743. [PubMed: 10619021]
- Kulkarni NN, Yi Z, Huehnken C, Agerberth B, Gudmundsson GH. 2015 Phenylbutyrate induces cathelicidin expression via the vitamin D receptor: Linkage to inflammatory and growth factor cytokines pathways. *Mol Immunol* 63(2):530–539. [PubMed: 25458314]
- Nagy L, Kao HY, Love JD, Li C, Banayo E, Gooch JT, Krishna V, Chatterjee K, Evans RM, Schwabe JW. 1999 Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes Dev* 13(24):3209–3216. [PubMed: 10617570]
- Narlikar GJ, Fan HY, Kingston RE. 2002 Cooperation between complexes that regulate chromatin structure and transcription. *Cell* 108(4):475–487. [PubMed: 11909519]

- Oelgeschlager M, Nuchprayoon I, Luscher B, Friedman AD. 1996 C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. *Mol Cell Biol* 16(9):4717–4725. [PubMed: 8756629]
- Pal S, Vishwanath SN, Erdjument-Bromage H, Tempst P, Sif S. 2004 Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. *Mol Cell Biol* 24(21):9630–9645. [PubMed: 15485929]
- Pal S, Yun R, Datta A, Lacomis L, Erdjument-Bromage H, Kumar J, Tempst P, Sif S. 2003 mSin3A/histone deacetylase 2- and PRMT5-containing Brg1 complex is involved in transcriptional repression of the Myc target gene cad. *Mol Cell Biol* 23(21):7475–7487. [PubMed: 14559996]
- Pan Z, Hetherington CJ, Zhang DE. 1999 CCAAT/enhancer-binding protein activates the CD14 promoter and mediates transforming growth factor beta signaling in monocyte development. *J Biol Chem* 274(33):23242–23248. [PubMed: 10438498]
- Pedersen TA, Kowenz-Leutz E, Leutz A, Nerlov C. 2001 Cooperation between C/EBPalpha TBP/TFIIB and SWI/SNF recruiting domains is required for adipocyte differentiation. *Genes Dev* 15(23):3208–3216. [PubMed: 11731483]
- Petrovick MS, Hiebert SW, Friedman AD, Hetherington CJ, Tenen DG, Zhang DE. 1998 Multiple functional domains of AML1: PU.1 and C/EBPalpha synergize with different regions of AML1. *Mol Cell Biol* 18(7):3915–3925. [PubMed: 9632776]
- Pham TH, Langmann S, Schwarzfischer L, El Chartouni C, Lichtinger M, Klug M, Krause SW, Rehli M. 2007 CCAAT enhancer-binding protein beta regulates constitutive gene expression during late stages of monocyte to macrophage differentiation. *J Biol Chem* 282(30):21924–21933. [PubMed: 17540774]
- Pike JW, Meyer MB. 2010 The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3). *Endocrinol Metab Clin North Am* 39(2):255–269, table of contents. [PubMed: 20511050]
- Rigo I, McMahon L, Dhawan P, Christakos S, Yim S, Ryan LK, Diamond G. 2012 Induction of triggering receptor expressed on myeloid cells (TREM-1) in airway epithelial cells by 1,25(OH)(2) vitamin D(3). *Innate Immun* 18(2):250–257. [PubMed: 21690199]
- Sanchez-Martinez R, Zambrano A, Castillo AI, Aranda A. 2008 Vitamin D-dependent recruitment of corepressors to vitamin D/retinoid X receptor heterodimers. *Mol Cell Biol* 28(11):3817–3829. [PubMed: 18362166]
- Seth-Vollenweider T, Joshi S, Dhawan P, Sif S, Christakos S. 2014 Novel mechanism of negative regulation of 1,25-dihydroxyvitamin D3-induced 25-hydroxyvitamin D3 24-hydroxylase (Cyp24a1) Transcription: epigenetic modification involving cross-talk between protein-arginine methyltransferase 5 and the SWI/SNF complex. *J Biol Chem* 289(49):33958–33970. [PubMed: 25324546]
- Seuter S, Neme A, Carlberg C. 2017 Epigenomic PU.1-VDR crosstalk modulates vitamin D signaling. *Biochim Biophys Acta* 1860(4):405–415.
- Singh H 2008 PU.1, a shared transcriptional regulator of innate and adaptive immune cell fates. *J Immunol* 181(3):1595–1596. [PubMed: 18641293]
- Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, Borregaard N. 2001 Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* 97(12):3951–3959. [PubMed: 11389039]
- Steinmann J, Halldorsson S, Agerberth B, Gudmundsson GH. 2009 Phenylbutyrate induces antimicrobial peptide expression. *Antimicrob Agents Chemother* 53(12):5127–5133. [PubMed: 19770273]
- Stopa N, Krebs JE, Shechter D. 2015 The PRMT5 arginine methyltransferase: many roles in development, cancer and beyond. *Cell Mol Life Sci* 72(11):2041–2059. [PubMed: 25662273]
- Sun Y, Sun J, Tomomi T, Nieves E, Mathewson N, Tamaki H, Evers R, Reddy P. 2013 PU.1-dependent transcriptional regulation of miR-142 contributes to its hematopoietic cell-specific expression and modulation of IL-6. *J Immunol* 190(8):4005–4013. [PubMed: 23509362]
- Teclé T, Tripathi S, Hartshorn KL. 2010 Review: Defensins and cathelicidins in lung immunity. *Innate Immun* 16(3):151–159. [PubMed: 20418263]
- Termen S, Tollin M, Rodriguez E, Sveinsdottir SH, Johannesson B, Cederlund A, Sjövall J, Agerberth B, Gudmundsson GH. 2008 PU.1 and bacterial metabolites regulate the human gene CAMP

- encoding antimicrobial peptide LL-37 in colon epithelial cells. *Mol Immunol* 45(15):3947–3955. [PubMed: 18657865]
- Tissieres P, Araud T, Ochoda A, Drifte G, Dunn-Siegrist I, Pugin J. 2009 Cooperation between PU.1 and CAAT/enhancer-binding protein beta is necessary to induce the expression of the MD-2 gene. *J Biol Chem* 284(39):26261–26272. [PubMed: 19632992]
- Tollin M, Bergman P, Svenberg T, Jornvall H, Gudmundsson GH, Agerberth B. 2003 Antimicrobial peptides in the first line defence of human colon mucosa. *Peptides* 24(4):523–530. [PubMed: 12860195]
- Trotter KW, Archer TK. 2008 The BRG1 transcriptional coregulator. *Nucl Recept Signal* 6:e004. [PubMed: 18301784]
- Vandamme D, Landuyt B, Luyten W, Schoofs L. 2012 A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cell Immunol* 280(1):22–35. [PubMed: 23246832]
- Villagra A, Cruzat F, Carvallo L, Paredes R, Olate J, van Wijnen AJ, Stein GS, Lian JB, Stein JL, Imbalzano AN, Montecino M. 2006 Chromatin remodeling and transcriptional activity of the bone-specific osteocalcin gene require CCAAT/enhancer-binding protein beta-dependent recruitment of SWI/SNF activity. *J Biol Chem* 281(32):22695–22706. [PubMed: 16772287]
- Wang L, Pal S, Sif S. 2008 Protein arginine methyltransferase 5 suppresses the transcription of the RB family of tumor suppressors in leukemia and lymphoma cells. *Mol Cell Biol* 28(20):6262–6277. [PubMed: 18694959]
- Weber G, Heilborn JD, Chamorro Jimenez CI, Hammarsjo A, Torma H, Stahle M. 2005 Vitamin D induces the antimicrobial protein hCAP18 in human skin. *J Invest Dermatol* 124(5):1080–1082. [PubMed: 15854055]
- Wei H, Mundade R, Lange KC, Lu T. 2014 Protein arginine methylation of non-histone proteins and its role in diseases. *Cell Cycle* 13(1):32–41. [PubMed: 24296620]
- Wei R, Christakos S. 2015 Mechanisms Underlying the Regulation of Innate and Adaptive Immunity by Vitamin D. *Nutrients* 7(10):8251–8260. [PubMed: 26404359]
- Yang Z, Wara-Aswapati N, Chen C, Tsukada J, Auron PE. 2000 NF-IL6 (C/EBPbeta) vigorously activates il1b gene expression via a Spi-1 (PU.1) protein-protein tether. *J Biol Chem* 275(28):21272–21277. [PubMed: 10801783]
- Yim S, Dhawan P, Ragunath C, Christakos S, Diamond G. 2007 Induction of cathelicidin in normal and CF bronchial epithelial cells by 1,25-dihydroxyvitamin D(3). *J Cyst Fibros* 6(6):403–410. [PubMed: 17467345]
- Zhang DE, Hetherington CJ, Tan S, Dziennis SE, Gonzalez DA, Chen HM, Tenen DG. 1994 Sp1 is a critical factor for the monocytic specific expression of human CD14. *J Biol Chem* 269(15):11425–11434. [PubMed: 7512565]
- Zhong Y, Armbrrecht HJ, Christakos S. 2009 Calcitonin, a regulator of the 25-hydroxyvitamin D3 1alpha-hydroxylase gene. *J Biol Chem* 284(17):11059–11069. [PubMed: 19261615]

Fig. 1.

Functional cooperation between PU.1, C/EBPα and VDR in the regulation of CAMP transcription **A**. qRT-PCR was performed using total RNA from NHBE cells treated with vehicle (basal; open bar) or 1,25(OH)₂D₃ for 24h. Results are reported a relative expression level and represent the mean ± SE of 3 separate experiments. * p < 0.05 compared to basal. **B**. PU.1 cooperates with C/EBPα and VDR in the regulation of CAMP transcription. Top panel CAMP promoter schematic showing the location of the C/EBP site (–627/–619), the VDRE (–615/–600) and the putative PU.1 site (–195 – 185). HEK cells, a PU.1, VDR and C/EBPα negative cell line, were transfected with the CAMP promoter (250 ng), VDR (50 ng) and CEBPα (100 ng) or PU.1 (6.25 ng) or VDR in the presence of PU.1 and/or C/EBPα expression plasmids. Transfected cells were treated with vehicle or 1,25(OH)₂D₃ (10 nM)

for 24h and luciferase activity was determined. The results represent the mean $\pm$ SE; n = 6-8 separate experiments. * p < 0.05 compared to basal. + p < 0.05 compared to C/EBP α alone. The induction of CAMP promoter activity by a combination of 1,25(OH) $_2$ D $_3$, C/EBP α and PU.1 is significantly greater than CAMP promoter activity in response to C/EBP α +D or C/EBP α + PU.1 (p < 0.01). **C.** Beas-2B cells were transfected with 250 ng CAMP promoter in the presence or absence of C/EBP α and/ or PU.1 DN expression vector. After 24h cells were treated with vehicle or 1,25(OH) $_2$ D $_3$ (10 nM) for 24h. CAMP promoter activity is expressed as fold induction by comparison to basal levels (open bar) (mean $\pm$ SE, n = 4-6 separate experiments) * p < 0.05 compared to 1,25(OH) $_2$ D $_3$ treated, + or compared to C/EBP α +D. Similar results were observed using A549 adenocarcinomic human alveolar basal epithelial cells (not shown). Note there was no effect of 0.1 μ g PU.1 DN on basal levels of CAMP transcription (open bar, PU.1 DN). **D.** qRT-PCR analysis of CAMP mRNA was performed using total RNA from NHBE cells. Cells were transfected using BioT reagent with empty vector, or C/EBP α , PU.1 or C/EBP α and PU.1. 24h post transfection cells were treated with vehicle or 1,25(OH) $_2$ D $_3$ for 24h. Results are reported as relative expression levels. Maximum expression is C/EBP α , PU.1 +D (maximum level =1) to which other samples were compared. *Significantly different from basal (p < 0.05); + Significantly different from C/EBP α alone; Significantly different from C/EBP α +D and C/EBP α + PU.1. n = 3 separate experiments. **E.** qRT-PCR analysis of CAMP mRNA was performed using total RNA from Beas-2B cells. Upper panel: Cells were transfected with empty vector or PU.1 DN. Cells were treated with vehicle (basal) or 1,25(OH) $_2$ D $_3$ (10 nM) in the absence or presence (last two lanes) of PU.1 DN. Results represent the mean $\pm$ SE of 3 separate experiments *p < 0.05 compared to 1,25(OH) $_2$ D $_3$ treatment in the absence of PU.1 DN. Lower panel: Cells were transfected with empty vector or C/EBP α (100ng) in the absence or presence (last two lanes) of PU.1 DN. Results represent the mean $\pm$ SE of 3 separate experiments. *p < 0.05 compared to C/EBP α alone. **F.** Representative Western blot of PU.1 in Beas-2B cells. Cells were treated with vehicle (C) or 1,25(OH) $_2$ D $_3$ (10 nM) for 24h. HEK cells were included as a negative control. Similar results were observed in two additional experiments.

Fig. 2.

Mutation of the PU.1 binding site (–195/–185) in the CAMP promoter inhibits the observed functional cooperation in the regulation of CAMP transcription. **A.** Upper panel: Schematic of luciferase constructs indicating the mutation of the PU.1 site. HEK cells were transfected with the CAMP promoter (250 ng), VDR (50 ng) in the absence or presence of C/EBPα (100ng) and PU.1 (6.25 ng) expression plasmids. Transfected cells were treated with vehicle (basal) or 1,25(OH)₂D₃ (10 nM) for 24h and luciferase activity was determined. The results represent the mean ± SE of 3–4 separate experiments. *p < 0.05 compared to WT promoter construct transfected with VDR, C/EBPα and PU.1; + p < 0.01 compared to WT promoter construct transfected with VDR, C/EBPα and PU.1 and 1,25(OH)₂D₃ treated. **B.** ChIP analysis indicates that PU.1 is recruited to the CAMP promoter by 1,25(OH)₂D₃ in lung epithelial cells. Beas-2B cells were treated with vehicle (basal) or 1,25(OH)₂D₃ (10 nM). Cells were cross linked and cell lysates were subjected to immunoprecipitation with PU.1 antibody. DNA was isolated and PCR, using primers designed against the PU.1 site, was

carried out in the linear range of DNA amplification. IgG was used as a control. Lower panel: graphic representation of the ChIP results. **C.** 1,25(OH)₂D₃ was similarly found to recruit PU.1 to the CAMP promoter using NHBE cells.

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Fig. 3.

Cooperation between BRG1, 1,25(OH)₂D₃ and C/EBPα in the regulation of CAMP. **A.** Beas-2B cells were transfected with the CAMP promoter luciferase construct (-693/+17) (250 ng) alone or with C/EBPα expression vector (100ng) in the absence or presence of increasing concentrations of dominant negative (DN) Brahm-related gene 1 (BRG1). Cells were treated with vehicle (basal, open bar) or 1,25(OH)₂D₃ (10 nM). BRG1 DN resulted in a significant decrease in the induction of CAMP transcription by 1,25(OH)₂D₃ (10 nM) or CEBPα (100 ng) + 1,25(OH)₂D₃. Results represent the mean ± SE of 3–4 separate experiments. *p<0.05 compared to cells treated with 1,25(OH)₂D₃ or transfected with C/EBPα and treated with 1,25(OH)₂D₃. Similar results were observed using A549 cells (not shown). **B.** qRT-PCR analysis of CAMP mRNA was performed using total RNA from Beas-2B cells. Upper panel: Cells were transfected with empty vector or BRG DN expression vector. 24h post transfection cells were treated with vehicle (basal) or with 1,25(OH)₂D₃ (10 nM) for another 24h. Results represent the mean ± SE of 3 separate experiments. *p < 0.05 BRG DN transfected 1,25(OH)₂D₃ treated compared to 1,25(OH)₂D₃ treated. Lower panel: Cells were transfected with vector or C/EBPα (100 ng) in the presence or absence of BRG DN. BRG DN resulted in a significant decrease in induction of CAMP mRNA by C/EBPα (mean ± SE of 3 separate experiments). * p < 0.05 BRG DN transfected compared to cells transfected with C/EBPα in the absence of BRG DN.

Fig. 4.

Treatment with HDACi butyrate relieves the inhibition of CAMP by BRG-DN. **A.** Beas-2B cells were treated with vehicle (basal) or increasing concentrations of HDACi butyrate and CAMP mRNA was measured using qRT-PCR. Results are reported as relative expression level (maximum expression level = 1 to which other samples were compared) and represent the mean $\pm$ SE of 3 separate experiments (* $p < 0.05$ compared to basal). **B.** Butyrate augments the effect of 1,25(OH)₂D₃ on CAMP transcription. Beas-2B cells were transfected with 250 ng CAMP promoter and treated with 1,25(OH)₂D₃ (10 nM), butyrate (2 mM) or

butyrate + 1,25(OH)₂D₃ and luciferase activity was determined. Results represent the mean ± SE of 3 separate experiments. * p < 0.05 compared to basal. + p < 0.05 compared to cells treated with 1,25(OH)₂D₃ alone or butyrate alone. pXP2; vector alone. Similar results were observed using A549 cells and A549 cells or Beas-2B cells treated with increasing concentrations of trichostatin A (an additional HDACi; 25 – 100 nM) (not shown). **C.** Butyrate relieves the inhibition by BRG DN of 1,25(OH)₂D₃ or C/EBPα + 1,25(OH)₂D₃ induced CAMP transcription. Beas-2B cells were transfected with the CAMP promoter luciferase construct (–693/+17; 250 ng) alone or with C/EBPα expression vector (100 ng) in the absence or presence of DN BRG1 (0.1 ug). Cells were treated with 1,25(OH)₂D₃ (10 nM) or 1,25(OH)₂D₃ + Butyrate (0.5, 1 or 2 mM). * p < 0.05 compared to 1,25(OH)₂D₃ treated in the presence of BRG DN without butyrate treatment (4C left panel) or compared to C/EBPα +D in the presence of BRG DN without butyrate treatment (4C right panel). **D.** qRT PCR analysis of CAMP mRNA was performed using total RNA from Beas-2B cells. Upper panel: Cells were transfected with empty vector or BRG DN (last 4 lanes). Cells were treated with vehicle (basal), 1,25(OH)₂D₃ (10 nM) or 1,25(OH)₂D₃ and increasing concentrations of butyrate (0.5 – 2 mM). Results represent the mean ± SE of 3 separate experiments. * p < 0.05 compared D + BRG DN in the in the absence of butyrate. Lower panel: Cells were transfected with empty vector, C/EBPα (100 ng), or C/EBPα +BRG DN (last 4 lanes). Cells were treated with vehicle or butyrate (0.5 – 2mM; last 3 lanes). Results represent the mean ± SE of 3 separate experiments. * p < 0.05 compared to C/EBPα and BRG DN transfected in the absence of butyrate. + p < 0.05 compared to C/EBPα transfected in the absence of butyrate. **E.** ChIP analysis shows that 1,25(OH)₂D₃ recruits BRG1 to the CAMP promoter which is associated with an increase in acetylated histone 4. NHBE cells were treated with vehicle or 1,25(OH)₂D₃ for 1h and cross linked by 1% formaldehyde for 15 min. Cross linked cell lysates were subjected to immunoprecipitation with BRG1 antibody (α-BRG1) or with antibody against acetylated histone 4 (α-AcH4). DNA was isolated and PCR using specific primers designed to amplify fragments in the CAMP promoter containing the C/EBP site (at –627/–619) was carried out in the linear range of DNA amplification. IgG was used as a control. Right panel: graphic representation of ChIP assay results.

Fig. 5.

PRMT5 represses CAMP mRNA and transcription. **A.** PRMT5 and BRG1 are components of the same complex in lung epithelial cells. Beas-2B cell lysates were used for immunoprecipitation with BRG1 antibody, PRMT5 antibody or control IgG antibody. Similar results were observed in 3 independent experiments. **B.** Beas-2B cells were transfected with the CAMP promoter luciferase construct (-693/+17; 250 ng) alone or with C/EBP α expression vector (100 ng) in the absence or presence of increasing concentrations of PRMT5. PRMT5 resulted in a significant decrease in the induction of CAMP

transcription by $1,25(\text{OH})_2\text{D}_3$ or C/EBP α + $1,25(\text{OH})_2\text{D}_3$. Results represent the mean $\pm$ SE of 3-4 separate experiments. * $p < 0.05$ compared to cells treated with $1,25(\text{OH})_2\text{D}_3$ or transfected with C/EBP α and treated with $1,25(\text{OH})_2\text{D}_3$ in the absence of PRMT5. Similar results were observed using A549 cells (not shown). **C.** qRT-PCR analysis of CAMP mRNA was performed using total RNA from Beas-2B cells. Upper panel: Cells were transfected with empty vector or PRMT expression vector. 24h post transfections cells were treated with vehicle (basal) or with $1,25(\text{OH})_2\text{D}_3$ (10 nM) for another 24h. Results represent the mean $\pm$ SE of 3 separate experiments. * $p < 0.05$ PRMT5 transfected $1,25(\text{OH})_2\text{D}_3$ treated compared to $1,25(\text{OH})_2\text{D}_3$ treated. Lower panel: Cells were transfected with vector or C/EBP α (100 ng) in the presence or absence of PRMT5. PRMT5 resulted in a significant decrease in induction of CAMP mRNA by C/EBP α (mean $\pm$ SE of 3 separate experiments). * $p < 0.05$ PRMT5 transfected compared to cells transfected with C/EBP α in the absence of PRMT5. **D.** PRMT5 inhibitor CMP5 enhances $1,25(\text{OH})_2\text{D}_3$ induction of CAMP mRNA. qRT-PCR analysis of CAMP mRNA in NHBE cells treated with $1,25(\text{OH})_2\text{D}_3$ (10 nM) or $1,25(\text{OH})_2\text{D}_3$ + CMP5 (40 μM) for 24h. * $p < 0.05$ compared to $1,25(\text{OH})_2\text{D}_3$ (+D) treated NHBE cells. Data represent the mean $\pm$ SE of 3 independent experiments. **E.** ChIP analysis of dimethylation of H4R3 at the CAMP promoter in response to $1,25(\text{OH})_2\text{D}_3$. Beas-2B cells were treated with vehicle or $1,25(\text{OH})_2\text{D}_3$ for 2 or 6h and cross linked by 1% formaldehyde for 15 min. Cross linked cell lysates were subjected to immunoprecipitation with H4R3Me2 antibody. DNA was isolated and PCR using specific primers designed to amplify fragments of the CAMP promoter containing the C/EBP site (at -627/-619) was carried out in the linear range of DNA amplification. IgG was used as a control. **F.** Time dependent modulation of H4R3Me2 (H4R3 is a histone substrate of PRMT5) by $1,25(\text{OH})_2\text{D}_3$ in Beas-2B cells. Cells were treated with $1,25(\text{OH})_2\text{D}_3$ (10 nM) and protein lysates were collected at specific times. Representative Western blot. Lower panel: Graphic representation of Western blot analysis. 6-24h after $1,25(\text{OH})_2\text{D}_3$ treatment there was a significant increase in H4R3Me2s ($p < 0.05$ compared to basal).

Fig. 6.

Mechanistic model depicting the regulation of the induction of CAMP in lung epithelial cells. Induction of CAMP transcription involved cooperation among VDR, C/EBPα, PU.1 and chromatin remodeling. 1,25(OH)₂D₃ induces C/EBPα in lung epithelial cells (Dhawan et al., 2015). 1,25(OH)₂D₃ or C/EBPα induce CAMP transcription. PU.1 alone does not enhance CAMP transcription. However, PU.1 together with C/EBPα results in an enhancement of CAMP transcription and together PU.1 and C/EBPα further enhance 1,25(OH)₂D₃/VDR regulation of CAMP. BRG1, which interacts with C/EBP and is recruited to the C/EBP site by 1,25(OH)₂D₃, acts cooperatively with histone acetylation to enhance CAMP transcription (cooperation between chromatin remodeling through BRG1 and histone acetylation in the enhancement of 1,25(OH)₂D₃ regulation of CAMP is shown in Fig. 4). PRMT5 negatively regulates 1,25(OH)₂D₃ induction of CAMP transcription at least in part via symmetrical dimethylation of H4R3 (see Fig. 5).