

## Vitamin D has wide regulatory effects on histone demethylase genes

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**Abbreviations:** EMT, epithelial-mesenchymal transition; HDM, histone demethylase; HMT, histone methyltransferase; H3K27, lysine 27 of histone H3; JmjC, Jumonji C; LPS, lipopolysaccharide; LSD, lysine-specific demethylase; miRNA, microRNA; PADI, peptidylarginine deiminase; shRNA, short-hairpin RNA; VDR, vitamin D receptor; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>.

## **Abstract**

Vitamin D from the diet or synthesized in the skin upon UV-B irradiation is converted in the organism into the active metabolite  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> ( $1,25(\text{OH})_2\text{D}_3$ , calcitriol), a pleiotropic hormone with wide regulatory actions. The classical model of  $1,25(\text{OH})_2\text{D}_3$  action implies the activation of the vitamin D receptor, which binds specific DNA sequences in its target genes and modulates their transcription rate. We have recently shown that  $1,25(\text{OH})_2\text{D}_3$  induces the expression of the *JMJD3* gene coding for a histone demethylase that is involved in epigenetic regulation. JMJD3 mediates the effects of  $1,25(\text{OH})_2\text{D}_3$  on a subset of target genes and affects the expression of *ZEB1*, *ZEB2* and *SNAIL*, inducers of epithelial-mesenchymal transition. Novel data indicate that  $1,25(\text{OH})_2\text{D}_3$  has an unanticipated wide regulatory action on the expression of genes coding for histone demethylases of the Jumonji C (JmjC) domain and lysine-specific demethylase (LSD) families. Moreover, *JMJD3* knockdown decreases the expression of *miR-200b* and *miR-200c*, two microRNAs targeting *ZEB1* RNA. This may explain the upregulation of this transcription factor found in JMJD3-depleted cells. Thus,  $1,25(\text{OH})_2\text{D}_3$  exerts an ample regulatory effect on the expression of histone modifying enzymes involved in epigenetic regulation that may mediate its actions on gene transcription and cell phenotype.

## **Vitamin D regulates gene expression**

1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>, calcitriol) is the most active vitamin D metabolite in the organism and a pleiotropic hormone with ample regulatory actions.<sup>1</sup> For decades, lack of adequate vitamin D levels has been linked to impaired intestinal calcium absorption and bone formation (rickets in children, osteomalacia in adults). However, during the past decades, 1,25(OH)<sub>2</sub>D<sub>3</sub> is receiving an increasing interest as epidemiological studies suggest that vitamin D deficiency is associated with a higher risk of developing colon cancer and perhaps other neoplasias, and autoimmune and infectious diseases such as multiple sclerosis and tuberculosis.<sup>2-4</sup> At the cellular level, 1,25(OH)<sub>2</sub>D<sub>3</sub> modulates proliferation, differentiation, survival, metabolism and physiology, and potentiates the antitumoral activity of certain chemotherapeutic agents, in a cell-type- and -context-dependent manner.<sup>5-10</sup>

According to the classical model of action, 1,25(OH)<sub>2</sub>D<sub>3</sub> binds to and activates a transcription factor of the nuclear receptor superfamily, the vitamin D receptor (VDR), and modulates the transcription rate of its target genes.<sup>11</sup> 1,25(OH)<sub>2</sub>D<sub>3</sub> is a major regulator of gene expression in higher organisms as VDR is expressed in at least 38 human cell types.<sup>12</sup> In line with this, genome-wide studies using chromatin immunoprecipitation coupled with ultra high-throughput sequencing (ChIP-Seq) have revealed a high number and variety of VDR binding sites in genes associated to many different cell functions and physiological and pathological conditions.<sup>13-16</sup>

This mechanism of action has progressively become more comprehensive and complex with the finding that VDR is partially located outside the cell nucleus where it mediates the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the opening of ion channels and in the activity of enzymes such as kinases, phosphatases, and phospholipases.<sup>17</sup> Remarkably, at least part of these extranuclear signaling pathways converges and contributes to the control of gene transcription by VDR.<sup>18,19</sup> Moreover, 1,25(OH)<sub>2</sub>D<sub>3</sub> regulates genes that seem to lack VDR

binding sites,<sup>15</sup> and exerts post-translational gene regulatory effects by controlling the expression of multiple proteases and protease inhibitors.<sup>20</sup> In addition, in certain systems VDR has been found to interact with other transcription regulators such as  $\beta$ -catenin and FoxO factors having ligand-dependent or -independent effects.<sup>21,22</sup>

### **Histone demethylation**

The key role of epigenetic regulation, defined as chromatin changes affecting nucleosomal histones or DNA methylation, on transcription activation and repression is well recognized. The tails of the four core histones exposed on the nucleosome surface are subjected to a variety of enzyme-catalyzed post-translational modifications including acetylation, methylation, ubiquitylation, ADP-ribosylation, phosphorylation and sumoylation, which may occur singly or in combination.<sup>23,24</sup> Histone methylation is more complex than any other post-translational histone modification. It can occur on either lysines or arginines residues, and the consequence of its methylation can be either positive or negative towards transcriptional expression depending on the type of residue and on its position within the histone. Further complexity results from the fact that there can be multiple methylated states on each residue, as lysines can be mono- (me1), di- (me2) or tri- (me3) methylated, and arginines can be mono- or dimethylated.<sup>25,26</sup> The methylation state can lead to different functional consequences, as effector proteins might recognize one modification and have little affinity for other on the same residue.<sup>27</sup> Histone methylation is a reversible process mediated by two antagonizing groups of enzymes: histone methyltransferases (HMT) and histone demethylases (HDM).<sup>27-29</sup> Misregulation of histone modifications may lead to deregulation of gene expression and perturbation of cellular identity, which may contribute to cancer initiation, progression and/or metastasis.<sup>30,31</sup>

There are three distinct classes of enzymes capable of removing methylation marks. The first class includes peptidylarginine deiminase (PADI) enzymes that convert methylated residues of arginine to citrulline through a demethylimination reaction. Besides their clear role antagonizing methylarginine modifications, PADI enzymatic activity does not result in the production of an unmodified arginine, and for that fail to meet the requirements of a true HDM.<sup>27,32</sup> Lysine specific demethylase (LSD)-1 was the founding member of the second class of enzymes and the first true demethylase to be described.<sup>33</sup> The demethylation reaction catalyzed by LSDs limits these enzymes to use only mono- and dimethylated lysine residues as substrates.<sup>27,34</sup> The third and largest group of HDM enzymes contains a Jumonji C (JmjC) domain and catalyzes site-specific demethylation of mono-, di- and trimethylated lysines and, in the case of one particular member (JMJD6), also the site-specific demethylation of mono- and dimethylated arginines.<sup>28,35,36</sup> There are 30 proteins with a JmjC domain annotated in the human genome, although some of them are unlikely to be involved in histone demethylation.<sup>28</sup>

### **Vitamin D modulates the expression of histone demethylase genes**

Recently, we have reported that 1,25(OH)<sub>2</sub>D<sub>3</sub> regulates the RNA expression of the genes encoding for the JmjC domain-containing enzymes JMJD3 and JMJD4 in SW480-ADH human colon cancer cells. The RNA expression of a few other members of this group of enzymes such as PHF2, UTX, UTY, KDM5C/2A/3A, JMJD1C and MINA was unaffected by 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment.<sup>37</sup> As there was no literature for JMJD4, we focused our studies on JMJD3. This enzyme, also known as KDM6B, was the first specific demethylase of the trimethylated lysine 27 of histone H3 (H3K27me3) described.<sup>38-41</sup> Together with another JmjC domain-containing enzyme, UTX, they are the only demethylases capable to eliminate the H3K27me3 mark.<sup>31,38-40</sup> The presence of H3K27me3 at transcriptional sites usually

correlates with gene repression and the deregulation of this histone mark has been associated with cancer.<sup>42-44</sup>

Notably, we found that JMJD3 is induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> and partially mediates the transcriptional upregulation of a subset of 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes including *CYP24A1*, the tumor invasion suppressor *CDHI*/E-cadherin and the putative tumor suppressor *CST5*/cystatin D.<sup>37</sup> Accordingly, JMJD3 depletion blocks the acquisition of an epithelial phenotype, the inhibition of cell proliferation and the antagonism of the Wnt/β-catenin signaling promoted by 1,25(OH)<sub>2</sub>D<sub>3</sub> in colon cancer cells.<sup>37</sup> In addition, JMJD3 seems to have a role in the process of epithelial-mesenchymal transition (EMT), that is a crucial early step in metastasis,<sup>45,46</sup> as JMJD3 depletion upregulates the expression of the EMT inducers SNAIL1, ZEB1 and ZEB2 and the mesenchymal markers fibronectin-1 and LEF1, while it downregulates the epithelial proteins claudin-1 and claudin-7.<sup>37</sup> Data obtained with samples from colon cancer patients indicate that *JMJD3* RNA was downregulated in a 56% of patients correlating with low *VDR* and high *SNAIL1* RNA expression.<sup>37</sup> Altogether, our data indicate that *JMJD3* is a 1,25(OH)<sub>2</sub>D<sub>3</sub> target gene that, in turn, partially mediates the antitumoral effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on human colon cancer cells.

Now, we have extended the study of 1,25(OH)<sub>2</sub>D<sub>3</sub> action to the LSDs and to all genes known to encode JmjC domain-containing enzymes. Quantitative RT-PCR analyses show that 1,25(OH)<sub>2</sub>D<sub>3</sub> induces the expression of *HSPBAP1*, *JARID2* and *KDM5B*, while it downregulates that of *KDM4A/4C/4D/5A/2B*, *HIF1AN*, *JMJD5/6*, *HR* and *PLA2G4B* in SW480-ADH cells (Figure 1). In contrast, *KDM4B/3B/5D*, *JMJD8*, *PHF8* and *NO66* are not affected by 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment (Figure 1). Additionally, the expression of *LSD1* and *LSD2*, the two members of the LSD family, is also induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> (Figure 1). De Santa et al.<sup>41</sup> analyzed the expression of 27 JmjC domain-containing proteins in mouse macrophages and found that only two, *JMJD3* and *JARID2*, that are induced by 1,25(OH)<sub>2</sub>D<sub>3</sub>

in SW480-ADH cells, were also upregulated upon bacterial lipopolysaccharide (LPS) stimulation. Several reports have shown that JMJD3 is regulated upon environmental stimuli (bacterial products, viruses, parasites and toxins),<sup>41,47-50</sup> suggesting that it may bridge extracellular signals with gene expression regulation. Moreover, *JMJD3* is also induced by retinoic acid, the ligand of another member of the nuclear receptor superfamily, in neural stem cells.<sup>51</sup>

Several findings indicate that 1,25(OH)<sub>2</sub>D<sub>3</sub>-regulated JmjC domain-containing proteins are interesting candidates for future studies. JARID2 is a novel subunit of the Polycomb repressive complex 2 that is essential for its binding to target genes and required for the recruitment of Polycomb repressive complex 1 in mouse embryonic stem cells.<sup>52-55</sup> HR functions as a VDR corepressor in the skin that interacts with histone deacetylases and regulates Wnt signaling.<sup>56-59</sup> JMJD5 is required for breast cancer cell proliferation, while JMJD6 overexpression enhanced angiogenic sprouting in endothelial cells.<sup>60,61</sup> There is scarce literature for several of these genes as the research on the biological role of this class of epigenetic modulators is still in its infancy. Overall, our results indicate a plausible combinatorial action of JMJD3 demethylase and other JmjC domain-containing enzymes on the fine-tuning of the gene regulatory activity of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

Given the key role played by ZEB1 in the induction of EMT in several cell systems,<sup>62,63</sup> we have investigated the mechanism of its regulation by JMJD3 in SW480-ADH cells. *ZEB1* RNA levels were higher in cells expressing a short-hairpin RNA (shRNA) against *JMJD3* (shJMJD3 cells) than in those expressing a scrambled shRNA (shControl cells) in both the absence and presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Figure 2A). Consistently, shJMJD3 cells had higher content of ZEB1 protein than shControl cells (Figure 2B). ZEB1 expression is strongly downregulated by microRNAs (miRNAs) of the *miR-200* family.<sup>64,65</sup> To explore whether these miRNAs could mediate the effect of JMJD3 on ZEB1 expression, we measured the

levels of *miR-200b* and *miR-200c* in shJMJD3 and shControl cells. Supporting this hypothesis, lower expression of both *miR-200b* and *miR-200c* was found in shJMJD3 cells compared to shControl cells (Figure 2C). As a negative control, the expression of *miR-128*, a miRNA unrelated to EMT, was unaffected by JMJD3 depletion (Figure 2C).

The regulatory effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the expression of JMJD3 suggests changes in H3K27 methylation. However, the analysis of the cellular levels of global H3K27me<sub>3</sub> did not reveal alterations upon 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment (Figure 3A) or following expression of a mutant JMJD3 (Figure 3B) or by *JMJD3* knockdown (Figure 3C). These results agree with those obtained for *Jmjd3* in mouse macrophages and for JMJD6 in human endothelial cells. Thus, De Santa et al.<sup>41</sup> demonstrated that although neither *Jmjd3* induction nor *Jmjd3* depletion caused any detectable modification in the global levels of H3K27me<sub>3</sub>, *Jmjd3* induction by LPS coincides with H3K27me<sub>3</sub> downregulation and with *Jmjd3* occupancy at the *Bmp2* gene promoter. Similarly, Boeckel et al.<sup>61</sup> did not observe changes in global histone methylation patterns in JMJD6-deficient cells, but they did not rule out the possibility that histone modifications might occur at specific promoters. Altogether, these results suggest that effects on H3K27 methylation must be analyzed at the single gene level. Furthermore, it must be considered that the level of H3K27 methylation also depends on the enzymes that methylate this residue: the HMTs of the Polycomb group.<sup>66,67</sup>

JMJD3 and, possibly, others HDMs may control 1,25(OH)<sub>2</sub>D<sub>3</sub> target gene expression and its antitumoral action by histone demethylation-dependent and/or -independent mechanisms (Figure 4). First, JMJD3 may contribute to a local chromatin environment that facilitates the expression of 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes *via* H3K27 demethylation. This has been previously demonstrated for the induction of the *Bmp2* gene by LPS in mouse macrophages.<sup>41</sup> Therefore, JMJD3 may be recruited to the regulatory region of 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes, such as *CYP24A1*, *CDH1*/E-cadherin and *CST5*/cystatin D, and execute



epigenetic changes that would facilitate gene transcription activation. In line with this, dynamic modification of H3K27me3 has recently been reported in the regulation of *CDKN1A/p21<sup>CIP1</sup>* by 1,25(OH)<sub>2</sub>D<sub>3</sub> in normal prostate cells.<sup>68</sup>

Supporting the second mechanism, a ChIP-Seq study conducted by Dr. Natoli's group suggested that the involvement of Jmjd3 in tuning transcriptional regulation by LPS in mouse macrophages is largely independent of its H3K27 demethylase activity.<sup>69</sup> Unexpectedly, Jmjd3 was found to be located preferentially at gene transcription start sites with upregulated H3K4me3, a histone mark associated with active genes or poised for activation. Importantly, only a minority of Jmjd3 peaks were associated with neighbouring H3K27me3 peaks, while Jmjd3 deletion resulted in defective recruitment of RNA polymerase II but not in altered H3K27me3 signals.<sup>69</sup> Moreover, the assumption that histones are the only substrate for HDMs may be incorrect, as it has been demonstrated that in human cancer cell lines LSD1 interacts with and dynamically regulates lysine methylation of the p53 protein, thus repressing p53-mediated transcriptional activation.<sup>70</sup> Therefore, the possibility that non-histone substrates and/or non-enzymatic activities of HDMs may mediate the biological actions of these enzymes should be considered.<sup>71</sup> It is possible that JMJD3, as well as other JmjC domain-containing enzymes, may engender demethylation of non-histone substrates and/or other actions than protein demethylation, and that these unknown functions could account for the observed effects in our studies.

Interestingly, we found that the overexpression of an inactive JMJD3 C-terminal region harbouring a point mutation in the iron-binding centre (MUT 1141-1641) in SW480-ADH cells has an inhibitory effect on the regulation of 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes similar to that observed in shJMJD3 cells.<sup>37</sup> Although these results would favor a demethylase-dependent mechanism, it is unknown whether the mutation generates allosteric changes in the JMJD3 protein that would circumvent and/or affect other putative unknown functions. In an

attempt to clarify this point and taking advantage of the available Jmjd3 ChIP-Seq data in mouse macrophages,<sup>69</sup> we analyzed in that system if Jmjd3 (upon induction by LPS) is recruited to *Cyp24a1* and *Cdh1* gene loci. These two genes are 1,25(OH)<sub>2</sub>D<sub>3</sub> targets whose induction is reduced by *JMJD3* knockdown or by the expression of JMJD3 MUT 1141-1641 in SW480-ADH cells.<sup>37</sup> We found that Jmjd3 binds to a region upstream (40,861) and to two regions upstream (41,835 and 39,560) of *Cyp24a1* and *Cdh1* transcription start sites, respectively (Figure 5). These results suggest that JMJD3 may execute epigenetic changes in the promoter of these genes to modulate gene expression. However, these data need further validation, as they were obtained in a totally different cell system and the number of tags in peaks is low.

Our results show that 1,25(OH)<sub>2</sub>D<sub>3</sub> exerts a complex regulatory effect on the RNA expression of a series de HDMs, some of which are induced while others are repressed. Furthermore, we have uncovered the epigenetic regulator *JMJD3* as a new 1,25(OH)<sub>2</sub>D<sub>3</sub> target gene that, in turn, integrates with the gene regulatory actions of this hormone. DNA methylation has been proposed as part of the mechanism of repression by 1,25(OH)<sub>2</sub>D<sub>3</sub> of certain target genes, such as *CYP27B1*.<sup>72</sup> Likewise, 1,25(OH)<sub>2</sub>D<sub>3</sub> induces the expression of *Gadd45α* that promotes epigenetic gene activation by DNA demethylation in *Xenopus laevis*.<sup>73</sup> However, our work demonstrates for the first time that a HDM modulates gene activation by this hormone.

We believe that the reciprocal regulation of JMJD3 and 1,25(OH)<sub>2</sub>D<sub>3</sub> may play a role during colorectal tumorigenesis, and potentially offer new therapeutic targets to delay tumor progression. Considering a classical epigenetic view, the regulation of JMJD3 by 1,25(OH)<sub>2</sub>D<sub>3</sub> may be a mechanism to guarantee the correct activation of genes through the loss of the repressive H3K27me3 mark. Having in mind the requirement of JMJD3 expression for an adequate 1,25(OH)<sub>2</sub>D<sub>3</sub> action, the deregulation in human colon cancer of *JMJD3*

(downregulation)<sup>37</sup> and of the Polycomb gene *EZH2* (overexpression)<sup>74-78</sup> may generate resistance to therapy with 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs.

## **Conclusions**

In summary, the new set of data showing the regulation by 1,25(OH)<sub>2</sub>D<sub>3</sub> of numerous HDMs may reflect a complex combinatorial modulation, *via* histone demethylation-dependent and/or -independent mechanisms, of the transcription of a wide range of target genes (Figure 4). HDMs controlled by 1,25(OH)<sub>2</sub>D<sub>3</sub> supposedly regulate the expression of many genes, of which only part might be 1,25(OH)<sub>2</sub>D<sub>3</sub> targets. This amplifies the regulatory action of this hormone and may contribute to explain the existence of a number of target genes that lack VDR binding sites. Additionally, data demonstrating the effect of JMJD3 depletion on the expression of the EMT inducer ZEB1, *via* the downregulation of *mi-R200b* and *miR-200c*, point out to a new mechanism by which 1,25(OH)<sub>2</sub>D<sub>3</sub> may inhibit colon cancer progression.

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## Figure legends

**Figure 1.** Effect of  $1,25(\text{OH})_2\text{D}_3$  on the expression of the genes encoding LSDs and nineteen JmjC domain-containing enzymes in SW480-ADH human colon cancer cells. qRT-PCR analysis of RNA levels in SW480-ADH cells treated with 100 nM  $1,25(\text{OH})_2\text{D}_3$  or vehicle for 4 or 48 h. qRT-PCR assays were performed as described in reference 37 and the sequences of the primers used are displayed in Supplementary Table 1. *SDHA* was used for normalization. Data are expressed as the fold-change over vehicle-treated cells. Mean  $\pm$  SEM ( $n = 3$ ). The statistical significance was assessed by the Student *t* test (\*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ ; n.s.,  $p > 0.05$ ).

**Figure 2.** *JMJD3* knockdown increases the expression ZEB1 and represses *miR-200b* and *miR-200c*. **(A)** qRT-PCR analysis of the RNA levels of *ZEB1* in SW480-ADH shControl and shJMJD3 cells treated with 100 nM  $1,25(\text{OH})_2\text{D}_3$  or vehicle for 8 or 48 h. qRT-PCR assays were performed as described in reference 37 and the sequences of the primers used are displayed in Supplementary Table 1. *SDHA* was used for normalization. Mean  $\pm$  SEM ( $n = 3$ ). **(B)** Western blot analysis of ZEB1 protein expression in SW480-ADH shControl and shJMJD3 cells treated with 100 nM  $1,25(\text{OH})_2\text{D}_3$  or vehicle for 8 or 48 h. Western blot assays were performed as indicated in reference 37 using antibodies against ZEB1 and lamin B (sc-10572 and sc-6216, Santa Cruz Biotechnology). Lamin B was used as a loading control. Graph shows the mean  $\pm$  SEM ( $n = 3$ ). **(C)** qRT-PCR analysis of the RNA levels of *miR-200b* and *miR-200c* in SW480-ADH shControl and shJMJD3 cells. RNA was extracted with the miRNeasy Mini Kit (217004, Qiagen) and reverse transcribed using the corresponding primer and the TaqMan MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems). The following TaqMan probes were used: *miR-200b* (002251), *miR-200c* (002300), *miR-128* (002216) and *RNU44* (001094) (Applied Biosystems). *RNU44* small nucleolar RNA was used

for normalization and *miR-128* as a negative control. Mean  $\pm$  SEM ( $n = 3$ ). **(A-C)** The generation of SW480-ADH shControl and shJMJD3 cells was previously described in reference 37. The statistical significance was assessed as indicated in the legend to Figure 1.

**Figure 3.** JMJD3 does not affect the global level of H3K27me3 in SW480-ADH cells. **(A)** Western blot analysis of H3K27me3 in SW480-ADH cells treated with the indicated concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> for 72 or 96 h. **(B)** Western blot analysis of H3K27me3 levels in Mock, WT 1141-1641 and MUT 1141-1641 SW480-ADH cells treated with 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> or vehicle for 48 h. The generation of Mock, WT 1141-1641 and MUT 1141-1641 SW480-ADH cells was previously described in reference 37. **(C)** Western blot analysis of H3K27me3 levels in SW480-ADH shControl and shJMJD3 cells treated with 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> or vehicle for 8 or 48 h. The generation of SW480-ADH shControl and shJMJD3 cells was previously described in reference 37. **(A-C)** Western blot assays were performed as indicated in reference 37 using antibodies against total H3 (ab1791, Abcam) and H3K27me3 (9733, Cell Signaling). Total histone H3 was used as a control. Graphs show the mean  $\pm$  SEM ( $n = 3$ ). The statistical significance was assessed as indicated in the legend to Figure 1. All comparisons were found not significant ( $p > 0.05$ ).

**Figure 4.** 1,25(OH)<sub>2</sub>D<sub>3</sub> regulates the expression of JMJD3 and other HDMs that, in turn, mediate the antitumoral effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in colon cancer. HDMs may modulate 1,25(OH)<sub>2</sub>D<sub>3</sub> action by demethylation-dependent and/or -independent effects on the expression of: (i) 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes, (ii) EMT inducers, and/or (iii) other unknown targets.

**Figure 5.** Jmjd3 binding to *Cyp24a1* (A) and *Cdh1* (B) gene loci in LPS-stimulated mouse macrophages. Jmjd3 ChIP-Seq peak data for mouse Chr 2 and 8 from reference 69 were converted to bedGraph format and overlaid on mouse genome assembly (mm8) as a custom track using the UCSC genome browser. Peak height represents the number of overlapping tags observed in that region and width of peak suggests the area covered by the tags.



**Tables**

**Supplementary Table 1.** Sequence of the primers used for qRT-PCR.

<b>Gene</b>	<b>Forward primer</b>	<b>Reverse primer</b>
<i>HIF1AN</i>	CCCCTTCTGCTTGTCTTCAG	GAAATGCCCTCAAAGTGCTC
<i>HR</i>	CACAGGACCAAGAGCATCAA	AGCAGTTCGCAGAGAGAAGG
<i>HSPBAP1</i>	TCAGAATGGGGATGAAAAGC	TGGTCACAGTTCCAGGTCAG
<i>JARID2</i>	AAGGAGGTGCTGATGGAGAA	AGAGGGTGGAACTTGTGGTG
<i>JMJD5</i>	ACATCAGCATCCCCGACTAC	TACTTCCTCCCCATCACCTG
<i>JMJD6</i>	CCAACTTCCCTGTGGTATGG	GGGTGCTCTTGCTTCAAAT
<i>JMJD8</i>	GCTTTGGAATCGCAGGAG	GCGTCTTCTCAGGTGGGTAA
<i>KDM2B</i>	AGTCACCAAGGAAGGCTGTG	AAAGGAAAGTGTCGGCTCAC
<i>KDM3B</i>	AACTTCCTCAAACCCCCTTG	TTGCTCTTCACTCCTGCTGA
<i>KDM4A</i>	GCAAGAAAAGAAACGGCAAC	ACCCTGGAAGCACCTACTCC
<i>KDM4B</i>	CGCTCTTCTACCCCTACTGC	GTCTTCTGACGGCTTTTGG
<i>KDM4C</i>	CACAAGCCAGATAGCAGCAA	CCCTCCGATGTAACGACTTC
<i>KDM4D</i>	TGAACGCTATGACCTGTGGA	GCTGCTCTCCTGGGTAAGT
<i>KDM5A</i>	TGAACGATGGGAAGAAAAGG	TGGGTAGAAAGGCTGGAATG
<i>KDM5B</i>	TGCTTTCGTCAGGGAATCTT	ATGATGTTGTGCCAGGAGGT
<i>KDM5D</i>	AAACATTGGCTCCCTGCTAC	GATGCTGTGGGGCTTGTATT
<i>LSD1</i>	GTGTGTGCCTGTGGCTTTAG	GACTCGTGGAGCGGGTATT
<i>LSD2</i>	CCTGGAAGCCAAAGACAGAA	CACGAGCGAGCAGATACTTG
<i>NO66</i>	CGAGGAACTGGCTCTGACAT	CCCGAGGAAAATACAGCAAA
<i>PHF8</i>	GGATTTGCCTGGATTTTGAA	AGGGTTATCTGCGTCATTGG
<i>PLA2G4B</i>	GCCTCCATTTCCACAAAGAC	AGGTAGCCAACATCCAGCAG
<i>SDHA</i>	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
<i>ZEB1</i>	GCCAATAAGCAAACGATTCTG	TTTGGCTGGATCACTTTCAAG

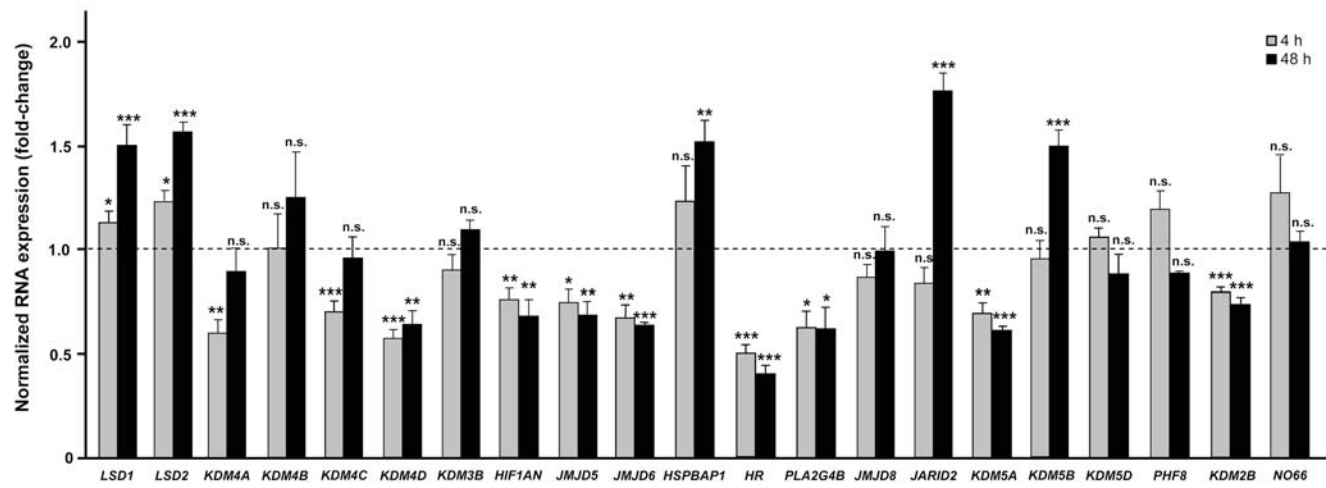
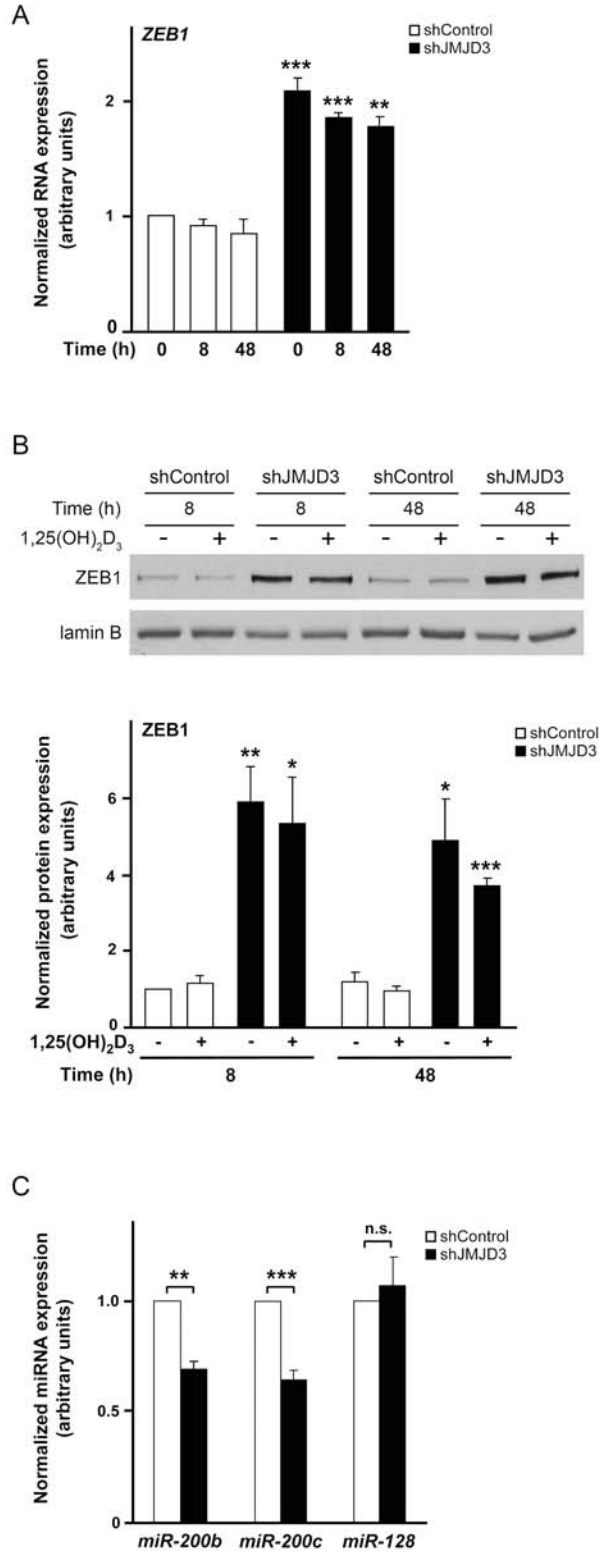


Figure 1



**Figure 2**

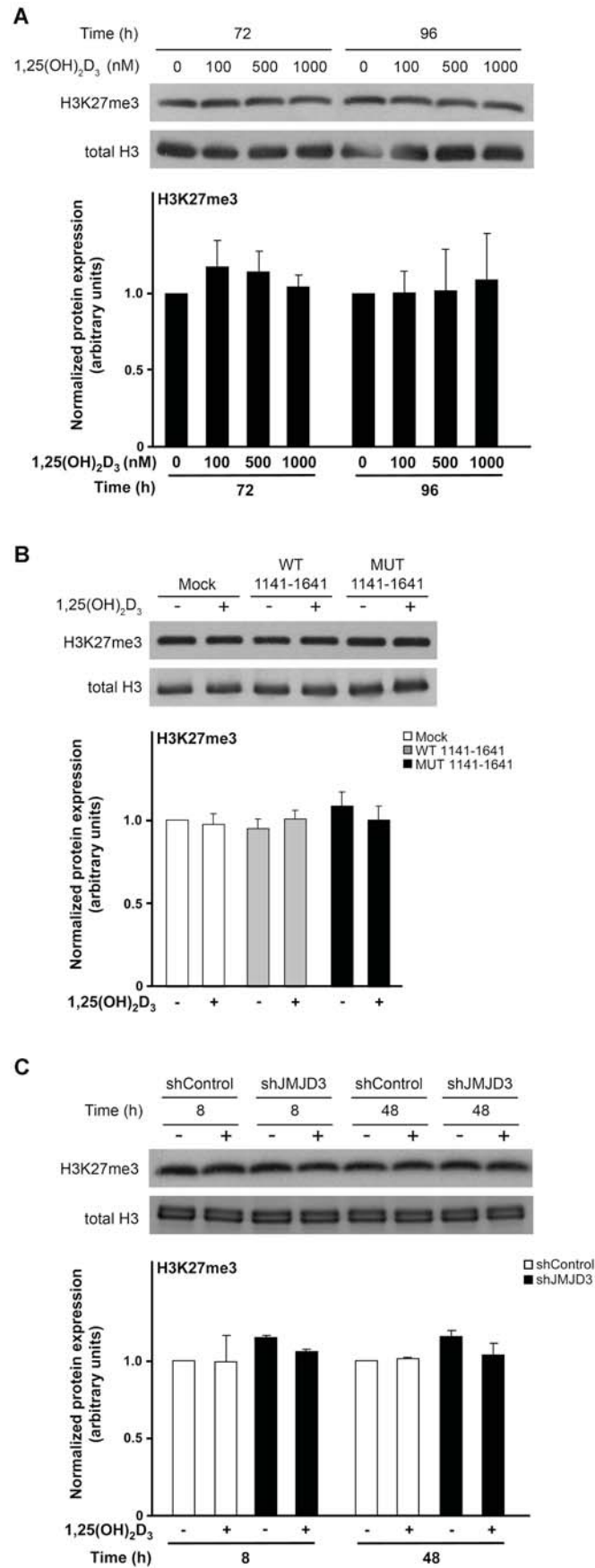


Figure 3

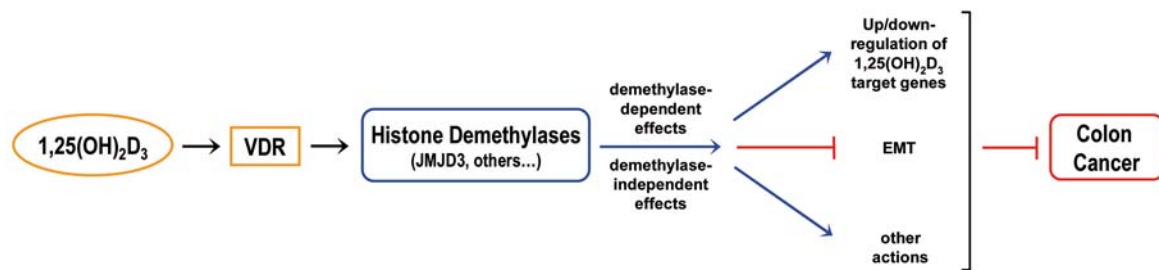
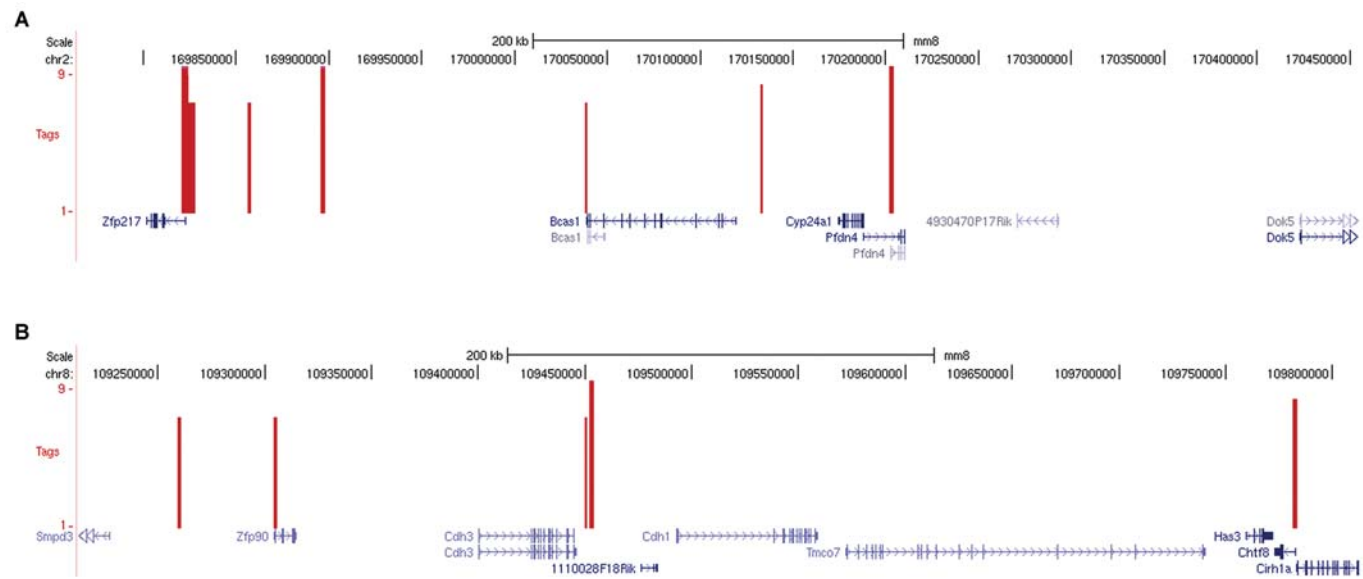


Figure 4



**Figure 5**