KDM6B/JMJD3 histone demethylase is induced by vitamin D and modulates its effects in colon cancer cells

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Abstract

KDM6B/JMJD3 is a histone H3 lysine demethylase with an important gene regulatory role in development and physiology. Here we show that human JMJD3 expression is induced by the active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and that JMJD3 modulates the gene regulatory action of this hormone. $1,25(OH)_2D_3$ activates the *JMJD3* gene promoter and increases the level of *JMJD3* RNA in human cancer cells. *JMJD3* upregulation was strictly dependent on vitamin D receptor (VDR) expression and was abolished by cycloheximide. In SW480-ADH colon cancer cells, *JMJD3* knockdown or expression of an inactive mutant JMJD3 fragment decreased the induction by $1,25(OH)_2D_3$ of several target genes and of an epithelial adhesive phenotype. Moreover, *JMJD3* knockdown upregulated the epithelial-to-mesenchymal transition inducers SNAIL1 and ZEB1 and the mesenchymal markers Fibronectin and LEF1, while it downregulated the epithelial proteins E-cadherin, Claudin-1 and Claudin-7. Additionally, *JMJD3* knockdown abolished the nuclear export of β-catenin and the inhibition of β-catenin transcriptional activity caused by $1,25(OH)_2D_3$. Importantly, the expression of *JMJD3* correlated directly with that of *VDR* and inversely with that of *SNAI1* in a series of 96 human colon tumours. Our results indicate for the first time that an epigenetic gene coding for a histone demethylase such as *JMJD3* is a VDR co-target that partially mediates the effects of $1,25(OH)_2D_3$ on human colon.

INTRODUCTION

Histone methylation on specific lysine residues is a crucial point for gene expression in eukaryotic cells that is controlled by the opposite action of histone methyltransferases (KMTs), such as Polycomb group proteins, and demethylases (KDMs), such as LSD1 and the family of Jumonji (Jmj) C domain-containing enzymes (1). Several KDMs have been implicated in development, differentiation or stem cell renewal, and their mutation or deregulation have been linked to cancer and other diseases (2-7).

JMJD3 (KDM6B) and UTX (KDM6A) specifically demethylates di- and tri-methyl-lysine 27 on histone H3 (H3K27me2/3) (8-10). The presence of H3K27me3 at transcriptional sites usually correlates with gene repression (7,11,12), and Polycomb-mediated H3K27 methylation pre-marks genes for DNA methylation and silencing in cancer (13). Thus, JMJD3 is expected to enable the activation of genes, which indeed occurs for those involved in animal body patterning and the inflammatory response (8,10,14,15). Moreover, JMJD3 is induced upon activation of the RAS-RAF signalling pathway and contributes to the transcriptional activation of the $p16^{INK4A}$ tumour suppressor in diploid fibroblasts (16,17), and it is aberrantly overexpressed and seems to be involved in Epstein-Barr virus-associated Hodgkin's lymphoma (18). In addition, JMJD3 appears to have transcriptional effects unrelated to the level of histone methylation at least in lipopolysaccharide-stimulated mouse macrophages, and the possibility of non-histone substrates and/or other activities of KDMs is emerging (19,20).

Many epidemiological and preclinical studies suggest a protective effect of vitamin D against several neoplasias, particularly colon cancer, whose confirmation is pending of adequate clinical trials (21-23). The active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a hormone with wide gene regulatory effects in higher organisms (24,25), which is in line with the expression of the vitamin D receptor (VDR) in over 30 different cell

types (26). VDR is a member of the nuclear hormone receptor superfamily of transcription factors modulated by ligand, phosphorylation, and perhaps other modifications (27,28). Although part of VDR is extranuclear and upon ligand binding can activate signalling pathways, these are thought to converge with the regulation of gene transcription as the major effect of DNA-bound VDR/1,25$(OH)_2D_3$ complexes (29,30).

1,25$(OH)_2D_3$ is believed to regulate the expression of hundreds of genes but only a subset of them seem to contain VDR binding sites (VDRE, vitamin D regulatory elements). In addition, very few genes respond to 1,25$(OH)_2D_3$ with drastic changes in their transcription rate; rather the hormone has a weak, moderate effect that is compatible with a permissive action. We hypothesized that this could be related to epigenetic changes: hormone-induced alterations at the chromatin level that could facilitate gene transcription regulation by other agents. Supporting this, transcriptomic studies by us and others have revealed *JARID2/JMJ*, *JARID1A/KDM5A* and *JMJD2B/KDM4B* as candidate 1,25$(OH)_2D_3$ targets in human SW480-ADH colon or SCC25 squamous cell carcinoma cells (31 and our own unpublished results). All this led us to investigate the possibility that 1,25$(OH)_2D_3$ affects the expression of genes controlling epigenetic processes such as those regulating histone modifications. As the majority of 1,25$(OH)_2D_3$ target genes are induced while only around one-third are repressed, we chose histone H3 lysine demethylases to study.

Our results show that 1,25$(OH)_2D_3$ induces the expression of JMJD3 in several types of human cancer cells. The regulation is transcriptional and probably mediated by one or more short-lived proteins. Importantly, a knockdown approach has shown that JMJD3 mediates in part the induction by 1,25$(OH)_2D_3$ of several target genes such as *CDH1*/E-cadherin and *CST5*/cystatin D and modulates the expression of the epithelial-to-mesenchymal transition (EMT) inducers SNAIL1 and ZEB1. Probably as a consequence of these actions, JMJD3 controls the phenotype of human colon cancer cells. Importantly, the level of expression of

VDR and *JMJD3* directly correlate in human colon tumours, suggesting that the regulation of JMJD3 by $1,25(OH)_2D_3$ may take place *in vivo* during colon cancer progression and mediates at least in part the protective action of $1,25(OH)_2D_3$ against this neoplasia.

RESULTS

JMJD3 gene is induced by $1,25(OH)_2D_3$

To study whether $1,25(OH)_2D_3$ could affect the expression of histone H3 lysine demethylases, we analyzed the RNA level of ten JmjC-domain proteins by qRT-PCR in SW480-ADH colon cancer cells treated for 4 or 48 h with the hormone. As control, we studied *CYP24A1* and *CDH1*/E-cadherin, known to be induced, and *CYP27B1*, which is repressed by $1,25(OH)_2D_3$. We found the strongest change (2 to 3-fold increase at 48 h treatment) for *JMJD3*, while *JMJD4* showed only a 25% decrease and the remaining genes were unaffected (Supplementary Material, Fig. S1). To investigate in depth the observed effect we first performed time-course and dose-curve experiments. A progressive increase in *JMJD3* RNA level was detected following hormone treatment (10^{-7} M) (Fig. 1A) that was dose-dependent at 48 h (Fig. 1B). The general validity and specificity of this finding was examined using a panel of human cancer cell lines, investigating the requirement of VDR, and analysing the putative effect of other nuclear receptors ligands. A common upregulation (2 to 3-fold) by $1,25(OH)_2D_3$ was found in several human colon (SW1417, SW48, KM12C), breast (MCF7, MDA-MB-453, MDA-MB-231) and skin (UACC-257) cancer cell lines (Supplementary Material, Fig. S2A). No effect of $1,25(OH)_2D_3$ was detected in other cancer cell lines such as HT29 or Caco-2 (colon), SK-MEL-28 (skin) or AGS (gastric) (not shown). Importantly, no regulation was detected in SW620 and SW480-R cells that do not express VDR (32) (Supplementary Material, Fig. S2A). Consistently, VDR knockdown by shRNA in SW480-ADH cells abolished the increase in *JMJD3* RNA expression caused by $1,25(OH)_2D_3$ (Fig. 1C). Finally, neither estradiol, progesterone, dexamethasone, triiodothyronine, *all*-trans- or *cis*-retinoic acid, or docosahexaenoic acid (ligands for estrogen, progesterone, glucocorticoid, thyroid, retinoic or

rexinoid receptors, respectively) changed basal *JMJD3* RNA expression nor affected the induction by $1,25(OH)_2D_3$ (Supplementary Material, Fig. S2B).

Next we studied the mechanism of *JMJD3* upregulation. To investigate whether the effect on *JMJD3* RNA level was due to the activation of the gene promoter we studied the effect of $1,25(OH)_2D_3$ in SW480-ADH cells that were transfected with a series of plasmids containing distinct sequences of the promoter upstream of the luciferase reporter gene (17). Promoter constructs P1-P5 containing -3,422, -2,439, -2,314, -2,224, and -2,173 bp immediately upstream of the *JMJD3* transcription start site active in macrophages (MF-TSS) were activated by the hormone (Fig. 1D). On the other hand, the activity of P6 and P7 constructs spanning -2,102 and -1,428 bp upstream of this site, or P8 containing -2,928 bp upstream the *JMJD3* start site active in embryonic stem cells (ESC-TSS), were not affected by $1,25(OH)_2D_3$ (Fig. 1D). This suggests that $1,25(OH)_2D_3$ induces *JMJD3* transcription *via* sequences located from -2,173 to -2,102 bp upstream the MF-TSS. This region of the promoter contains sites for RAP1, Sp1, Krox-20, ETF, C/EBPα, and AP-1 transcription factors and is crucial for the induction of *JMJD3* promoter by BRAF (17). However, the involvement of other sequences not contained in the constructs studied cannot be ruled out. The protein synthesis inhibitor cycloheximide (CHX) blocked the increase in *JMJD3* RNA by $1,25(OH)_2D_3$ (Fig. 1E). The long treatment with CHX that is needed because of the slow kinetics of *JMJD3* induction makes the requirement of intermediary short-lived protein(s) suggestive, but not definitive. In addition, no statistically significant VDR binding to three putative VDREs revealed by *in silico* analysis of the *JMJD3* gene (-28697-AGGCCATTTAGTTCA; -1073-TGACCTCTACCACCT; +9183-TGACCCAGCCGACCC respect to the ESC-TSS) was found by means of chromatin immunoprecipitation assays (Supplementary Material, Fig. S3A and B). This negative result further supports an indirect mechanism of regulation.

JMJD3 modulates the gene regulatory effects of $1,25(OH)_2D_3$

To study the importance of JMJD3 in $1,25(OH)_2D_3$ action we generated SW480-ADH cells stably expressing *JMJD3* shRNA (shJMJD3) or a non-targeting shRNA (shControl). First, we confirmed a stable downregulation of *JMJD3* expression, which did not affect *VDR* RNA expression (Fig. 2A).

As a major effect of $1,25(OH)_2D_3$ in human colon and breast carcinoma cells is the induction of E-cadherin protein, which causes a morphological change to an adhesive epithelial phenotype (32-34) we analyzed these biological parameters in shJMJD3 cells. The increase in both intercellular adhesion and E-cadherin protein expression by $1,25(OH)_2D_3$ was partially inhibited in shJMJD3 cells (Fig. 2B), and was co-incident with changes in the tubulin cytoskeleton and cell morphology (Fig. 2B). In line with this, the induction of *CDH1*/E-cadherin RNA and also that of *CYP24A1* and *CST5*/cystatin D, two other $1,25(OH)_2D_3$ targets (35,36), was inhibited in shJMJD3 cells (Fig. 2C). *JMJD3* knockdown decreased also the basal expression of *CDH1*/E-cadherin and *CST5*/cystatin D (Fig. 2C). These effects were reproduced at the promoter and protein levels: the activity of the *CDH1*/E-cadherin gene promoter was lower in shJMJD3 than in shControl cells (Fig. 2D); likewise, the basal and induced expression of E-cadherin and cystatin D proteins was lower in shJMJD3 cells (Fig. 2E). In contrast, however, *JMJD3* knockdown did not affect the expression of VDR protein (Fig. 2E), indicating that JMJD3 is not equally required for the activation of all genes and that the inhibition of the gene regulatory action of $1,25(OH)_2D_3$ in shJMJD3 cells is not a consequence of diminished VDR expression.

To exclude off-target effects of the knockdown approach and confirm the role of JMJD3 mediating $1,25(OH)_2D_3$ action, we generated cells expressing ectopically a HA-tagged inactive JMJD3 C-terminal region harbouring a His to Ala1388 mutation in the iron-binding

center (MUT 1141-1641) (10). As control, we generated also cells expressing the same wild-type JMJD3 polypeptide (WT 1141-1614) or and empty vector (Mock). Expression of the corresponding exogenous polypeptides was confirmed by qRT-PCR and Western blot (Supplementary Material, Fig. S4A and B). As happened for *JMJD3* knockdown, neither MUT 1141-1614 nor WT 1141-1641 JMJD3 polypeptides altered the expression of VDR RNA or protein (Supplementary Material, Fig. S4A and B). Importantly, MUT 1141-1641 cells behaved as shJMJD3 cells in terms of $1,25(OH)_2D_3$-induced phenotype change, basal and $1,25(OH)_2D_3$-induced *CDH1*/E-cadherin, *CST5*/cystatin D and *CYP24A1* RNA and protein expression, and *CDH1*/E-cadherin promoter activity (Supplementary Material, Fig. S4C-F). These effects were specific, as they did not take place in cells expressing the WT 1141-1641 JMJD3 polypeptide (Supplementary Material, Fig. S4C-F).

JMJD3 knockdown enhances the expression of epithelial-to-mesenchymal transition inducers

The finding that *JMJD3* knockdown affected *CDH1*/E-cadherin expression and cell phenotype led us to investigate the possibility that these effects could be due to the regulation of genes such as *SNAI1*, *SNAI2*, *ZEB1* or *ZEB2* encoding transcriptional repressors (SNAIL1, SNAIL2, ZEB1, ZEB2) of *CDH1*/E-cadherin that are EMT inducers (37). Interestingly, *JMJD3* knockdown increased the expression of *SNAI1*, *ZEB1* and *ZEB2* RNA without affecting that of *SNAI2* RNA (Fig. 3A), and increased also SNAIL1 and ZEB1 protein levels (Fig. 3B). Consistent also with a role of JMJD3 preventing EMT, shJMJD3 cells had higher RNA levels of the mesenchymal genes Lymphoid Enhancer-binding Factor 1 (*LEF1*) and Fibronectin (*FN1*), and lower RNA and protein levels of the epithelial genes *CLDN1*/Claudin-1, *CLDN7*/Claudin-7 and *CDH1*/E-cadherin (Fig. 3C and D).

JMJD3 knockdown inhibits the antagonism of the Wnt/β-catenin pathway by $1,25(OH)_2D_3$

We and others have reported that $1,25(OH)_2D_3$ antagonizes the Wnt/β-catenin signalling pathway by promoting a direct VDR-β-catenin interaction and nuclear export of β-catenin, which together lead to inhibition of the transcriptional activity of β-catenin/T-cell factor (TCF) complexes (32,33,38-40). Further emphasizing the requirement for JMJD3 in $1,25(OH)_2D_3$ action, both β-catenin nuclear export and inhibition of its transcriptional activity by $1,25(OH)_2D_3$ were blocked in shJMJD3 cells (Fig. 4A and B). Expectedly from the effect of Wnt/β-catenin pathway promoting cell proliferation, $1,25(OH)_2D_3$ caused a stronger inhibition of the proliferation of shControl cells than of shJMJD3 cells (45% and 29% respectively; $P < 0.001$) (Fig. 4C). In line with the reduced proliferation of cells that express SNAIL1 and undergo EMT (41,42), shJMJD3 cells grew less than shControl cells (19%; $P < 0.001$) (Fig. 4C).

Analysis of the expression of *JMJD3*, *VDR* and *SNAI1* in human colon tumours

To translate the findings in cultured cells to the *in vivo* setting, we studied the expression of *JMJD3*, *VDR* and *SNAI1* genes in matched normal and tumour tissue samples of a series of 96 colon cancer patients.

Lower *JMJD3* RNA expression in tumour *versus* normal tissue was found in 54/96 (56.3%) patients. No significant correlation was detected between the level of *JMJD3* expression and any clinical parameter, but a tendency existed between low *JMJD3* expression and poor tumour differentiation (ANOVA test, $P = 0.082$). In line with previous studies (43-45), *VDR* expression was downregulated in tumour *versus* normal tissue in two-thirds (64/96, 66.7%) of patients while, in contrast, *SNAI1* was upregulated in a similar percentage of cases (62.2%).

Interestingly, a significant direct correlation was found between the RNA levels of *JMJD3* and *VDR* (Pearson correlation coefficient $r = 0.245$, $P = 0.016$) (Fig. 5A). In contrast, the expression of *JMJD3* and *SNAI1* RNA showed a strong inverse trend with borderline significance (ANOVA test, $P = 0.057$) in 82 informative cases (Fig. 5B).

DISCUSSION

In this study we demonstrate that the human histone H3 lysine demethylase *JMJD3* gene is induced by $1,25(OH)_2D_3$ in a panel of cancer cell lines. Data obtained using a series of *JMJD3* promoter constructs suggest that $1,25(OH)_2D_3$ induces *JMJD3* transcription *via* sequences located from -2,173 to -2,102 bp upstream the MF-TSS. The blockade of *JMJD3* induction using CHX and the absence of VDR binding to three putative VDREs present in the *JMJD3* gene suggest an indirect mechanism of regulation. However, the long CHX treatments needed due to the slow kinetics of *JMJD3* regulation, could affect VDR expression and therefore the possibility of a direct regulation of *JMJD3* by $1,25(OH)_2D_3$ can not be excluded. Also, other putative VDREs may exist. We show that *JMJD3* is implicated in the regulation of several $1,25(OH)_2D_3$ target genes such as *CDH1*/E-cadherin and *CST5*/cystatin D. Moreover, knockdown experiments indicate that *JMJD3* contributes to maintain the adhesive epithelial cell phenotype, as its downregulation augments the expression of SNAIL1 and ZEB1 transcription factors. The study of *JMJD3* expression in normal and tumour tissue of colon cancer patients strongly supports its role during the progression of this neoplasia. Furthermore, the direct correlation between the expression levels of *JMJD3* and *VDR* suggests that $1,25(OH)_2D_3$ regulates *JMJD3* in the human colon.

Recent studies have shown the importance of histone methyltransferases and demethylases in the gene regulatory effects of several nuclear receptors (28). Transcriptional activation of nuclear receptor target genes requires changes in the local chromatin environment that are at least partially consequence of the direct or indirect recruitment by the receptors of histone methylases and demethylases. In addition, several histone-modifying enzymes target estrogen and retinoic acid nuclear receptors and/or their coactivators and corepressors (SRC3, CBP/p300...) (28). Altogether, these findings suggest strongly that histone methyltransferases

and demethylases mediate the ligand-dependent regulation of gene expression by some nuclear receptors (46). For VDR, dynamic modification of H3K27me3 has recently been reported in the regulation of *CDKN1A* by $1,25(OH)_2D_3$ in normal prostate cells (47). In line with this and in a cancer context, our results demonstrate for the first time that a KDM modulates ligand-activated VDR effects on gene transcription. From the screening of a large number of JmjC-containing proteins (Supplementary Material, Fig. S1), it is conceivable that KDMs others than JMJD3 can be also involved and thus, that the gene regulatory activity of VDR/$1,25(OH)_2D_3$ can be fine tuned by the combinatorial action of several histone modifying enzymes, probably in a cell-type dependent manner.

Mechanistically, the induction of JMJD3 may have several different effects on $1,25(OH)_2D_3$ action that are not mutually exclusive. First, to contribute to a local chromatin environment that facilitates the expression of target genes via H3K27me2/3 demethylation. *CDH1*/E-cadherin and *CST5*/cystatin D may be examples. Second, to demethylate VDR and/or any of its coregulators, although no data supporting this exist. Third, to alter chromatin of genes other than those transcriptionally-regulated by $1,25(OH)_2D_3$ that may, however, act additively or synergistically with this hormone. Finally, the proposed existence of JMJD3 effects unrelated to histone demethylation (20) opens the possibility to distinct, unknown mechanism(s) of modulation of $1,25(OH)_2D_3$ action.

Interestingly, the first genome-wide studies performed to identify VDR binding sites by ChIP-Seq have revealed a huge number of them in the human and mouse genomes (48,49). As it happens with other transcription factors, only a subset of VDR binding sites are expected to be functional, and this is thought to depend on the epigenetic status of the cell (50). Conceivably, JMJD3 may contribute to generate a chromatin structure favourable to gene activation by $1,25(OH)_2D_3$ and so, its induction by $1,25(OH)_2D_3$ would be a pre-requisite to ensure an optimal gene regulatory action of the hormone.

Studies by Kato's group have proposed DNA methylation as part of the mechanism of gene repression by $1,25(OH)_2D_3$ (51), while *Gadd45a*, a well-known $1,25(OH)_2D_3$ target gene, promotes epigenetic gene activation by repair-mediated DNA demethylation in *Xenopus laevis* (52). Our work, however, links a histone demethylase to gene activation by this hormone.

Moreover, the analysis of global transcriptomic studies available in the Oncomine database (http://www.oncomine.org) has previously evidenced reduced *JMJD3* expression in several human cancers relative to normal tissues including several hematopoietic malignancies and lung and liver carcinomas (16,17). Our study, however, is the first one to measure *JMJD3* expression individually in a series of human cancer patients under well-controlled conditions, and together with the results generated in cultured colon cancer cells, it strengthens the previously suggested nature of JMJD3 as a tumour suppressor (16,17).

In conclusion, our study uncovers the epigenetic regulator *JMJD3* as a new $1,25(OH)_2D_3$ target gene that in turn integrates with the gene regulatory actions of this hormone. Disruption of this link in human cancer cells may play a role during colon tumourigenesis and potentially offer new therapeutic targets to delay tumour progression.

MATERIALS AND METHODS

Cells and cell culture

Human cancer cell lines SW480-ADH and SW480-R (32), SW620, SW1417, HCT116, HT29, KM12C, Caco-2, MCF7, MDA-MB-231, MDA-MB-453, UACC-257, SK-MEL-28 and AGS were cultured in DMEM plus 10% foetal bovine serum (Invitrogen, Paisley, UK), except MDA-MB-453 that was cultured in DMEM:F12 (Invitrogen). SW480-ADH shVDR and shControl cells were described previously (29). All experiments using $1,25(OH)_2D_3$ were performed in medium supplemented with charcoal-treated serum. Phase-contrast images of cultured cells were captured with a Leica DC300 digital camera mounted on an inverted Leitz Labovert FS Microscope. All images were processed using Adobe Photoshop CS5 software (Adobe Systems, San José, CA).

Antibodies and reagents

We used primary mouse monoclonal antibodies against E-cadherin, β-catenin (BD Biosciences, San Jose, CA), β-tubulin (Sigma-Aldrich, St. Louis, MO) and HA (Covance, Princeton, NJ); rat monoclonal antibodies against VDR (Millipore, Billerica, MA); rabbit polyclonal antibodies against Claudin-1 and Claudin-7 (Zymed-Invitrogen) and goat polyclonal antibodies against ZEB1, β-actin and Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies used: Alexa 488-conjugated goat anti-mouse (Molecular Probes-Invitrogen); HRP-conjugated anti-rabbit (MP Biomedicals, Solon, OH), anti- mouse (Promega, Madison, WI), anti-goat and anti-rat (Santa Cruz Biotechnology). F-actin was stained with Phalloidin-TRITC and nuclei using DAPI (Molecular Probes-Invitrogen). Cycloheximide, estradiol, dexamethasone, progesterone, triiodothyronine, *all-trans*-retinoic acid, *cis*-retinoic acid and docosahexaenoic acid were from Sigma-Aldrich.

Patients and tumour samples

Normal and tumour tissue samples from 96 colon cancer patients were obtained immediately after surgery, immersed in RNA later (Applied Biosystems, Carlsbad, CA), snap-frozen in liquid nitrogen and stored at -80ºC until processing. Tumours were considered sporadic cases because no clinical antecedents of *Familial Adenomatous Polyposis* (FAP) were reported and those with clinical criteria of hereditary non-polyposis colorectal cancer (HNPCC) (Amsterdam criteria) were excluded. Tumours were examined by two different pathologists to: (a) confirm adenocarcinoma diagnostic and presence of at least 75% of tumour tissue in the sample, (b) establish the histological level of the tumour, and (c) verify the absence of tumour cells in normal tissue. All patients gave written informed consent. The protocol was approved by the Research Ethics Board of the Hospital Universitario Puerta de Hierro, Majadahonda, Madrid, Spain.

Transfection

Cells were transfected using the jetPEI reagent (PolyPlus Transfection, Illkirch, France). *Firefly* (Luc) and *Renilla reniformis* luciferase (Rluc) activities were measured separately using the Dual Luciferase kit (Promega). Luc activity was normalized to the Rluc activity. All experiments were performed at least in triplicate. The -987/+92 promoter construct for *CDH1*/E-cadherin was previously reported (32). The 4X-VDRE-DR3-tk-Luc construct was provided by Dr. C. Carlberg (Kuopio, Finland). To study β-catenin/TCF transcriptional activity we used the TOP-Flash and FOP-Flash plasmids provided by Dr. H. Clevers, (Utrecht, The Netherlands). *JMJD3* gene promoter constructs were provided by Dr. K. Helin (Copenhagen, Denmark).

SW480-ADH cells stably expressing JMJD3 were generated by transfection with pRC-JMJD3-1141-1641-WT-HA, pRC-JMJD3-1141-1641-MUT-HA or the empty vector (kindly provided by Dr. G. Natoli, Milan, Italy) followed by selection with 0.5 mg/ml of G418 (Sigma-Aldrich) for two weeks. HA expression was confirmed by Western blot and that of *JMJD3* RNA by qRT-PCR using primers for a common sequence to JMJD3 1141-1641 WT and MUT (Forward: GGAGACCTTTATCGCCTCTG; Reverse: TCCCTTTCACCTTGGCATT).

Gene silencing

To knockdown *JMJD3* expression, SW480-ADH cells were seeded in 150 mm dishes and infected with lentiviral particles containing a U6 promoter driving an short hairpin (shRNA) targeting *JMJD3* RNA. Control cells were infected with lentivirus bearing a non-targeting shRNA that activates the RISC complex and the RNA interference pathway but that contains at least five mismatched nucleotides compared with any human gene. Mission shRNA lentiviral particles against human *JMJD3* and scramble negative control (Sigma-Aldrich) were used. After infection cells were selected with 1 μg/μl puromycin (Sigma-Aldrich) for one week.

Quantitative RT-PCR

RNA was extracted from ~30 mg of tumour or normal tissue and from cultured cells using RNeasy mini kit (Qiagen, Hilden, Germany). RNA was retrotranscribed using the High Capacity cDNA Archive Kit (Applied Biosystems). Primers used for qRT-PCR are listed in Supplementary Material, Table S1. We also used the following TaqMan probes: *JMJD3* (Hs00389749_m1), *CYP24A1* (Hs00167999_m1), *VDR* (Hs01045840_m1), *SNAI1* (Hs00195591_m1), *CST5* (Hs00983867_m1), *UTX* (Hs00958902_m1), *18S* (4310893E) (Applied Biosystems). RNA expression values were normalized *versus* the housekeeping gene succinate dehydrogenase complex subunit A (*SDHA*) or with the 18S ribosomal RNA (*18S*). In

experiments with cell lines we used the 7500 StepOne Plus Real-Time PCR System using the TaqMan or SYBR Gene Expression Master Mix (Applied Biosystems). Thermal cycling was initiated with a denaturation step of 95ºC for 10 min and consisted of 40 cycles (denaturation at 95ºC 10 s, annealing and elongation at 60ºC for 60 s). RNA expression analysis for human colon samples was performed in a LightCycler apparatus using the LightCycler-FastStart DNA MasterPLUS SYBR Green I Kit (Roche Diagnostics, Basel, Switzerland). Thermal cycling was initiated with a denaturation step of 95ºC for 10 min and consisted of 40 cycles of denaturing at 94ºC 0 s; annealing at 59ºC (*JMJD3*) 60ºC (*VDR*) 68ºC (*SNAI1*) or 59ºC (*SDHA*) for 5 s and elongation at 72ºC for 5 s. All experiments were performed in triplicate.

Western blot

Proteins were separated by SDS-PAGE. After blotting onto a PVDF membrane, proteins were revealed following the ECL technique (GE Healthcare, Chalfont St. Gills, UK). Different exposure times of the films were used to ensure that bands were not saturated. Quantification of the films was done by densitometry using ImageJ software.

Immunofluorescence and confocal microscopy

Cells were fixed in methanol at -20ºC for 1 min (E-cadherin, β-Tubulin and β-Catenin staining) or in *p*-formaldehyde (F-Actin staining) for 15 min and permeabilized in 0.2% Triton X-100, and then washed four times in PBS. Cells were incubated with the primary antibodies diluted in PBS for 1 h at 37^{o}C. After four washes in PBS, cells were incubated with secondary antibodies for 45 min at room temperature and washed three times in PBS. For F-Actin staining cells were incubated with Phalloidin-TRITC for 15 min at 37ºC and washed in PBS. Finally, coverslips containing the cells were mounted using Prolong Gold antifade reagent (Molecular Probes-Invitrogen). Cell imaging was performed on a Leica TCS SP5 DMI6000

microscope using argon ion (488 nm), HeNe (543 nm) and violet diode (405 nm) lasers. Images were acquired sequentially by direct register using Leica Confocal Software (LAS AF).

Cell proliferation assay (MTT)

This assay is based on the cleavage of the yellow [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl] tetrazolium bromide salt (MTT) to purple formazan crystals by metabolic active cells. Ten thousand cells were seeded in 24-well plates and treated with $1,25(OH)_2D_3$ or vehicle. At indicated times cells were incubated with the MTT solution (final concentration of 0.5 mg/ml, Roche Diagnostics) for 4 h at 37ºC. After this incubation period, a water-insoluble formazan dye is formed. After solubilization with 500 µl of 0.04 M HCl isopropanol during 30 min at R/T, the formazan dye was quantified using a scanning microplate spectrophotometer (VersaMax, Molecular Devices. Sunnyvale, CA). The absorbance was measured as 570-630 nm. All experiments were performed using triplicates.

***In silico* screening of VDREs and chromatin immunoprecipitation assays**

The sequence surrounding the transcription start site (TSS) of the human *JMJD3* gene was obtained from the ENSEMBL database. This TSS was coincident with the ESC-TSS described by Agger *et al.* (17). A region spanning 50 kb upstream and 50 kb downstream of the TSS was analyzed for putative VDREs as previously described (53,54). The effect of single nucleotide variations on the classical VDRE sequence (AGTTCAnnnAGTTCA) suggested RGKTCA (R=A/G and K=G/T) as the consensus hexameric core sequence for VDREs. We screened for hexamers pairs in DR3, DR4 and ER6 to ER9 orientation fitting the RGKTCA consensus sequence or having at most two deviations from it. We selected the three theoretically best VDREs (-28697, -1073 and +9183) based on their predicted binding strength, location relative to the TSS and conservation across multiple species.

X-ChIP was used to measure the association of VDR and RNA polymerase II to the three VDREs and the TSS of the *JMJD3* gene as previously described (55). Rabbit polyclonal anti-VDR and anti-RNA polymerase II antibodies (both from Abcam, Cambridge, UK) were used for immunoprecipitation. q-PCR was performed in the 7900 HT Sequence Detection System (Applied Biosystems) using SensiMix (dT) Kit and SYBR Green (both from Quantace, London, UK). Primers used were as follows: -28697 (Forward: AGGGCAACTATATCCCTAAGGT; Reverse: TGGAGCGTTTTAATGCTGTC), -1073 (Forward: CTAGCCTGAAGCTCCCTTCC; Reverse: ACCAAGGCCTCCTTCCTAAG), +9183 (Forward: CTTGCGAGACCCTTGTGG; Reverse: AGTCGCTCAGTCCCACTGTC) and TSS (Forward: CCCCACCTCTAACTGACTTTTT; Reverse: TGGGGTACAACAGACCACAG). Thermal cycling was initiated with a denaturation step at 95ºC for 10 min and consisted of 40 cycles of denaturing at 95ºC 15 s; annealing at 58ºC (-28697, TSS) or 62ºC (-1073, +9183) for 30 s and elongation at 72ºC for 30 s. All experiments were performed in triplicate.

Statistical analysis

Results are expressed as mean ± SEM unless otherwise specified. Statistical significance was assessed by two-tailed unpaired Student's *t* test using the GraphPad Instat3 program. Differences were considered significant when $P < 0.05$. The single asterisk indicates $P < 0.05$, the double asterisk $P < 0.01$, and the triple asterisk $P < 0.001$.

The correlation between the tumour versus normal ratio (T/N) of *JMJD3* RNA expression and that of *VDR* in samples from colon cancer patients was studied using the Pearson correlation coefficient. Since *SNAI1* RNA was not detected in any normal tissue, the T/N cannot be calculated and its expression was evaluated as presence or absence in tumour tissue. Therefore, correlation between *SNAI1* tumour expression and T/N of *JMJD3* expression

was studied using ANOVA test. Associations between clinicopathological parameters and *JMJD3* gene expression were studied using ANOVA test. Statistical analysis of gene expression data from colon cancer patients was performed using SPSS software (SPSS, Chicago, IL). Differences were considered statistically significant when $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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LEGENDS TO FIGURES

Figure 1. $1,25(OH)_2D_3$ induces *JMJD3* expression in colon cancer cells. (**A**) qRT-PCR analysis of *JMJD3* RNA levels in SW480-ADH cells treated with 10^{-7} M $1,25(OH)_2D_3$ for the indicated times. (**B**) *JMJD3* RNA expression in SW480-ADH cells treated with the indicated doses of $1,25(OH)_2D_3$ for 48 h. (**C**) *JMJD3* RNA levels in SW480-ADH cells expressing shVDR or shControl that were treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. (**D**) Activity of a series of *JMJD3* promoter constructs (P1-P8) (17) in SW480-ADH cells. Cells transfected with the indicated construct were incubated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h and the expression of luciferase in cell extracts was estimated. Inset: Scheme of the *JMJD3* promoter constructs used. Constructs are represented as gray boxes and arrows indicate the position of the transcription start site active in embryonic stem cells (ESC-TSS) and in macrophages (MF-TSS). (**E**) *JMJD3* RNA levels in SW480-ADH cells pretreated 30 min with 8 μg/ml cycloheximide (CHX) as indicated and then with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 24 or 36 h. In (**A**-**C** and **E**) *18S* rRNA expression was used for normalization. Data are expressed as the mean ± SEM of three independent experiments (**A**-**D**) or as mean ± SD of a representative experiment performed in triplicate (**E**).

Figure 2. *JMJD3* knockdown inhibits the induction of an adhesive epithelial phenotype and target genes by $1,25(OH)_2D_3$ without affecting VDR expression. (**A**) qRT-PCR analysis of the RNA levels of *JMJD3* and *VDR* in SW480-ADH shControl and shJMJD3 cells. *SDHA* was used for normalization. Mean ± SEM (n = 3). (**B**) Upper panels, representative phase-contrast images of SW480-ADH shControl and shJMJD3 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. Bar: 10 μm. Lower panels, representative immunofluorescence and confocal microscopy images of E-cadherin and β-Tubulin expression in SW480-ADH shControl and

shJMJD3 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. Bar: 10 μm. (**C**) qRT-PCR analysis of the RNA levels of *CDH1*/E-cadherin, *CYP24A1* and *CST5*/cystatin D in SW480-ADH shControl and shJMJD3 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 8 h. *18S* was used for normalization. Mean ± SEM (n = 3). (**D**) Activity of the *CDH1*/E-cadherin promoter (-987 to +92 bp) in SW480-ADH shControl and shJMJD3 cells. The cells were transfected with the *CDH1*/E-cadherin promoter construct and the expression of luciferase in cell extracts was estimated 48 h later. Mean ± SEM (n = 4). (**E**) Western blot analysis of E-cadherin, Cystatin D and VDR protein expression in SW480-ADH shControl and shJMJD3 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 8 or 48 h. β-Actin was used as control. Numbers refer to the fold-change with respect to untreated shControl cells. The Western blot shown correspond to a representative experiment of the three performed.

Figure 3. *JMJD3* knockdown increases the expression EMT inducers and mesenchymal markers and represses epithelial markers. (**A**) qRT-PCR analysis of *SNAI1, SNAI2, ZEB1* and *ZEB2* RNA expression in SW480-ADH shControl and shJMJD3 cells. *SDHA* was used for normalization. Mean ± SEM (n = 3). (**B**) Western blot analysis of SNAIL1 and ZEB1 protein expression in SW480-ADH shControl and shJMJD3 cells. β-Actin and Lamin B were used as controls. Numbers show the quantification of two experiments. (**C**) qRT-PCR analysis of *LEF1, FN1, CLDN-1*/Claudin-1 and *CLDN-7*/Claudin-7 RNA expression in SW480-ADH shControl and shJMJD3 cells. *SDHA* was used for normalization. Mean ± SEM (n = 3). (**D**) Western blot analysis of Claudin-1, Claudin-7 and E-cadherin protein expression in SW480-ADH shControl and shJMJD3 cells. β-Actin and Lamin B were used as controls. Numbers show the quantification of two experiments.

Figure 4. *JMJD3* knockdown abrogates the inhibition of Wnt/β-catenin signalling and cell proliferation by $1,25(OH)_2D_3$. (**A**) Representative immunofluorescence and confocal microscopy images of β-catenin and F-actin expression in SW480-ADH shControl and shJMJD3 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. Bar: 10 μm. (**B**) Transcriptional activity of β-catenin/TCF complexes in SW480-ADH shControl and shJMJD3 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 72 h. The cells were transfected with the TOP-Flash and FOP-Flash reporter plasmids and the ratio of luciferase expression (TOP-Flash/FOP-Flash) in cell extracts was calculated. Mean ± SEM (n = 4). (**C**) Proliferation of SW480-ADH shControl and shJMJD3 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for the indicated times was estimated by MTT assays. Mean ± SEM (n = 4).

Figure 5. Expression levels of *JMJD3, VDR* and *SNAI1* genes in human colon cancer patients. RNA levels of *JMJD3, VDR* and *SNAI1* were analyzed by qRT-PCR in normal and tumour tissue samples of 96 colon carcinoma patients. Quantification was performed as described in Material and Methods. (**A**) Scattergram showing the relation between *JMJD3* and *VDR* RNA levels in each patient. The *P* value was calculated using the Pearson test. (**B**) Box-plot of *JMJD3* expression in tumours expressing or not *SNAI1* RNA. Boxes in the plots include values in the 25%-75% interval, internal lanes represent the median, and values higher than 1.5 box lengths were represented as outliers (o). The *P* value was calculated using the ANOVA test.

LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure 1. Effect of $1,25(OH)_2D_3$ on the expression of ten JmjC-domain proteins in colon cancer cells. qRT-PCR analysis of RNA levels in SW480-ADH cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for the indicated times. *CYP24A1* and *CDH1*/E-cadherin genes that are induced, and *CYP27B1* gene that is repressed by $1,25(OH)_2D_3$ were used as controls. *SDHA* was used for normalization. Mean ± SEM (n = 3).

Supplementary Figure 2. $1,25(OH)_2D_3$ induces *JMJD3* expression in a panel of human cancer cell lines of different origin. (**A**) qRT-PCR analysis of *JMJD3* RNA levels in colon (SW1417, SW48, KM12C), breast (MCF7, MDA-MB-453, MDA-MB-231) and skin (UACC-257) cancer cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. SW620 and SW480-R cell lines that lack VDR expression and do not respond to $1,25(OH)_2D_3$ were used as control. (**B**) qRT-PCR analysis of *JMJD3* RNA levels in SW480-ADH cells treated with 10^{-7} M of the indicated agents for 48 h. (**A** and **B**) *18S* rRNA was used for normalization. Data (mean ± SD) correspond to one representative experiment performed in triplicate.

Supplementary Figure 3. VDR does not bind to three putative VDREs in the human *JMJD3* gene. (**A**) Genomic location of three putative VDREs (R1, R2 and R3) present in the human *JMJD3* gene revealed by *in silico* analysis. VDREs are represented as red boxes and their sequence is indicated. Blue boxes correspond to exons (small boxes: noncoding exons; large boxes: coding exons). ESC-TSS: transcription start site active in embryonic stem cells; MF-TSS: transcription start site active in macrophages. (**B**) X-ChIP analysis of the association of VDR (left panels) or RNA polymerase II (right panels) to the three VDREs and the transcription start site (TSS) of the human *JMJD3* gene in SW480-ADH cells treated with 10^{-7}

M $1,25(OH)_2D_3$ or vehicle for the indicated times. Enrichment was measured by q-PCR with specific primers to each region and normalized over input. Mean ± SEM (n = 3).

Supplementary Figure 4. Ectopic expression of the mutant inactive JMJD3 MUT 1141-1641 polypeptide inhibits the induction of an adhesive epithelial phenotype and target genes by $1,25(OH)_2D_3$ without affecting VDR expression. (**A**) qRT-PCR analysis of the RNA levels of *JMJD3* and *VDR* in Mock, WT 1141-1641 and MUT 1141-1641 SW480-ADH cells. *SDHA* and *18S* were used for normalization. Data (mean ± SD) correspond to one representative experiment performed in triplicate. (**B**) Western blot analysis of the ectopic expression of WT 1141-1641 and MUT 1141-1641 JMJD3 polypeptides and of VDR in Mock, WT 1141-1641 and MUT 1141-1641 SW480-ADH cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. β-Actin and Lamin B were used as controls. (**C**) Left panels, representative phase-contrast images of Mock, WT 1141-1641 and MUT 1141-1641 SW480-ADH cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. Bar: 15 μm. Right panels, representative immunofluorescence and confocal microscopy images of E-cadherin protein expression in the same cells. Bar: 10 μm. (**D**) qRT-PCR analysis of the RNA levels of *CDH1*/E-cadherin, *CYP24A1* and *CST5*/cystatin D in Mock, WT 1141-1641 and MUT 1141-1641 SW480-ADH cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 8 h. *18S* was used for normalization. Mean ± SEM (n = 3). (**E**) Activity of the *CDH1*/E-cadherin promoter in SW480-ADH Mock, WT 1141-1641 and MUT 1141-1641 cells. The cells were transfected with the *CDH1*/E-cadherin promoter construct (-987 to +92 bp) and the expression of luciferase in cell extracts was estimated 48 h later. Mean ± SEM (n = 3). (**F**) Western blot analysis of E-cadherin and Cystatin D protein expression in SW480-ADH Mock, WT 1141-1641 and MUT 1141-1641 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. β-Actin was used as control. Numbers refer to the fold-

change with respect to untreated Mock cells. The Western blot shown correspond to a representative experiment of the three performed.

TABLES

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

Gene	Forward Primer	Reverse Primer
KDM2A	GAAGAGGAGGAGGAGGAGGA	GCTGAGGTAGCGGAAGACAG
KDM3A	TCAAGAAAACCCAGCAGACC	CACCACATCCCCAAGAAACT
JMJD1C	TACATCACGACGCAGGTCTC	CTTGGGAGTTCATTGCTGGT
JMJD3	TGCTCCGTCAACATCAACAT	CTTCTGCTCCGTCAACATCA
JMJD4	GTGGGGAATGGACTCTCGT	GACTCTCGGTCAAGGACAGG
MINA	TCAGAAAAGGGCAACGATTC	CCAACCAAGGAGCCAAAGTA
PHF2	GAGGCAAGAAGAAAGGGAAGA	GGGCACACTCAGGACACTCT
UTY	CCACCAACTTCACCATACCC	GGAGGAAAGAAAGCATCACG
KDM5C	GTGGTGGAGGAAGGTGGTTA	AGCGTAGCAAGGAGCCAATA
CYP27B1	CCTCAGCCACTGTTCTGTCC	CCAGCGTATTTTTGGGGATA
VDR	TTGCCATACTGCTGGACGC	GGCTCCCTCCACCATCATT
FN1	GGGAGCCTCGAAGAGCAAG	AACCGGGCTTGCTTTGAC
LEF1	CGAAGAGGAAGGCGATTTAG	GTCTTGGCCACCTCGTGTC
CLDN1	TTGGTCTCTATCTCCTGAATC	ATTGTGTGGGAAGGTCAA
CLDN7	AGCTTGCTCCTGGTATGGC	CAATAAAGAGGCAGGGCC
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
CDH1	AGAACGCATTGCCACATACACTC	CATTCTGATCGGTTACCGTGATC
SNAI1	CACTATGCCGCGCTCTTTC	GGTCGTAGGGCTGCTGGAA
SNAI2	GGCAAGGCGTTTTCCAGAC	GCTCTGTTGCAGTGAGGGC
ZEB1	GCCAATAAGCAAACGATTCTG	TTTGGCTGGATCACTTTCAAG
ZEB2	TATGGCCTACACCTACCCAAC	AGGCCTGACATGTAGTCTTGTG

ABBREVIATIONS

$1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; JMJD3, jumonji domain containing 3; KDM6B, lysine (K)-specific demethylase 6B