LETTERS

DNA demethylation in hormone-induced transcriptional derepression

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Epigenetic modifications at the histone level affect gene regulation in response to extracellular signals[1,2]. However, regulated epigenetic modifications at the DNA level, especially active DNA demethylation, in gene activation are not well understood[3–5]. Here we report that DNA methylation/demethylation is hormonally switched to control transcription of the cytochrome p450 27B1 (*CYP27B1*) gene. Reflecting vitamin-D-mediated transrepression of the *CYP27B1* gene by the negative vitamin D response element (nVDRE)[6,7], methylation of CpG sites (^{5m}CpG) is induced by vitamin D in this gene promoter. Conversely, treatment with parathyroid hormone, a hormone known to activate the *CYP27B1* gene[8], induces active demethylation of the ^{5m}CpG sites in this promoter. Biochemical purification of a complex associated with the nVDRE-binding protein (VDIR, also known as TCF3)[6,7] identified two DNA methyltransferases, DNMT1 and DNMT3B, for methylation of CpG sites[9], as well as a DNA glycosylase, MBD4 (ref. 10). Protein-kinase-C-phosphorylated MBD4 by parathyroid hormone stimulation promotes incision of methylated DNA through glycosylase activity[11], and a base-excision repair process seems to complete DNA demethylation in the MBD4-bound promoter. Such parathyroid-hormone-induced DNA demethylation and subsequent transcriptional derepression are impaired in *Mbd4*$^{-/-}$ mice[12]. Thus, the present findings suggest that methylation switching at the DNA level contributes to the hormonal control of transcription.

CYP27B1 is the final enzyme in vitamin D biosynthesis, and it is primarily expressed in the renal proximal tubule[6–8]. Two calcemic hormones strictly regulate *CYP27B1* gene transcription[6,7]. Parathyroid hormone (PTH) induces CYP27B1 expression by activating protein kinase A and C (PKA and PKC, respectively)[8,13]. 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2$D3), a hormonally active form of vitamin D3, is a repressive signal that binds to and activates the nuclear vitamin D receptor (VDR)[14–16]. A basic helix–loop–helix transcriptional activator (VDR interacting repressor, VDIR) regulates the transcription of *CYP27B1* by the negative vitamin D response element (nVDRE)[6,7]. Heterodimers of vitamin-D-bound VDR and retinoid X receptor (RXR) repress the activation of VDIR that is bound upon the nVDRE by means of the histone deacetylase (HDAC) co-repressor complex[6].

We found that the HDAC inhibitor tricostatin A (TSA) did not fully abrogate vitamin-D-induced transrepression in either 293F cells or mouse cortical tubular (MCT) cells (Supplementary Fig. 2). Using newly established stable 293F and MCT transformants expressing Flag–VDIR, we tested for other factor(s)/complex(es) that co-repressed transcription[17,18] (Supplementary Fig. 3a). The VDIR and VDR interactants consisted of several complexes when fractionated on an ion-exchange column (Supplementary Fig. 3b) and a glycerol gradient (data not shown)[17,18]. We identified DNA methyltransferases 1 and 3B (DNMT1 and DNMT3B)[9] as VDIR and VDR interactants (Fig. 1a) with DNMT activity (Fig. 1g). Generally, DNMT family members methylate cytosines at specific DNA sequences to repress gene expression[19]. In a luciferase assay in 293F cells, both DNMT1 and DNMT3B acted as co-repressors for vitamin-D-induced transrepression of the *CYP27B1* promoter (Supplementary Fig. 5). Vitamin-D-induced DNA methylation of cytosines (^{5m}C) was found in the CpG regions of the promoter in 293F cells (see Fig. 1b) and MCT cells (data not shown), as assessed by bisulphite sequencing (Fig. 1c and Supplementary Fig. 6), methylation-specific PCR (Supplementary Fig. 7), and chromatin immunoprecipitation and quantitative PCR (ChiP-qPCR) with an anti-^{5m}C antibody (Fig. 1d). This methylation step required DNMT1 and DNMT3B (Fig. 1d and Supplementary Fig. 8) based on knockdown assays (Supplementary Fig. 4). The knockdown of DNMTs abrogated vitamin-D-induced transrepression of endogenous *CYP27B1* (Fig. 1e), and a DNMT inhibitor, 5-azacytidine, plus TSA, abolished vitamin-D-induced transrepression (Supplementary Fig. 2). Vitamin-D-induced recruitment of DNMT1 and DNMT3B was detected at the *CYP27B1* promoter by ChIP analysis (Fig. 1f and Supplementary Fig. 9), presumably as direct VDR interactants as observed in an *in vitro* glutathione *S*-transferase (GST)-pull-down assay (Supplementary Fig. 10). DNA methylation of the CpG sites in the promoter and coding regions (Fig. 1c, d), as well as histone deacetylation (Fig. 1d, f), were induced by vitamin D, but a heterochromatin marker, HP1α[18,20], was not detected (Supplementary Fig. 11). Upregulation of DNMT activity of the VDIR immunocomplex by vitamin D was reduced in the presence of PTH (Fig. 1g). Continued PTH treatment of cells that had been pre-incubated with vitamin D induced demethylation of the ^{5m}CpGs (Figs 1c, 2d and Supplementary Fig. 7).

Next, we assayed for a PTH effect in DNA replication. In proximal renal tubule cells expressing the *Cyp27b1* gene in mice, bromodeoxyuridine (BrdU) incorporation (Supplementary Fig. 12a)[20] was not affected by either 48 h PTH treatment or VDR deficiency ($Vdr^{-/-}$)[21] (Fig. 2a). Likewise, PTH-induced DNA demethylation of the *CYP27B1* promoter in 293F cells was detected despite 24 h arrest of the cell cycle after serum depletion or treatment with aphidicolin[22] (Fig. 2b and Supplementary Fig. 12b, c). It was thus unlikely that ^{5m}C replaces C in the newly synthesized DNA during DNA replication.

We characterized MBD4 (refs 10, 12) further because MBD4 recruitment to the promoter coincided with the state of DNA methylation (Fig. 2c, d and Supplementary Fig. 13). MBD4 belongs to the 5-methyl-CpG binding domain (MBD) family, which is implicated in

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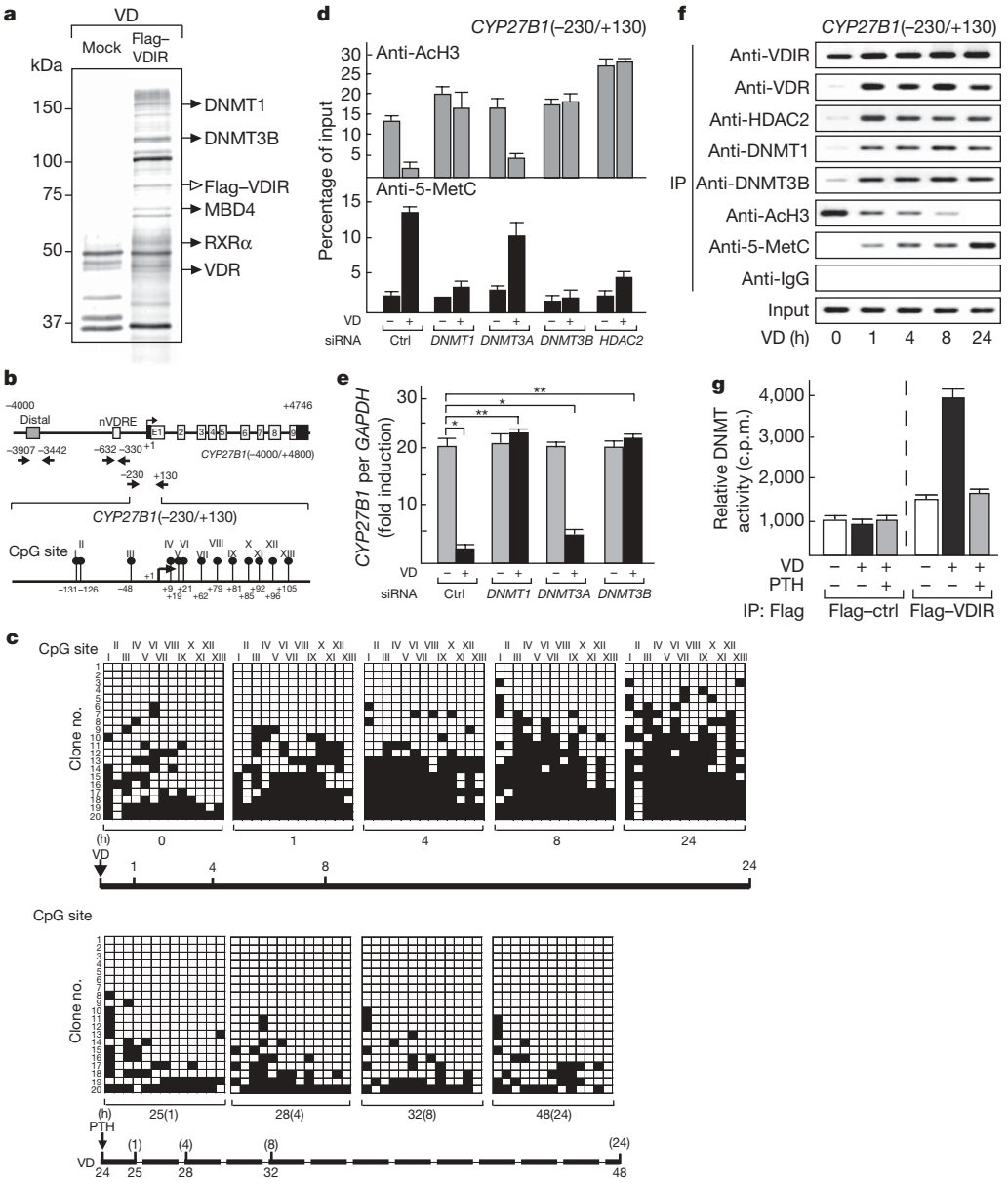

Figure 1 | Hormonal control of DNA methylation/demethylation of the *CYP27B1* gene promoter. **a**, Silver staining of anti-Flag-affinity purification, followed by anion-exchange column chromatography using vitamin D (VD; 100 nM)-treated MCT cells expressing Flag–VDIR. **b**, Schematic representation of the CpG sites in the *CYP27B1* promoter and coding region. Black filled circles indicate CpG sites. **c**, Time course of DNA methylation/demethylation in *CYP27B1*(−230/+130) region. Bisulphite sequencing was performed using vehicle-treated, vitamin D (100 nM)-treated and vitamin D/PTH (1 μM)-treated 293F cells for the indicated time. Numbers in parentheses denote time after PTH treatment. White and black squares indicate unmethylated and methylated CpGs, respectively. **d**, **e**, ChIP–qPCR analyses of 293F cells transfected with indicated short interfering RNAs (siRNAs), treated with vitamin D (100 nM) for 24 h (means ± s.d., $n = 3$) (**d**) and qPCR (means ± s.d., $n = 3$, $^{*}P < 0.005$, $^{**}P > 0.2$) (**e**). Ctrl, control. **f**, Time-dependent ChIP analyses using 293F cells with vitamin D (100 nM) treatments for the indicated times. **g**, DNMT activity using Flag–VDIR immunoprecipitants (IP) in vitamin D/PTH-treated 293F cells. Activity (means ± s.d., $n = 3$) is shown as c.p.m. of *S*-adenosyl-L-[methyl-^{3}H]-methionine incorporated into oligonucleotide substrate. Background activity was measured in the control experiments performed with Flag-alone immunoprecipitants.

transcriptional repression[23]. Unlike other MBD family members, MBD4 functions in DNA repair as a thymine glycosylase to remove T/G mismatches generated after the deamination of ^{5m}C (refs 10, 24). ChIP analyses showed that MBD4 was the only MBD protein identified at the *CYP27B1* promoter (Fig. 2c). MBD4 was co-immunoprecipitated with VDIR in the presence of vitamin D, and remained associated after co-treatment of vitamin D with PTH independent of the dissociation of DNMTs from VDIR (Fig. 2e). These hormonal effects were not seen in the reported MBD4-binding ^{5m}CpGs sites in the multidrug resistance (*MDR*, also known as *ABCB1*) gene promoter[25] (Supplementary Fig. 14). VDIR seemed to be indispensable for MBD4 recruitment to the promoter (Fig. 2f and Supplementary Fig. 15), presumably through physical interaction (Supplementary Fig. 16). It was recently reported that in transcriptionally active promoters, demethylation of ^{5m}CpGs requires DNMT3A/B for deamination of ^{5m}CpGs for further T/G mismatch repair[26]. However, in 293F cells pretreated with vitamin D for 24 h, DNMT1, DNMT3B or thymine-DNA glycosylase (TDG) was dispensable for PTH-induced demethylation of ^{5m}CpGs within the *CYP27B1* promoter (Fig. 2g). Knockdown of *MBD4*, but not *MBD2*, blocked PTH-induced demethylation of ^{5m}CpGs (Fig. 2h, i and Supplementary Fig. 17). Given the efficient binding of MBD4 to ^{5m}CpG (ref. 10), we proposed that the DNA glycosylase activity of MBD4 induced active DNA demethylation of the ^{5m}CpG sites. We tested this idea with an MBD4 mutant with a deletion in the putative

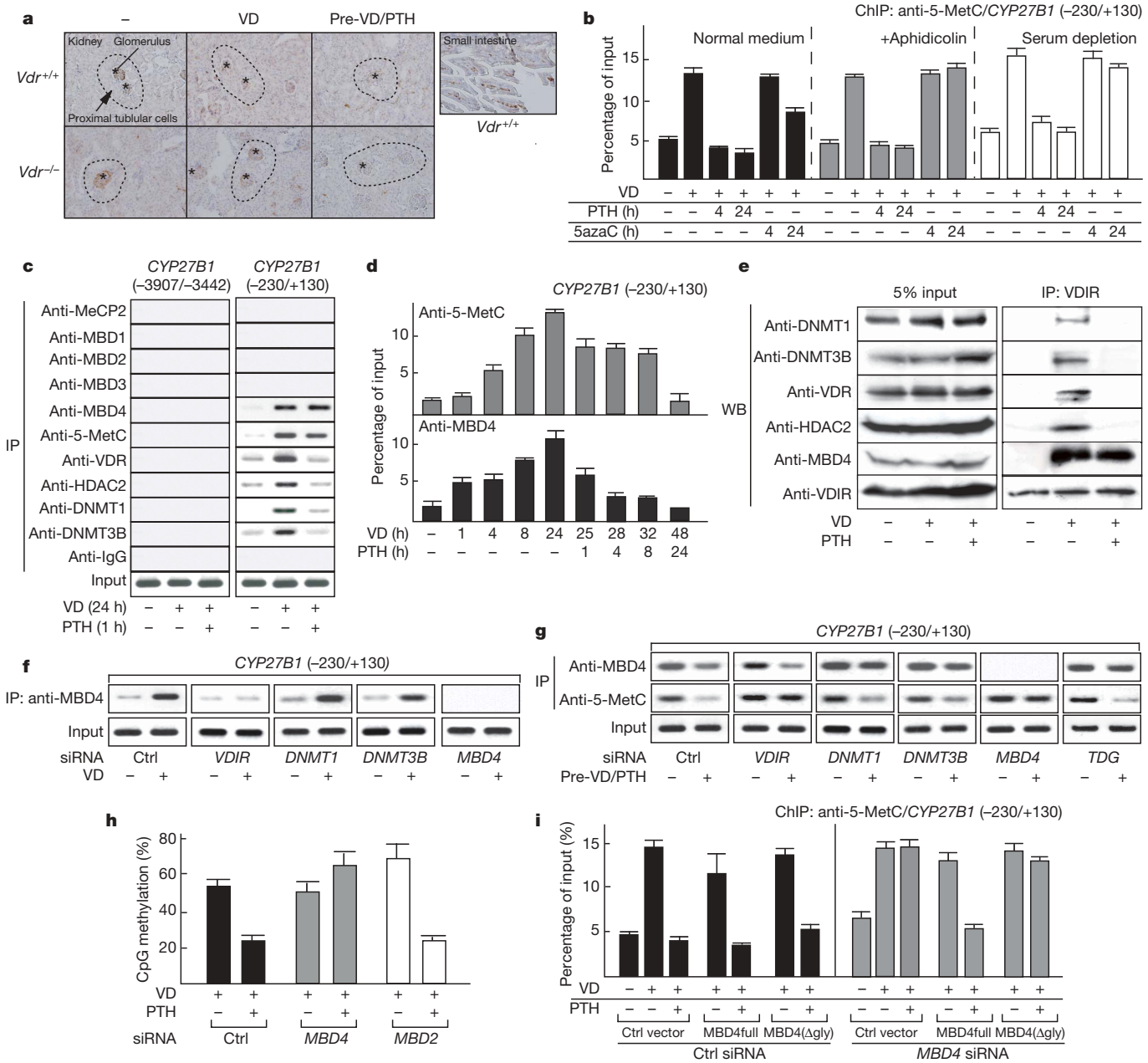

Figure 2 | MBD4 is indispensable for PTH-induced DNA demethylation in the *CYP27B1* promoter. **a**, *In vivo* BrdU incorporation in the kidney and small intestine (control) of mice with indicated genotypes. Proximal tubular cells surrounding the glomerulus (asterisk) are circled by a dashed line. Original magnification, ×200. Experimental details are presented in Supplementary Fig. 11a. **b**, ChIP–qPCR analyses using 293F cells treated with aphidicolin (5 μM) or cultured with serum-free DMEM (means ± s.d., $n = 3$). 5azaC, 5-azacytidine. **c**, **d**, ChIP analyses (**c**) and ChIP–qPCR analyses (means ± s.d., $n = 3$) (**d**) using vitamin D/PTH-treated 293F cells. **e**, Western blotting (WB) using immunoprecipitants with anti-VDIR antibody in vitamin D/PTH-treated MCT cells. **f**, ChIP analyses using 293F cells transfected with indicated siRNAs for 24 h, and then treated with vitamin D (24 h). **g**, ChIP analyses using vitamin-D-treated 293F cells transfected with indicated siRNAs for 24 h, then further treated with PTH (24 h). **h**, Bisulphite sequencing using 293F cells transfected with indicated siRNAs for 24 h, then further treated with vitamin D and PTH (24 h) (means ± s.d., three independent experiments, $n = 15$). **i**, ChIP–qPCR analyses of vitamin D/PTH-treated 293F cells transfected with siRNAs and rescue vectors containing wild-type MBD4 (MBD4full) or a MBD4(Δgly) for 48 h in the presence or absence of vitamin D (48 h) and/or PTH (24 h) (means ± s.d., $n = 3$).

glycosylase catalytic domain (Δgly) (Supplementary Figs 19a and 21a). This mutant was defective in PTH-induced DNA demethylation in cells deficient of endogenous MBD4 (Fig. 2i and Supplementary Fig. 18).

To test the idea that downstream signalling of PTH activates MBD4-mediated DNA demethylation, phosphorylation of putative sites on MBD4 by PKA and PKC (downstream signalling factors of PTH)[13] was measured with recombinant proteins (Supplementary Fig. 19). MBD4 was phosphorylated by PKC *in vitro* (Fig. 3a) and in 293F cells (Fig. 3b and Supplementary Fig. 20). With MBD4 recombinant mutants, serine residues (165 and 262) were mapped as PKC-phosphorylation sites (Fig. 3c). We addressed whether MBD4 has DNA glycosylase activity with mismatched and methylated CpG oligonucleotides, using TDG as a control, in *in vitro* assays[23,26]. Strand incisions by unphosphorylated MBD4 were clearly seen in T/G mismatched oligonucleotides, confirming the reported glycosylase activity of MBD4 for T/G mismatch (Fig. 3d, e and Supplementary Fig. 21)[10,26]. Although the direct DNA binding of MBD4 to both oligonucleotides was detected on electrophoretic mobility shift assays (EMSA) (Supplementary Fig. 22), strand incision of the methylated CpG oligonucleotides was less pronounced. However, strand incision of methylated CpG oligonucleotides was

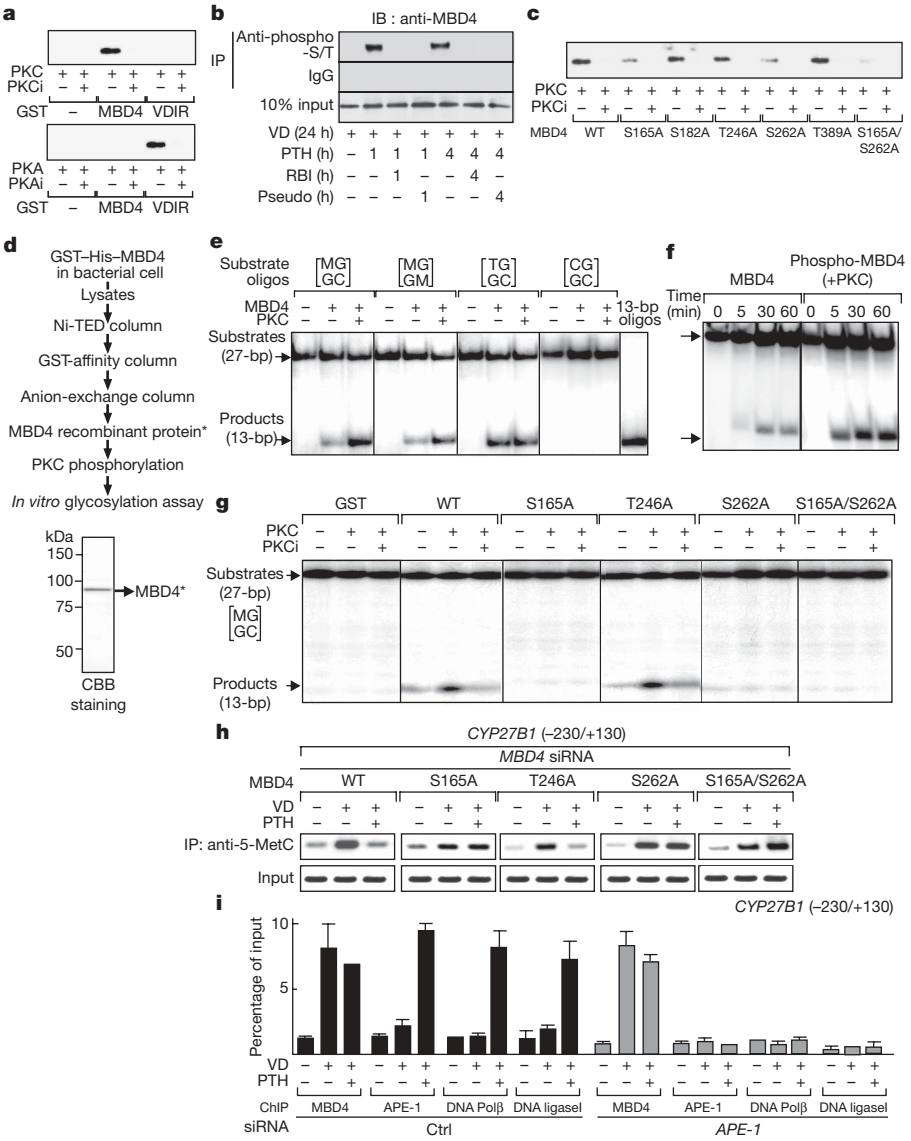

Figure 3 | The DNA glycosylase activity of MBD4 is potentiated by PKC-phosphorylation. a, *In vitro* kinase assay using GST–MBD4 and GST–VDIR with recombinant PKC or PKA. PKC inhibitors (PKCi; RBI and PKC pseudosubstrate) or a PKA inhibitor (H89) were added to the reactions as indicated. **b**, Western blotting with anti-MBD4 using immunoprecipitants with anti-phospho-S/T antibody on the treatments as indicated. **c**, *In vitro* kinase assay using recombinant MBD4 mutants, in which alanine replacements were introduced into the serine or threonine residues. **d**, *In vitro* glycosylation assay protocol (top) and CBB staining of human recombinant MBD4 (bottom). Experimental details of recombinant MBD4 preparations are presented in Supplementary Fig. 21. **e**, *In vitro* glycosylation assay with 0.5 μg of recombinant MBD4 and 5 nM of indicated oligonucleotides: hemi-methylated CpG [MG/GC], symmetrical methylated CpG [MG/GM], mismatched [TG/GC] and unmethylated CpG [CG/GC]. The top arrow indicates substrates (27-base pair (bp) oligonucleotides) and the bottom arrow indicates breakage products (13-bp oligonucleotides). **f**, The kinetics of the PKC-mediated ^{5m}CpG glycosylation activity of MBD4 with MG/GC. **g**, *In vitro* glycosylation assay using phosphorylation-defective MBD4 mutants. **h**, ChIP analyses of 293F cells transfected with *MBD4* siRNA and rescue vectors containing indicated MBD4 derivatives. **i**, ChIP–qPCR analyses of vitamin D/PTH-treated 293F cells transfected with *APE-1* siRNAs (means ± s.d., $n = 3$).

significant when MBD4 was phosphorylated by PKC[22] (Fig. 3e, f and Supplementary Fig. 23). A PKC inhibitor attenuated the enzymatic activity of MBD4 (Fig. 3g). Consistent with this, phosphorylation mutants attenuated PTH-induced DNA demethylation in 293F cells deficient in endogenous MBD4 (Fig. 3h), although their DNA-binding activities were retained (Supplementary Fig. 24). We then evaluated whether major factors responsible for DNA repair were involved in this mechanism[11,26,27]. ChIP analyses showed that apurinic/apyrimidinic (AP) endonuclease-1 (APE-1, also known as APEX1), DNA ligase I and polymerase (Pol) β—components of the base-excision repair process—were recruited simultaneously to the promoter together with MBD4 (Fig. 3i and Supplementary Fig. 25). APE-1 was pivotal in the recruitment of these DNA repair factors (Fig. 3i). These data indicate that DNA demethylation is completed through a base-excision repair process after glycosylation by MBD4. Consistent with the role of MBD4 in PTH-induced DNA demethylation, MBD4 knockdown reversed the effects of PTH on the *CYP27B1* promoter (Fig. 4a) and endogenous gene expression (Supplementary Fig. 26). Neither the MBD4 phosphorylation mutants nor the catalytic domain deletion mutant (Δgly) conferred a response to PTH (Fig. 4b and Supplementary Fig. 27). In ChIP analyses of the *CYP27B1* promoter, PTH-induced DNA demethylation was coupled to histone acetylation, H3K4 methylation and Ser-5-phosphorylated RNA Pol II recruitment[3] (Fig. 4c and Supplementary Fig. 28). Such PTH-induced alterations were abolished by depletion of MBD4 (Fig. 4c).

Finally, the physiological role of MBD4 in PTH-induced derepression was tested in *Mbd4*$^{-/-}$ mice. Vitamin D treatment for 3 days effectively suppressed endogenous *Cyp27b1* gene expression (Fig. 4d,

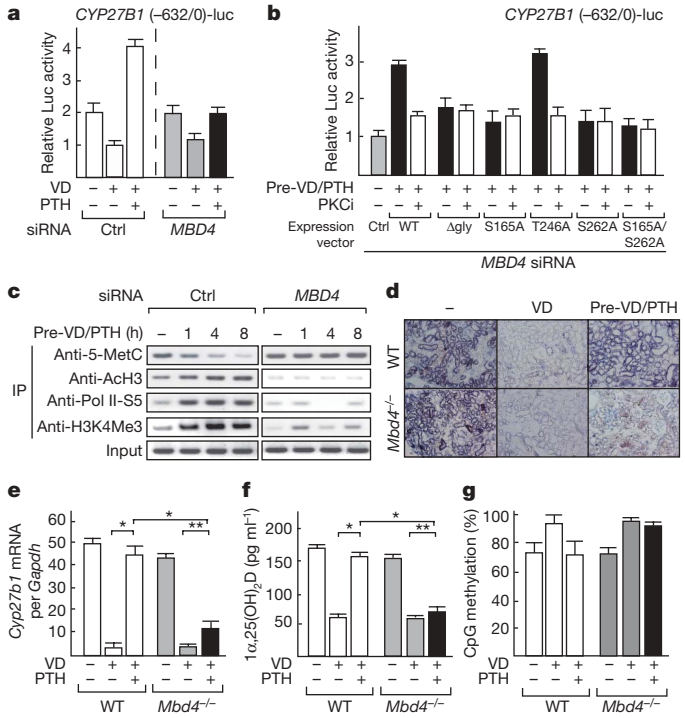

Figure 4 | MBD4-mediated DNA demethylation derepresses transcription of the vitamin D-transrepressed *CYP27B1* gene. **a**, Luciferase assay using 293F cells. The cells were transfected with *MBD4* siRNA for 24 h, and luciferase reporter containing the *CYP27B1*(−632/0) promoter, in the presence or absence of vitamin D (48 h) and/or PTH (last 24 h) (means ± s.d., $n = 3$). **b**, Luciferase assay using MBD4-knockdown 293F cells, transfected with phosphorylation-defective MBD4 mutants. **c**, ChIP analyses of vitamin D/PTH-treated 293F cells transfected with indicated siRNAs. **d**, *In situ* hybridization analysis of murine kidneys using antisense riboprobe for *Cyp27b1*. The *Cyp27b1* mRNAs (dark blue) are broadly localized in the uriniferous tubule cells. Original magnification, ×200. **e**, **f**, qPCR from total RNAs isolated from murine kidneys (**e**) and serum levels of $1\alpha,25(OH)_2D$ (**f**) with the indicated genotypes (wild type, $Mbd4^{-/-}$ medium-chain triglyceride vehicle-treated group, $n = 4$; wild type, $Mbd4^{-/-}$ vitamin-D-treated group, $n = 3$; wild type, $Mbd4^{-/-}$ pre-vitamin-D-PTH-treated group, $n = 7$) (means ± s.d., $*P < 0.001$, $**P > 0.2$). **g**, Bisulphite sequencing using whole murine kidneys (means ± s.d., three independent experiments, $n = 30$).

e) and induced DNA methylation in the kidneys of wild-type and $Mbd4^{-/-}$ mice (Fig. 4g and Supplementary Fig. 29). Administration of PTH to vitamin-D-pretreated, wild-type mice derepressed *Cyp27b1*, as determined by qPCR with reverse transcription (qRT–PCR; Fig. 4e) and *in situ* hybridization (Fig. 4d). The recovery of serum $1\alpha,25(OH)_2D$ levels (Fig. 4f) as well as DNA demethylation (Fig. 4g) were consistently seen. In $Mbd4^{-/-}$ mice, PTH effects were significantly impaired (Fig. 4d–g), confirming the *in vitro* observations.

Active DNA demethylation has been implicated in the derepression of silenced gene promoters during early development[3–5]. The involvement of active DNA demethylation of gene promoters for regulated transcription is, however, largely unknown. Here we found that DNA methylation/demethylation that determined the function of the *CYP27B1* gene promoter was regulated by hormonal switching (see Supplementary Fig. 1). Transcriptional derepression of the *CYP27B1* gene by active DNA demethylation was hormonally inducible. Thus, epigenetic switching at the DNA level reflects, at least in part, well-established hormonal actions on gene regulation.

Hormonally regulated DNA demethylation of the *CYP27B1* gene promoter is mediated by glycosylase activity of MBD4 by PKC-mediated phosphorylation. Although MBD4 is involved in repairing mismatched T/G, structural alteration of MBD4 caused by phosphorylation may shift its substrate specificity. Considering the association of base-excision repair factors with MBD4 on ^{5m}CpG sites in the *CYP27B1* gene promoter, it is likely that this form of active DNA demethylation involves a base-excision repair process. The dissociation of DNMTs from VDIR and MBD4 in the presence of PTH seems to preclude re-methylation of the repaired cytosine. Thus, DNA repair-mediated DNA demethylation in metazoans is probably accomplished by diverse processes that are dependent on the epigenetic context[25,28–30].

METHODS SUMMARY

Biochemical purification of VDIR-associated proteins. Preparation of the nuclear extracts, anti-Flag affinity purification, and mass spectrometry were performed as previously described[17]. Details are provided in Supplementary Methods.

***In vitro* glycosylation assay.** *In vitro* glycosylation assays were performed essentially as previously described[10,23,26]. Recombinant GST–MBD4 and its derivatives were purified from *Escherichia coli*, and subjected to a phosphorylation reaction by PKC. 5′-end [γ-^{32}P]-ATP-labelled DNA substrates were incubated with phosphorylated or non-phosphorylated GST–MBD4 as detailed in the Supplementary Methods.

Bisulphite sequencing and methyl-specific PCR. Genomic DNA was isolated from cell lines and wild-type or *Mbd4*-deficient mice, treated with vehicle, vitamin D or PTH as indicated. Bisulphite treatment and sequencing were performed as described. Results are representative of at least three independent experiments. Details are provided in Supplementary Methods.

DNMT assay. The VDIR complex was purified by immunoprecipitation. 293F cells were transfected as indicated with Flag-tagged VDIR, and cultured in the presence or absence of vitamin D for 24 h, and then PTH for 24 h. After 48 h, the cells were collected, and the Flag-tagged VDIR complex was purified by Flag M2 agarose (Sigma). DNA methyltransferase activity was found in the VDIR immunoprecipitates from the 293F cells only after treatment with vitamin D. Activity is displayed as counts per minute (c.p.m.) of *S*-adenosyl-L-[methyl-^{3}H]-methionine incorporated into an oligonucleotide substrate.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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