

The Polycomb group protein EZH2 directly controls DNA methylation

Emmanuelle Viré¹, Carmen Brenner¹, Rachel Deplus¹, Loïc Blanchon¹, Mario Fraga², Céline Didelot¹, Lluís Morey³, Aleyde Van Eynde⁴, David Bernard¹, Jean-Marie Vanderwinden⁵, Mathieu Bollen⁴, Manel Esteller², Luciano Di Croce³, Yvan de Launoit^{1,6} & François Fuks¹

The establishment and maintenance of epigenetic gene silencing is fundamental to cell determination and function¹. The essential epigenetic systems involved in heritable repression of gene activity are the Polycomb group (PcG) proteins^{2,3} and the DNA methylation^{4,5} systems. Here we show that the corresponding silencing pathways are mechanistically linked. We find that the PcG protein EZH2 (Enhancer of Zeste homolog 2) interacts—within the context of the Polycomb repressive complexes 2 and 3 (PRC2/3)—with DNA methyltransferases (DNMTs) and associates with DNMT activity *in vivo*. Chromatin immunoprecipitations indicate that binding of DNMTs to several EZH2-repressed genes depends on the presence of EZH2. Furthermore, we show by bisulphite genomic sequencing that EZH2 is required for DNA methylation of EZH2-target promoters. Our results suggest that EZH2 serves as a recruitment platform for DNA methyltransferases, thus highlighting a previously unrecognized direct connection between two key epigenetic repression systems.

The PcG protein EZH2 is a histone methyltransferase associated with transcriptional repression. EZH2 catalyses the addition of methyl groups, at least partly, to histone H3 at lysine 27 (H3K27)^{6–9}. Methylated K27 serves as an anchorage point for the recruitment of additional PcG proteins^{6–8}, the binding of which contributes to formation of a repressive chromatin state. Besides EZH2, DNMTs are also key epigenetic regulators involved in transcriptional repression^{10–12}. In the work presented here, we examined whether EZH2 and DNMTs might function through a common mechanistic pathway to silence gene expression. As shown in Fig. 1a, DNA methyltransferase activity was purified from HeLa nuclear extracts in ‘pull-down’ experiments using EZH2 fused to glutathione S-transferase (GST) (Fig. 1a, lane 3). The association of EZH2 with DNMT enzymatic activity was specific, since only background activity was measured in control experiments performed with GST alone, or with another GST-fused protein (PLZF, for promyelocytic leukaemia zinc finger) (Fig. 1a, lanes 1 and 2, respectively). GST–SUV39H1 (a H3K9 methyltransferase known to interact with DNMT¹³) was used as a positive control for the assay (Fig. 1a, lane 4). Furthermore, an antibody raised against EZH2 specifically precipitated DNA methyltransferase activity from nuclear extracts of untransfected HeLa cells (Fig. 1b, lane 3).

One or more among several DNA methyltransferases might be responsible for the EZH2-bound DNMT activity. To address this issue, we performed GST pull-downs with GST–EZH2, incubated with *in vitro* translated (IVT) ³⁵S-labelled DNMT1, DNMT3A or DNMT3B. As shown in Fig. 2a, all three DNMTs were able to bind to full-length GST–EZH2 (Fig. 2a, lane 3), in contrast to GST alone

(Fig. 2a, lane 2). GST–SUV39H1 was again used as a positive control (Fig. 2a, lane 7). Using various GST-fused EZH2 fragments, we found that EZH2 associates with DNMTs primarily via its amino-terminal portion (Fig. 2a, lanes 4–6). In the reciprocal experiment, IVT EZH2 was found to interact with both the amino-terminal and carboxy-terminal parts of DNMT1, and with the conserved PHD (plant homeodomain)-like motif in DNMT3A and DNMT3B (Supplementary Fig. S1). To demonstrate an *in vivo* interaction between EZH2 and DNMTs, we performed co-immunoprecipitations from extracts of untransfected cells. Endogenous EZH2 co-immunoprecipitated with endogenous DNMT1, DNMT3A and DNMT3B (Fig. 2b). The interaction of EZH2 with DNMTs was further confirmed by reverse endogenous co-immunoprecipitation of DNMTs with EZH2 (Fig. 2c, lane 2). EZH2 is known to associate with other PcG proteins, EED and SUZ12, within the context of the PRC2/3 complexes¹⁴. In GST pull-down experiments, we found that DNMTs can interact with EED and that endogenous EED and SUZ12 both associate with DNMT enzymatic activity (Supplementary Fig. S2). EED also co-immunoprecipitated with DNMTs from extracts of untransfected cells (Fig. 2c, lane 3). Taken together, these results indicate that native EZH2 interacts with DNMTs in the context of the PRC2/3 complexes and associates with DNA methyltransferase activity *in vivo*.

Next, we probed the functional relationship between EZH2 and DNA methyltransferases. As these proteins act as transcriptional repressors^{11,12,15}, we investigated whether they can silence a common target gene. Recent work has identified several EZH2-target genes, including the *MYT1* gene¹⁶. We first examined whether *MYT1* could be reactivated by addition of the DNA methylation inhibitor 5-aza-deoxycytidine (5-aza-dC). Measuring levels of messenger RNA in U2OS cells by reverse transcription followed by polymerase chain reaction analysis (RT–PCR), we observed elevated transcript levels of *MYT1* after cell treatment with 5-aza-dC (Fig. 3a). In contrast, 5-aza-dC treatment did not affect the expression of an unrelated house-keeping gene (*actin*) (Fig. 3a). Thus, the EZH2-regulated gene *MYT1* is specifically reactivated by 5-aza-dC.

We next investigated whether DNA methyltransferases might contribute with EZH2 to *MYT1* silencing. For this, we performed RNA interference (RNAi) experiments in U2OS cells to knock down EZH2, DNMT1, DNMT3A or DNMT3B (Supplementary Fig. S3). As illustrated in Fig. 3b (lane 2), *MYT1* mRNA levels were markedly elevated in cells with reduced EZH2 levels (RNAi EZH2), in keeping with previous observations¹⁶. Similarly, knockdown of any one of the three DNMTs resulted in higher *MYT1* expression (Fig. 3b, lanes 3–5). These data indicate that silencing of *MYT1* gene expression requires both EZH2 and DNA methyltransferases.

¹Free University of Brussels, Faculty of Medicine, Laboratory of Molecular Virology, 808 route de Lennik, 1070 Brussels, Belgium. ²CNIO, Cancer Epigenetics Group, C/- Melchor Fernández Almagro 3, 28029 Madrid, Spain. ³ICREA and CRG, Passeig Marítim 37-49, E-08003 Barcelona, Spain. ⁴Division of Biochemistry, Faculteit Geneeskunde, Katholieke Universiteit Leuven, Herestraat 49, 3000 Leuven, Belgium. ⁵Free University of Brussels, Faculty of Medicine, Laboratory of Neurophysiology, 808 route de Lennik, 1070 Brussels, Belgium. ⁶UMR 8117, CNRS, Institut Pasteur de Lille, Université de Lille 1, Institut de Biologie de Lille, 1 rue Calmette, 59021 Lille, Cedex, France.

The results described above suggest that both EZH2 and DNMTs might bind to the *MYT1* promoter. To test this possibility, we performed chromatin immunoprecipitation (ChIP) experiments. As shown in Fig. 4a (left panel, lane 1, 'RNAi control'), we observed binding of EZH2 to the *MYT1* promoter. DNMT1, DNMT3A and DNMT3B were also found to bind to this promoter (Fig. 4a, left panel, lane 1, 'RNAi control'). Thus, both EZH2 and DNMTs associate with the *MYT1* promoter.

Next, we tested whether EZH2 is required for DNMT binding to the *MYT1* promoter using the RNAi EZH2 cells. In control experiments, EZH2 depletion resulted, as expected, in reduced EZH2

binding, concomitant with reduced H3K27 trimethylation (H3K27me3) (Fig. 4a, left panel, compare lane 1 with lane 2). More importantly, the decrease in EZH2 and H3K27me3 occupancy at the promoter coincided with a significant reduction of DNMT1, DNMT3A and DNMT3B binding to *MYT1* (Fig. 4a, left panel, compare lane 1 with lane 2). It is noteworthy that RNA polymerase II (RNA pol II) was detected at the *MYT1* promoter after EZH2 knockdown (Fig. 4a, left panel, lane 2), but not when EZH2 and DNMTs were bound to the promoter (Fig. 4a, left panel, lane 1). This is consistent with the reactivation of *MYT1* gene expression, highlighted in Fig. 3. These findings can be extended to additional known EZH2-target promoters—*WNT1*, *KCNA1* and *CNR1* (Fig. 4a, right panel; ref 16): in keeping with our observations with *MYT1*, we found that binding of DNMTs to these promoters is also

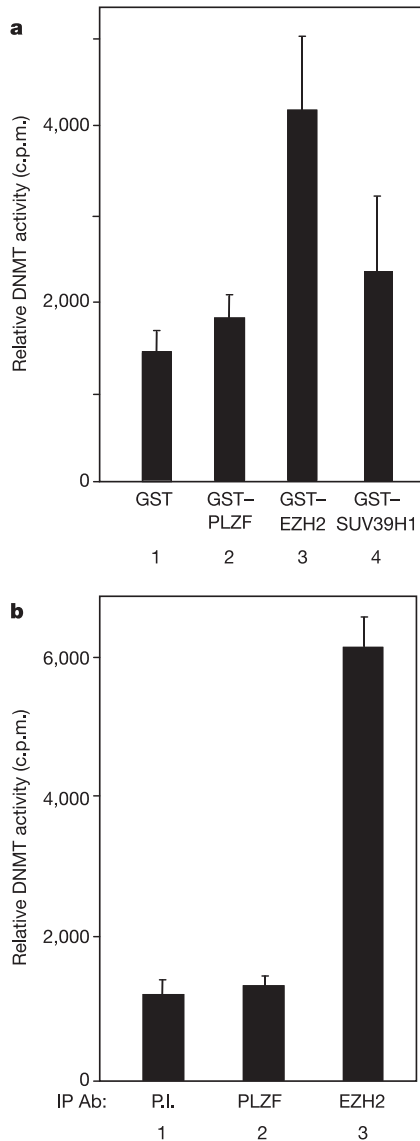


Figure 1 | The EZH2 Polycomb group protein associates with DNA methyltransferase activity. **a**, A GST-fused EZH2 protein was used to purify DNA methyltransferase activity from nuclear extracts. GST-SUV39H1 and GST-PLZF were also used as a positive¹³ and a negative²⁷ control, respectively. After incubation, the beads were washed and assayed for DNA methyltransferase activity read as c.p.m. of *S*-adenosyl-L[methyl-³H]methionine incorporated into an oligonucleotide substrate¹¹. **b**, Endogenous EZH2 associates with DNA methyltransferase activity. HeLa nuclear extracts were immunoprecipitated (IP) with either pre-immune serum (P.I.), a control antibody (ab) against PLZF²⁷, or an antibody against EZH2, and tested for associated DNA methyltransferase activity. The results are averages of at least three independent experiments with error bars showing standard deviations (s.d.).

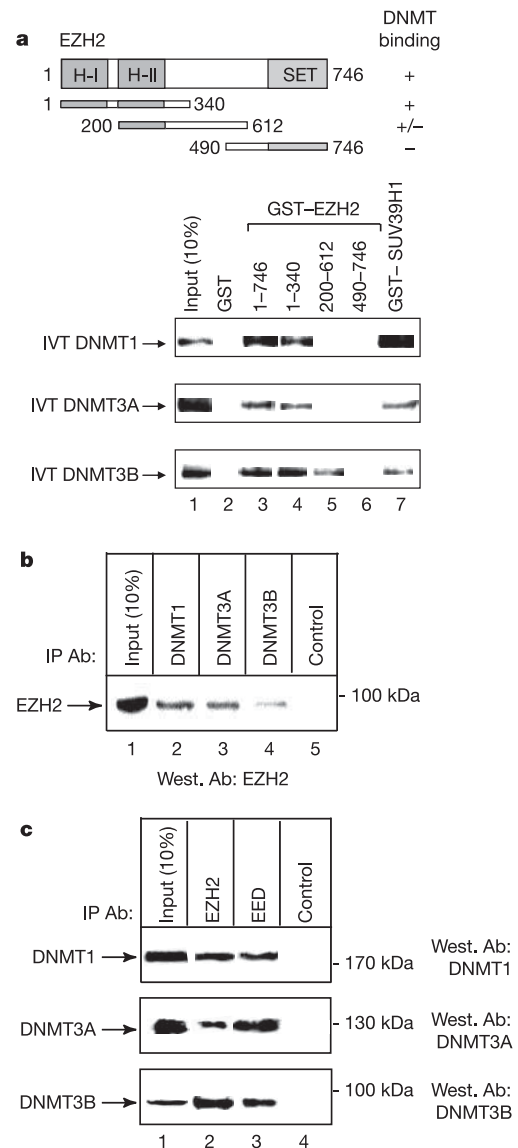


Figure 2 | EZH2 interacts with DNA methyltransferases *in vitro* and *in vivo*. **a**, *In vitro* translated (IVT) full-length DNMT1, DNMT3A or DNMT3B was incubated with the indicated GST fusions of EZH2. GST-SUV39H1 was used as a positive control for association with IVT DNMT proteins. **b**, EZH2 co-immunoprecipitates with DNA methyltransferases from untransfected cells. HeLa nuclear extracts were immunoprecipitated with the indicated antibodies. The presence of EZH2 in immunoprecipitates was visualized by western (west.) blot analysis using an antibody against EZH2. **c**, Endogenous co-immunoprecipitations of DNMTs with EZH2 and EED.

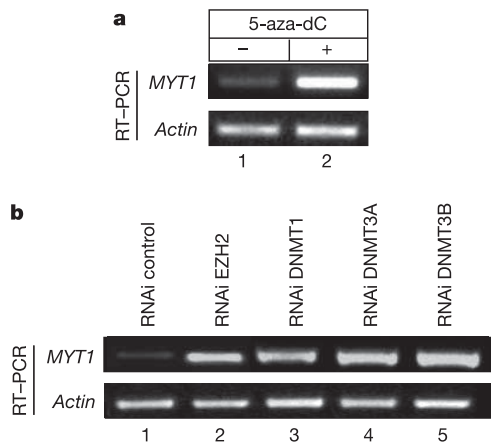


Figure 3 | Both EZH2 and DNA methyltransferases are required to repress the endogenous *MYT1* gene. **a**, The DNA methylation inhibitor 5-aza-dC reactivates *MYT1* expression. U2OS cells were either treated (lane 2) or not treated (lane 1) with 5-aza-dC (1 μ M). RT-PCR was performed to detect the presence of *MYT1* and *actin* transcripts. **b**, EZH2 or DNA methyltransferase depletion resulted in de-repression of endogenous *MYT1* expression. RT-PCR analysis of *MYT1* expression was performed on control cells and on the indicated single-gene knockdown cells.

compromised when association of EZH2 with the promoters is reduced (Fig. 4a, right panel). Together, these data indicate that the presence of EZH2 is necessary for binding of DNA methyltransferases to EZH2-target promoters. In contrast to these observations, DNA methyltransferases do not seem to be required for EZH2 or H3K27me3 binding to *MYT1* (Supplementary Fig. S4).

Having shown that EZH2 interacts physically with the DNA methyltransferases and facilitates their binding to EZH2-target promoters, we next examined whether EZH2 might influence their DNA methylation status. For this, we carried out bisulphite genomic sequencing on *MYT1* and *WNT1* in EZH2-overexpressed cells or in EZH2-knockdown cells. Overexpression of wild-type EZH2 was

found to increase CpG methylation of the promoters significantly, whereas overexpression of an EZH2 mutant, lacking the conserved SET domain (for Suppressor of variegation 3–9, Enhancer of Zeste, Trithorax), failed to induce DNA methylation (Supplementary Fig. S5). As shown in Fig. 4b, we found that a number of CpGs located within *MYT1* or *WNT1* were significantly less methylated in EZH2-depleted cells than in RNAi control cells (CpGs situated outside the regions presented showed no significant difference in DNA methylation status; data not shown). Taken together, these results indicate that EZH2 is needed for CpG methylation of EZH2-target promoters.

The results presented here reveal that the Polycomb group protein EZH2 controls CpG methylation through direct physical contact with DNA methyltransferases (Fig. 4c). It is well established that in various organisms H3K9 methylation can be a prerequisite to DNA methylation^{17–19}, which in mammals involves the SUV39H enzymes and also probably the HP1 (heterochromatin protein 1) adaptor^{13,19}. It should be noted, however, that there are situations in which H3K9 and H3K27 methylation silence gene expression without the involvement of DNA methylation^{20,21}. Nevertheless, our finding that EZH2-mediated H3K27 methylation can dictate CpG methylation adds complexity and versatility to the emerging picture in which methylation of histones and DNA can work hand-in-hand as part of an epigenetic program integrating gene-silencing networks within the cell. Our finding that the Polycomb group protein EZH2 associates directly with DNA methyltransferases may be relevant to many different processes. It may relate to the *de novo* methylation activity of DNMT3 proteins involved in regulating gene expression^{10,12}. In addition, it may involve the maintenance functions which all three DNMTs possess^{22,23} and which may be necessary for the stable inheritance of silenced epigenetic states. More precisely, our discovery suggests a possible mechanism for epigenetic memory, in which an ‘epigenetic memory module’ consisting of both EZH2 and DNMTs might allow coordinated and heritable transmission of silenced epigenetic states through DNA replication. The close connection identified here between EZH2 and DNA methyltransferases might also be relevant to X-chromosome inactivation in female mammals, where both EZH2 and DNMTs are known to contribute

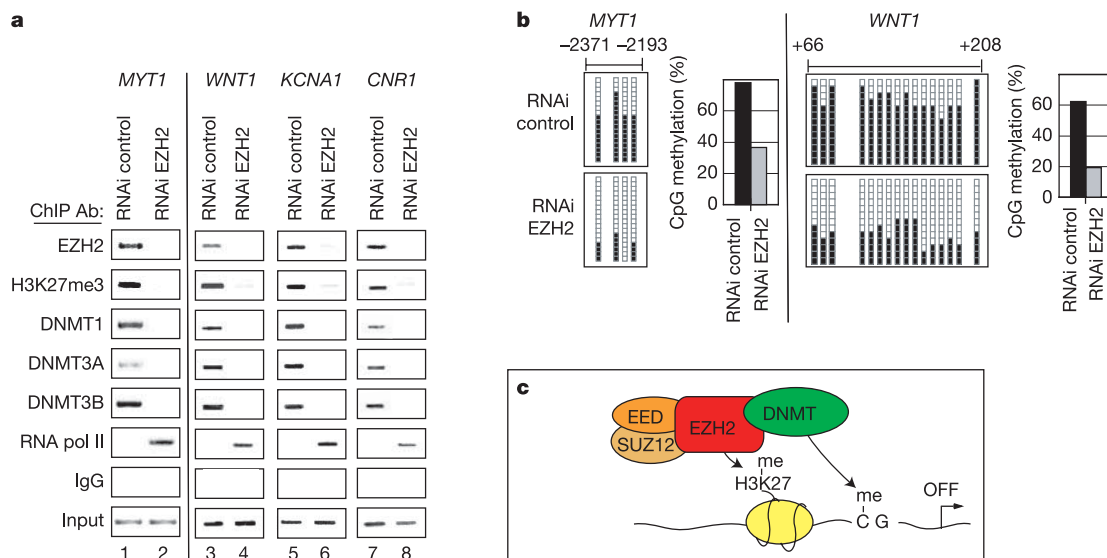


Figure 4 | EZH2 is required for binding of DNA methyltransferases and facilitates CpG methylation of EZH2-target promoters. **a**, ChIPs show that association of DNA methyltransferases with *MYT1* (left panels), as well as with other known EZH2-target genes (right panels; *WNT1*, *KCNA1* and *CNR1*; ref. 16), depends on EZH2. Cross-linked chromatin from mock-knockdown U2OS cells (RNAi control, lane 1) or EZH2-knockdown cells (RNAi EZH2, lane 2) was immunoprecipitated with the indicated

antibodies. **b**, Bisulphite sequencing shows that depletion of EZH2 (RNAi EZH2) leads to decreased methylation at a number of CpG sites within the *MYT1* (left panel) or the *WNT1* (right panel) promoter. Each row represents a single sequence analysed, and each square is a single CpG site. White and black squares represent unmethylated and methylated CpGs, respectively. **c**, EZH2 controls CpG methylation, in the context of the PRC2/3 complexes, through direct physical contact with DNA methyltransferases.

to maintaining the inactive state^{24–26}. Taken together, our results provide the first glimpse of a mechanistic link between two essential epigenetic systems: the Polycomb group proteins and the DNA methylation systems.

METHODS

GST fusion, pull-down assays and DNA methyltransferase assays. GST pull-downs and DNA methyltransferase assays were carried out as previously described^{11,27}. Plasmid constructs and antibodies used are detailed in the Supplementary Methods.

Immunoprecipitations and western blot analysis. Standard procedures were used for endogenous immunoprecipitations and western blotting¹³. See the Supplementary Methods for antibodies used.

Cell cultures and RNA interference. U2OS cells and the packaging cell line 293 GP were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 8% fetal bovine serum and grown at 37 °C under 5% CO₂. See the Supplementary Methods for RNA interference.

RNA purification and RT-PCR analysis. RT-PCR was performed as described previously²⁷. Primer sequences are available on request.

Chromatin immunoprecipitation. ChIP assays were performed as described previously²⁷, except that sonication of chromatin was performed with 30-s on-and-off cycles at high settings (Bioruptor, Diagenode). Details of antibodies used and PCR conditions can be found in the Supplementary Methods.

Bisulphite genomic sequencing. Bisulphite genomic sequencing was performed essentially as described²⁷. Detailed PCR amplification conditions and primer sequences are available on request.

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

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CORRIGENDUM

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Emmanuelle Viré, Carmen Brenner, Rachel Deplus, Loïc Blanchon, Mario Fraga, Céline Didelot, Lluís Morey, Aleyde Van Eynde, David Bernard, Jean-Marie Vanderwinden, Mathieu Bollen, Manel Esteller, Luciano Di Croce, Yvan de Launoit & François Fuks

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It has been drawn to our attention that in this Letter some of the lanes in Figures 2a, 2c, 4a and Supplementary Fig. S1 were inadvertently missing. A description of how the figures were made is provided in Supplementary Information to this Corrigendum, together with the corrected figure panels. Our results and conclusions are unaffected by these oversights.

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

ADDENDUM

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Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor

Marcos H. Hatada, Xiaode Lu, Ellen R. Laird, Jeremy Green, Jay P. Morgenstern, Meizhen Lou, Chris S. Marr, Thomas B. Phillips, Mary K. Ram, Kelly Theriault, Mark J. Zoller & Jennifer L. Karas

Nature 377, 32–38 (1995)

The Protein Data Bank ID at the RCSB is 2OQ1 for the ZAP70 coordinates.