DNA methylation reprogramming of human cancer cells by expression of a plant 5-methylcytosine DNA glycosylase

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Abstract

Patterns of DNA methylation, an important epigenetic modification involved in gene silencing and development, are disrupted in cancer cells. Understanding the functional significance of aberrant methylation in tumors remains challenging, due in part to the lack of suitable tools to actively modify methylation patterns. DNA demethylation caused by mammalian DNA methyltransferase inhibitors is transient and replication-dependent, whereas that induced by TET enzymes involves oxidized 5-meC derivatives that perform poorly understood regulatory functions. Unlike animals, plants possess enzymes that directly excise unoxidized 5-meC from DNA, allowing restoration of unmethylated C through Base Excision Repair. Here we show that expression of *Arabidopsis* 5-meC DNA glycosylase DEMETER (DME) in colon cancer cells demethylates and reactivates hypermethylated silenced loci. Interestingly, DME expression causes genome-wide changes that include both DNA methylation losses and gains, and partially restores the methylation pattern observed in normal tissue. Furthermore, such methylome reprogramming is accompanied by altered cell-cycle responses and increased sensibility to anti-tumor drugs, decreased ability to form colonospheres, and tumor growth impairment *in vivo*. Our study shows that it is possible to reprogram a human cancer DNA methylome by expression of a plant DNA demethylase.

Keywords: Epigenetics, colon cancer, DNA methylation, DNA demethylation, Base Excision Repair.

Introduction

DNA methylation at the C5 position of cytosine (5-methylcytosine, 5-meC) is an epigenetic mark with important roles in embryonic development, X-chromosome inactivation, imprinting and control of transposon activity [1, 2]. Altered methylation patterns are common in a growing number of human diseases, including disorders of the immune system [3] and cancer [4]. Tumor cells display local hypermethylation of gene promoters and global hypomethylation of gene-poor regions and repetitive sequences [5]. It has been proposed that the former leads to transcriptional silencing of tumor suppressor genes, whereas the latter contributes to genome instability [6, 7]. However, elucidation of the functional significance of aberrant DNA methylation in cancer and its causal relationship to tumorigenesis remains difficult, due in part to the lack of appropriate tools to actively modify methylation patterns in human cells.

DNA hypermethylation in cancer cells may be reversed by DNA methyltransferase inhibitors, such 5-aza-2'-deoxycytidine (5-azaCdR), that cause passive genome-wide DNA demethylation and have antitumoral properties *in vivo* [8]. However, demethylation is followed by re-methylation after drug withdrawal [9], which limits their usefulness for functional studies. An alternative option are enzymes involved in DNA demethylation. The ten-eleven translocation (TET) proteins TET1, TET2 and TET3 are alpha-ketoglutarate-dependent dioxygenases that catalyze conversion of 5-meC to 5-hydroxymethylcytosine (5-hmeC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC) by consecutive oxidation reactions [10, 11]. TET proteins have been proposed as promising candidates for mediating DNA demethylation in mammalian cells either via passive dilution, since 5-hmeC prevents maintenance DNA methylation, or in an active pathway, because 5-fC and 5-caC are substrates of the thymine DNA glycosylase (TDG)

that initiates a Base Excision Repair (BER) that would replace them with unmethylated cytosines [12-14]. However, some oxidized derivatives of 5-meC have been found to be stable and recognized by specific readers [15], and accumulating evidence suggests that they perform specific regulatory functions [16, 17]. Therefore, the use of TET proteins for functional studies on the role of aberrant DNA methylation in cancer remains problematic.

DNA demethylation mechanisms have independently evolved in plants and animals. In plants, a family of 5-meC DNA glycosylases directly excise unoxidized 5-meC, initiating restoration of unmethylated C through BER [18-20]. These enzymes, with no counterparts in animal cells, are typified by *Arabidopsis thaliana* DEMETER (DME), and its paralogs REPRESSOR OF SILENCING 1 (ROS1), and DEMETER-LIKE 2 and 3 (DML2 and DML3) [20-23]. We have recently shown that a fusion protein containing the catalytic domain of ROS1 and the DNA binding domain of yeast GAL4 specifically demethylates and reactivates a methylation-silenced reporter gene in human cells [24]. Here, we show that overexpression of DME in human colorectal cancer (CRC) DLD-1 cells causes genome-wide DNA methylation changes, including both gains and losses, that partially revert aberrant methylation to normal levels. Furthermore, the DNA methylome reprogramming induced by DME is accompanied by altered cell-cycle responses and enhanced sensitivity to chemotherapeutic agents *in vitro,* and reduced tumorigenesis *in vivo*.

1 # Results

2 **Expression of DME in DLD-1 cells demethylates and reactivates hypermethylated**
3 **silenced loci**

4 We stably transfected DLD-1 cells with the pcDNA3.1 vector either containing a
5 wild-type DME cDNA or a mutant version (D1562A) encoding a catalytically-inactive
6 enzyme [20]. Following transfection, G418-resistant transfectants were selected. Stable
7 transfectants containing the empty vector were also obtained. DME expression was
8 detected by RT-PCR and protein expression was confirmed by western blot using nuclear
9 extracts **(Fig. S1)**.

10 We first used bisulfite pyrosequencing and bisulfite sequencing of multiple clones to
11 examine the methylation status of three loci reported to be hypermethylated in DLD-1
12 cells: *ROR2* [25], *p14* [26], and *p16* [27]. Bisulfite pyrosequencing revealed that methylation at
13 promoters of *ROR2* and *p14* in cells expressing active DME was reduced to nearly
14 undetectable levels, whereas demethylation of the *p16* promoter was about 50 percent
15 **(Fig. 1A-B)**. Bisulfite sequencing of multiple clones confirmed that hypermethylation of
16 *ROR2* promoter was virtually abolished by DME expression, and that demethylation at
17 *p14* and *p16* was partially extended to gene body regions **(Fig. S2)**. We next tested
18 whether DNA demethylation was accompanied by gene reactivation. We re-analyzed the
19 methylation status of *ROR2*, *p14* and *p16* by quantitative methylation-specific PCR
20 (qMSP) analysis, and in parallel we measured their expression levels by quantitative RT-
21 PCR (qRT-PCR). The three genes were reactivated in cells expressing DME, but not in
22 non-transfected cells or in control transfectants with the mutant version or the empty
23 vector (**Fig. 1C-D**). DNA demethylation and transcriptional activation induced by DME
24 expression are either comparable or stronger than those achieved by treatment with 5-aza-

1 CdR (**Fig. S3**). However, whereas 5-aza-CdR-induced demethylation is transient, DME-
2 induced demethylation is stable over time (**Fig. S4**). Altogether, these results indicate that
3 expression of DME in DLD-1 cells causes loss of methylation at hypermethylated
4 silenced loci and the concomitant reactivation of their expression. Furthermore, both
5 effects are dependent on the catalytic activity of the 5-meC DNA glycosylase.

6 **DME-induced DNA demethylation is dependent upon the Base Excision Repair**
7 **pathway**

8 We employed a methylated GFP reporter plasmid to examine whether DME-induced
9 demethylation involves the Base Excision Repair pathway. Cells were transfected with
10 *SssI*-methylated or unmethylated CMV-GFP plasmid, and fluorescence was analyzed
11 after 48 h (**Fig. 2A-B**). The expression of the methylated GFP reporter gene was
12 specifically reactivated in DME- expressing cells, but not in either untransfected cells or
13 in cells expressing a catalytically-inactive version of the DNA glycosylase. Next, we
14 analyzed the effect of two different BER-inhibitors on such DME-dependent reactivation
15 (**Fig. 2C**). DME is a bifunctional DNA glycosylase that generates as a final product a
16 single-nucleotide gap flanked by 3´-phosphate and 5´-phosphate termini [20]. In
17 mammalian cells, processing of such intermediates during BER does not requires AP
18 endonuclease activity [28] but involves PARP [29]. We found that reactivation of the silenced
19 GFP reporter gene was significantly reduced by treatment with the poly (ADP-ribose)
20 polymerase (PARP) inhibitor ABT, but was not affected by the AP endonuclease
21 inhibitor CRT **(Fig. 2C**). Altogether, these results suggest that expression of DME in
22 DLD-1 cells initiates an active DNA demethylation process that involves the BER
23 pathway.

1 **Effect of DME expression on hypomethylated loci and repetitive DNA sequences**

2 We next asked whether DME expression has any effect on hypomethylated genes. To
3 this end, we specifically compared the effect of DME expression on three loci (*DNMT3b*,
4 *CSF2* and *BRD4*) that display different types of methylation changes in DLD-1 cells (**Fig.**
5 **3**). *DNMT3b* and *CSF2* are hypermethylated and hypomethylated, respectively, compared
6 to normal colon cells [30, 31]. *BRD4* is hypermethylated in most colon cancer cells lines, but
7 is unmethylated in both DLD-1 and normal colon cells [32]. Bisulfite pyrosequencing
8 confirmed that, as previously observed with *ROR2*, *p14* and *p16*, hypermethylation of
9 *DNMT3b* was virtually abolished in DME-expressing cells. Unexpectedly, however, both
10 *CFS2* and *BRD4* became methylated (**Fig. 3A**). Analysis of mRNA levels by qRT-PCR
11 confirmed that demethylation of *DNMT3b* was accompanied by reactivation, whereas
12 methylation gain at *CFS2* and *BRD4* was concomitant with silencing (**Fig. 3B**). We also
13 examined the effect of DME expression on three different types of DNA repetitive
14 elements (**Fig. S5**). We found that DME expression caused a small, but significant,
15 increase in DNA methylation levels at all three types of repeated sequences. Altogether,
16 these results indicate that DME expression causes complex effects on the methylome of
17 DLD-1 cells.

18 **DME causes genome-wide DNA methylation losses and gains that partially restore**
19 **normal patterns**

20 In order to analyze the effects of DME expression on the methylome of DLD-1 cells,
21 we performed a genome-wide DNA methylation analysis. We first examined global DNA
22 methylation levels by UPLC-MS/MS (**Table S1**). The results show that there are no
23 significant differences in 5-meC global content between control cells and cells expressing
24 DME. Next, we used the Illumina Infinium 450K array to analyze the methylation status

at > 480,000 CpG sites in DLD-1 cells transfected with the empty vector and cells expressing either active DME or a catalytically-inactive mutant version. We found that cells expressing active DME displayed statistically significant differences in methylation levels for about 38.000 CpG sites (8 % of analyzed sites), when compared either to cells transfected with the empty vector or cells expressing the mutant protein (**Fig. 4A**). Surprisingly, we found that expression of active DME was accompanied by both methylation gains and losses. The effect was very similar in the two independent transfectants analyzed, which shared a high proportion (around 90%) of both hypermethylated and hypomethylated CpG sites (**Table S2**). These results suggest that methylation changes observed upon DME expression are not stochastic in nature and depend upon the catalytic activity of the protein.

We next examined the genomic distribution of the 35,212 CpG sites (27,175 hypermethylated and 8,037 hypomethylated relative to control samples) showing differential methylation in both transfectants expressing WT DME when compared to cells expressing the mutant protein. Hypomethylated sites are enriched in promoters and 5´-UTRs ($p < 0.0001$ when compared to array background), with a pronounced representation of CpG islands (CGI) in those promoter regions ($p < 0.0001$). In contrast, hypermethylated sites are enriched in intergenic regions ($p < 0.0001$) characterized by the presence of non-CGIs ($p < 0.0001$) (**Fig. 4B**). Very similar results were obtained when comparing DME-expressing cells with cells transfected with the empty vector (**Fig. S6**). We also found that, at promoters, absolute methylation change values were somewhat higher for hypomethylated sites as compared to hypermethylated regions ($p < 0.0001$). Moreover, these differences are more pronounced according to their genomic location, as significant changes are observed for hypomethylated promoters and hypomethylated 3'-

UTRs ($p < 0.0001$), but not between hypermethylated promoters and hypermethylated 3'-UTRs (**Fig. 4C**).

The 35,212 CpG sites showing differential methylation upon DME expression are associated with 9,918 genes subdivided in three groups: 7,135 genes with only hypermethylated sites, 1,610 genes with only hypomethylated sites, and 1,173 genes including both hyper- and hypomethylated sites (**Fig. S7A**). Interestingly, in the latter group hypomethylation preferentially occurs at promoters, 5´-UTRs and first exon, whereas hypermethylation takes place at the remaining of the gene body and 3´-UTRs (**Fig. 4D; Table S3**). In genes only containing either hyper- or hypomethylated sites, no significant differences in absolute methylation changes were detected across the different gene regions (**Fig. S7B-C**). However, in genes containing only hypomethylated sites these were enriched in proximal promoters ($\leq$ 1kb) and regions close to the TSS (**Fig. S7D-E**) ($p < 0.0001$). Taken together, these results suggest that methylation gains and losses observed upon expression of DME are not randomly distributed over the genome.

A Gene Ontology (GO) analysis on the three gene groups showed that each one was enriched for genes involved in different cellular pathways (**Fig. S8**). The group of genes with both hyper- and hypo-methylated sites was enriched for genes associated with regulation of development. Although less noticeable, a similar result was found for the group of genes containing only hypomethylated sites. In contrast, the group of genes with only hypermethylated sites was enriched for genes involved in cell-cell adhesion and signaling.

Next, we performed unsupervised clustering analysis for healthy colon tissues, non-transfected DLD-1 cells, and several transfectants using the 3.000 probes with the highest variance (**Fig. 4E-F**). The two DLD-1 lines expressing WT DME clustered with normal

tissues on the x-axis (**Fig. 4E**). On the y-axis, hypermethylated CpG sites that upon DME expression reverted to normal levels (cluster 1) are enriched in promoters and CGIs ($p < 0.0001$), whereas hypomethylated sites that gained methylation to reach normal levels (cluster 2) are enriched in intergenic ($p < 0.05$) and intronic regions and non-CGIs ($p < 0.0001$) (**Fig. 4F**). Sites unmethylated in both normal tissues and DLD-1 cells but hypermethylated in DME-expressing cells (cluster 3), as well as hypermethylated sites that do not revert to normal levels upon DME expression (cluster 4) are also enriched in promoters and CGIs, although to a less extent. These results suggest that DME expression partially restores the methylation pattern observed in normal tissue.

DME expression alters the cell cycle response to anti-tumor agents

We next evaluated the effect of DME expression on the phenotype of DLD-1 cells. Measurements with the trypan blue exclusion assay showed that expression of DME increased cell proliferation *in vitro* (**Fig. S9**). We also analyzed the effect of DME expression on the response of DLD-1 cells to two drugs clinically effective against colorectal cancers: oxaliplatin and 5-fluorouracil (5-FU) (**Fig. 5A**). Cells expressing active DME displayed increased sensitivity to both oxaliplantin and 5-FU at all concentration tested. Pre-incubation of non-transfected cells with 5-aza-CdR also increased sensitivity to oxaliplatin, although to a lesser extent than DME expression, but did not have any significant effect on the sensitivity to 5-FU.

To define the potential mechanisms underlying the sensitivity of DME-expressing cells to anti-tumor drugs, we evaluated their cell cycle profile in the absence and presence of either oxaliplatin or 5-FU (**Fig. 5B**). In the absence of anti-tumor drugs, no obvious differences in cell cycle phase distribution were observed between cells expressing active DME and control cells. However, significant differences were observed in drug-treated

cells. Oxaliplatin induced S-phase arrest in control cells, but caused G2/M arrest in cells expressing active DME. On the other hand, exposure to 5-FU did not alter the phase distribution in control cells, but induced S-phase arrest in cells expressing DME. We also examined the phosphorylation status of Cdk2 at Tyr15 (Cdk2pTyr15) by immunoblot analysis (**Fig. 5C**). Cdk2, which operates in the transition from G1 to S phase and also in S phase progression, is subject to inhibitory phosphorylation at Thr14 and Tyr15 in proliferating cells [33]. We found that treatment with either oxaliplatin or 5-FU caused loss of inhibitory Tyr15 phosphorylation of Cdk2 in DME-expressing cells, but not in control cells. Altogether, these results indicate that expression of active DME alters the cell cycle response to both oxaliplatin and 5-FU.

DME expression inhibits colonosphere formation *in vitro* and cancer growth *in vivo*

The majority of tumors, including CRC, are heterogeneous cell populations comprising a small sub-population of poorly differentiated cancer stem-like cells (CSCs). It has been proposed that CSCs are responsible for a cancer's tumor-initiating and metastatic ability [34, 35]. CRC-CSCs can be grown to form floating spheroids (known as colonospheres) when cultured under anchorage-independent conditions in a serum-free defined medium [36]. We compared the colonosphere forming ability of cells expressing active DME and control cells (**Fig. 6A-B**). There was no significant difference in the number of colonospheres, but primary and secondary colonospheres formed by DME-expressing cells were significantly smaller than those formed by control cells (**Fig. 6B**). These results indicate that DME-expressing cells have a reduced ability to form colonospheres, and suggest an inhibition of their stemness properties.

We next examined whether DME expression has an effect on tumor growth *in vivo* by injecting cells expressing active DME or its catalytically inactive mutant version in

1 nude mice. The size of xenografts expressing active DME was significantly smaller than
2 those expressing the inactive version (**Fig. 6C-D**). Importantly, the growth rate of tumors
3 expressing active DME was strongly inhibited from early stages. These results indicate
4 that DME expression inhibits cancer growth *in vivo*.

5 ## Discussion

6 In this study we show that stable expression of a plant 5-meC DNA glycosylase
7 induces genome-wide changes in the methylome of CRC cells and important alterations
8 of their phenotype. There are few reports analyzing genome-wide effects upon expression
9 of enzymes involved in DNA demethylation. Two recent studies reported genome-wide
10 induction of 5hmeC in HEK293T cells upon transient expression of the TET1 catalytic
11 domain (TET1-CD) [37] and full-length TET2 [38]. However, methods used in both studies do
12 not allow unambiguous distinction between genuine DNA demethylation and 5-meC
13 oxidation.

14 One unexpected result of our study is that DME expression is associated with both
15 losses and gains of DNA methylation. Interestingly, demethylated regions are enriched in
16 promoters and CGIs, whereas hypermethylated sites tend to be located at intergenic
17 regions and non-CGIs. This result suggests that two different mechanisms are involved.
18 Although most hypomethylation changes are likely to be a direct effect of DME catalytic
19 activity, it is more difficult to explain the extensive hypermethylation changes observed.
20 A possible explanation is that demethylation initiated by DME leads to activation of
21 DNA methylation pathways. In agreement with this idea, we found that *DNMT3b*, which
22 is hypermethylated and silenced in DLD-1 cells [30] becomes demethylated and reactivated
23 upon DME expression. Interestingly, *DNMT3b* is required for remethylation observed
24 after drug withdrawal in 5-aza-CdR-treated HCT116 colon cancer cells [39].

1 Locations of DNA methylation changes induced by DME expression are mostly
2 reciprocal to those observed in tumor cells. In fact, our unsupervised clustering analysis
3 indicates that DME expression partially reverts the aberrant methylation patterns of DLD-
4 1 cells. The mechanisms leading to the simultaneous appearance of focalized DNA
5 hypermethylation and global DNA hypomethylation in cancer cells are poorly understood
6 [7]. However, there are hints that both processes are linked through developmentally-
7 regulated silencing programs, as suggested by methylation profiling of colorectal cancer
8 and normal tissues [40]. In this respect, it is interesting that genes whose promoters become
9 hypomethylated upon DME expression are enriched in functions related to development
10 regulation, whereas those hypermethylated are enriched for functions related to cell-cell
11 signaling. Genomic distribution of active DNA demethylation mediated by DME and
12 passive DNA demethylation induced by either mutation or inhibition of DNMTs is
13 apparently different. HCT116 double-knockout cells for DNMT1 and DNMT3B
14 (HCT116 DKO) show hypomethylation in 37 % of CpG sites, equally distributed across
15 promoter, gene body and intergenic regions [41]. By other hand, 5-aza-CdR-demethylated
16 sites in HCT116 cells are enriched in gene body regions, but not promoters [39].
17 Interestingly, however, genes that remain demethylated 68 days after 5-aza-CdR
18 withdrawal in HCT116 cells are, like those demethylated by DME in DLD-1 cells,
19 enriched in functions related to developmental processes [39].

20 A second key observation in our study is that DNA methylome-reprogramming
21 induced by DME is accompanied by important changes in tumor-related properties. This
22 result is perhaps not unexpected, since CpG sites differentially-methylated upon DME
23 expression cover about half of the genes examined (9,918 out of 21,231). Notably, DME
24 expression causes increased sensitivity to chemotherapy agents such as oxaliplatin and 5-

1 FU, concomitant with specific alterations of the cell-cycle response to these anti-tumor
2 agents. These results suggest that such response is under epigenetic control. In fact, DME
3 expression demethylates and reactivates two important cell-cycle regulators, such as *p14*
4 and *p16*. Interestingly, it has been reported that induced Pluripotent Stem (iPS)
5 reprogramming of DLD-1 cells is accompanied by *p16* reactivation and increased
6 sensitivity to 5-FU [42]. Another important feature of the altered phenotype of DME-
7 expressing cells is the reduced size of the spheres formed under unchorage-independent
8 conditions. This result suggests that methylation control is important for sphere formation
9 and, presumably, for CSCs subpopulation maintenance in colon tumors. In fact, it has
10 been previously reported that DNA methylation is essential for maintenance of human
11 colon CSCs [43].

12 A significant result is that DME expression inhibits the growth of tumor xenografts.
13 In this respect, it is interesting that overexpression of TET1 in colorectal cancer cells also
14 inhibits *in vivo* growth of tumors derived of colorectal cancer cells, as well as spheroid
15 formation [44]. The effect of TET1 has been linked to its capacity to de-repress inhibitors of
16 the Wnt pathway by DNA hydroxymethylation [44]. Indeed, our study shows that DME
17 expression demethylates and reactivates *ROR2*, which inhibits the canonical β-catenin
18 dependent Wnt signaling [45]. These results agree with the notion that epigenetic disruption
19 of the Wnt/β-catenin signaling pathway is implicated in numerous malignancies,
20 including colorectal cancer [25, 46]. Interestingly, DME expression increases cell
21 proliferation *in vitro* but decreases tumor growth *in vivo*. Such discrepancy emphasizes
22 the fact that tumor growth *in vivo* is affected by multiple factors, such as extracellular
23 matrix and stromal cells, that cannot be recreated *in vitro*. Our observations also suggest

1 that *in vitro* and *in vivo* growth are differentially affected by stable changes in the cancer
2 cell methylome.

3 In summary, our data show that expression of a plant 5-meC DNA glycosylase in
4 human CRC cells partially reverts their aberrant methylation and alters their tumoral
5 phenotype. Plant DNA demethylases, with no counterpart in human cells, may provide
6 new options to study the functional role of DNA methylation cancer. We have previously
7 shown that a paralog of DME (ROS1) may be targeted to demethylate specific DNA
8 sequences in human cells by fusion to a DNA binding domain [24]. Therefore, plant DNA
9 demethylases may be an useful addition to the growing collection of effectors used for
10 epigenetic editing.

11 ## Materials and Methods

12 ### Ethics statement

13 All experiments and procedures involving animals were conducted in accordance
14 with guidelines approved by the Animal Experimentation Service (SAEX) of the
15 University of Cordoba (UCO), with current legislation (Directive 2010/63/EU, belong
16 Commission Recommendation 2007/526 / EC and Royal Decree 53/2013).

17 ### Cell culture

18 Cells were grown in Dulbecco's modified eagle's medium–high glucose (DMEM,
19 4.5 g/L d-glucose) (Sigma), supplemented with 10% fetal bovine serum (FBS, Sigma)
20 and 1% penicillin/streptomycin (Sigma). Cells were maintained in a humidified
21 atmosphere at 37°C and 5% CO_2. After cultures became 80% confluent (usually 4 days),
22 cells were trypsinized, centrifuged and suspended in fresh medium.

Transfection and generation of stable cell lines

Wild-type DME cDNA and a catalytically inactive mutant version (D1562A) were subcloned into pcDNA3.1 vector (Invitrogen) from pET30b-DME and pET30b-DMED1562A, respectively [20], by *KpnI*/*NotI* digestion. DLD-1 cells were transfected with the empty plasmid or the different constructs using Lipofectamine LTX (Invitrogen), following the manufacturer´s recommendations. Stable transfectants were obtained after 2 weeks selection with 1 mg/ml G418 (Calbiochem).

RNA Purification and Reverse Transcriptase-Polymerase chain reaction (RT-PCR)

Total RNA from cell lines was extracted using the RNeasy Minikit (Qiagen). Residual genomic DNA was removed by DNAse I digestion with RNase-free (Thermo Scientific) and cDNA was synthesized using the qScript™ cDNA Synthesis Kit (Quanta). Quantitative real time RT-PCR (qRT-PCR) analysis was performed in a CFX Connect™ Real-Time PCR Detection System (Biorad) by mixing cDNA (from 1 µg total RNA) with iQ™ SYBR Green Supermix (Biorad) and specific primers (**Table S4**). Data were normalized using *GAPDH* and quantification of relative expression was determined by the $2^{-\Delta\Delta Ct}$ method [47].

Western blot analysis

For DME detection, nuclear extracts prepared with the Qproteome Cell Compartment Kit (Qiagen) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with a rabbit polyclonal antibody generated against the C-terminal region of *Arabidopsis thaliana* ROS1 (1:500); an anti-Histone H3 antibody (1:5000, Millipore) was used for loading control. For Cdk2 Tyr15 detection, cell lysates were prepared in RIPA Buffer (Sigma) and incubated with a monoclonal primary antibody against phosphor-

Cdk2 Tyr15 (1:250, Abcam), using an anti-Actin antibody (1:2000, Sigma) for loading control. All immunocomplexes were detected by the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences), using the appropriate horseradish peroxidase-conjugated secondary antibodies. Images were captured on a ChemiDoc™ XRS Imaging System (Biorad).

DNA methylation analysis

DNA was purified following standard protocols and 750 ng of each sample were bisulfite-modified with the EZ DNA Methylation Gold Kit (Zymo Research). Quantitative methylation-specific PCR (qMSP) analysis was performed using allele-specific primers complementary to methylated promoter target sequences (**Table S4**). To quantify input DNA after bisulfite treatment, a region of the *AluC4* locus free of CpG sites was amplified. The relative level of methylation at a specific locus was calculated by the $2^{-\Delta\Delta Ct}$ method [47]. Bisulfite sequencing of multiple clones was performed using primers shown in **Table S4**. At least twelve independent clones were sequenced for each sample. Bisulfite pyrosequencing was performed with primers shown in **Table S4**. The biotinylated PCR product was purified and made single-stranded to act as a template in a pyrosequencing reaction using the Pyrosequencing Vacuum Prep Tool (Qiagen). Methylation density was quantified using Pyrosequencing Analysis and the PyroMark Q24 system (Qiagen).

Global DNA methylation analysis by UPLC-MS/MS

Global 5-meC levels were analyzed by high-performance liquid chromatography (UPLC 1290 Infinity System, Agilent Technology) coupled to a triple-quadrupole mass spectrometer (6460 Jet Stream Series, Agilent Technologies). The chromatographic conditions and the optimized source parameters were based on those described by Le et

al. (2011) [48] with some modifications. Briefly, a reverse phase column (Zorbax Eclipse plus C18, 2.1x50 mm, 1.8 µm particle size) was used with a mobile phase gradient of formic acid and methanol (up to 80% in 8 min) flowing at 200 µL/min. Multiple reaction monitoring mode transition pairs of deoxycytidine (dC) and 5-meC were set as m/z 228.1→112.1 and m/z 242.1→126.1, respectively. The dwell times per channel were set at 100 ms and collision energy at 5V, for both analytes in positive polarity mode. Previous to this analytical procedure, DNA samples were hydrolyzed to nucleosides with Degradase Plus (Zymo Research). The DNA methylation level was expressed as [5-meC] / ([5-meC] + [dC]).

HumanMethylation450 BeadChip data preprocessing

IDAT files from the HumanMethylation450 BeadChip were processed using the R/Bioconductor package minfi (R package version 1.14.0). In order to adjust for the different probe design types present in the HumanMethylation450 BeadChip architecture, red and green signals from the IDAT files were corrected using the SWAN algorithm [49]. No background correction or control probe normalization was applied. Probes where at least two samples had detection P-values > 0.01 were filtered out. Beta-values were computed and employed across the analysis pipeline.

Transient transfection with CMV-GFP reporter plasmid

In vitro methylation of pAcGFP1-C1 plasmid DNA (Clontech) was performed using M.*SssI* methyltransferase (New England Biolabs) and verified by *HpaII* digestion (New England Biolabs). DME stable cell lines were transfected with either non-methylated or *in vitro* methylated GFP plasmid using Lipofectamine LTX (Invitrogen). After 48 hours cells were visualized in a Nikon ECLIPSE Ti inverted microscope and the numbers of

GFP$^+$ cells under different conditions were quantified using a FACSCalibur flow cytometer (BD Biosciences).

Drug treatments

Cells were seeded and let to attach for 24 h before adding the drug(s) at the indicated concentrations, and incubation continued for different time periods. 5-aza-2´-deoxycytidine, oxaliplatin, 5-fluorouracil, and CRT 0044876 were from Sigma, and ABT-888 was from Enzo Life Sciences.

Cell proliferation and cytotoxicity analysis

Cell proliferation rate was established by cell counting on consecutive days. 7.5×10^5 cells were seeded in T75 culture flasks and collected daily for 4 days. Viable cells, as assessed by trypan blue staining, were counted under a microscope in a hemocytometer. Cytotoxicity was assessed using a WST-1 colorimetric assay (Roche Applied Science). Cells were seeded on 96-well plates at a density of 8,000 cells/well and let to attach for 24 hours. After treatment at indicated times and doses, the WST-1 assay was performed following the protocol supplied by the manufacturer. The dye produced by viable cells was spectrophotometrically (420-480 nm) detected using an Imark™ Microplate Reader (Biorad).

Cell cycle analysis

Cell cycle phase distribution was measured by cytometry. Briefly, 2.5×10^5 cells were recovered by centrifugation and fixed with 70% ethanol for at least 24 h at 4ºC. Fixed cells were stained with 50 μg/ml propidium iodide (Sigma) and treated with 50 μg/ml RNase A (Sigma) for 20 minutes at room temperature protected from light.

Analysis and measurement of propidium iodide fluorescence were performed on a FACSCalibur flow cytometer (BD Biosciences).

Colonosphere formation assay

The colonosphere formation assay was performed as previously described [50].

Mouse xenograft model

Five-week-old athymic Nude-Foxn1$_{nu/nu}$ mice (Janvier Labs) were used for tumour xenograft experiments with pcDNA-DME and pcDNA-DME mutant transfected DLD-1 cells. Ten mice were used. Both flanks of each animal were injected subcutaneously with 2.5×10^6 cells in 100 µl Matrigel ® Matrix (Corning); DME-transfected cells were injected into the left flank and DME mutant-transfected cells into the right flank. Tumor width (W) and length (L) were measured every 5 days. Tumor volume was estimated according to the formula $V = 0.4x\ LxW^2$ (L = maximum length; W = maximum width).

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Conflict of Interest

The authors declare no conflict of interest.

Supporting Information

Supporting Information accompanies this paper on the Epigenetics website (http://www.tandfonline.com/loi/kepi20/).

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1 # Figure Legends

2 **Figure 1. DNA demethylation of *ROR2*, *p14* and *p16* in DME-expressing cells is**
3 **accompanied by gene reactivation.** **(A)** Schematic diagram of analyzed genes. Each
4 vertical bar represents a CpG dinucleotide. Position of ATG codon is indicated as a red
5 rectangle. Green arrows show the location of pyrosequencing primers and yellow arrows
6 the location of qMSP primers. **(B)** Methylation levels analyzed by bisulfite
7 pyrosequencing; CpG sites are shown as bars filled with black to represent percentage
8 methylation. **(C)** Methylation levels analyzed by qMSP **(D)** Gene expression levels
9 analyzed by qRT-PCR. Analyses were performed in non-transfected DLD-1 cells and
10 independent transfectants expressing WT DME (DME 2, DME 10 and DME 13), a
11 catalytically inactive mutant version (mut 7 and mut 13) or cells transfected with the
12 empty vector. Values are shown relative to those detected in non-transfected cells. Data
13 are the mean ± SE of three independent experiments.

14 **Figure 2. DME-induced DNA demethylation is dependent upon the Base**
15 **Excision Repair (BER) pathway**. **(A)** Non transfected cells and stably-transfected lines
16 were transiently transfected with Sss I-methylated (right) or unmethylated (left) CMV-
17 GFP reporter plasmid. Fluorescence microscopy images were taken 48 h after
18 transfection. **(B)** Flow cytometry measurements of GFP expression. Values are shown
19 relative to those detected in cells transiently transfected with unmethylated plasmid. Data
20 are the mean ± SE of five independent experiments. **(C)** Effect of BER inhibitors on GFP
21 reactivation. Cells transiently transfected with methylated CMV-GFP plasmid were
22 plated either in the absence or presence of BER inhibitor (ABT or CRT; 50 mM), and
23 GFP expression was measured after 24 h by flow cytometry. Values are shown relative to
24 those detected in cells transiently transfected with unmethylated plasmid. Data are the

mean ± SE of three independent experiments. Asterisks indicate statistical significance as determined by Student's *t*-test (* $P < 0.05$).

Figure 3. DME expression on three loci displays different types of methylation changes in DLD-1 cells. (A) Methylation levels analyzed by bisulfite pyrosequencing; Each vertical bar represents a CpG dinucleotide, and position of ATG codon is indicated as a red rectangle; green arrows show the location of pyrosequencing primers. Analyzed CpG sites are shown as bars filled with black to represent percentage methylation. **(B)** Gene expression levels analyzed by qRT-PCR. Values are shown relative to those detected in non-transfected cells. Data are the mean ± SE of three independent experiments.

Figure 4. DME expression causes both losses and gains of DNA methylation, partially restoring the pattern of normal tissue. (A) Barplot depicting the number of CpG sites displaying differential methylation in several pairwise comparisons. Cell lines are DLD-1 cells transfected with the empty vector and transfectants expressing WT DME or a mutant version. Hyper-/hypomethylation indicates a statistically significant methylation increase/decrease observed in the first member of each pair. **(B)** Distribution of differentially-methylated CpG sites according to their genomic location (inner ring) and CpG class (outer ring). The left plot represents the distribution of all probes in the 450K methylation array. Center and right plots depict sites displaying hyper- or hypomethylation in both DME 2 and DME 13 lines when compared to cells expressing a mutant DME version. Colors indicate the percentage of sites at each location, as indicated at the bottom. **(C)** Overall methylation changes in CpG sites displaying differential methylation according to their respective genomic locations. Absolute beta values are means for sites displaying hyper-or hypomethylation in both DME 2 and DME 13 lines

1 when compared to cells expressing a mutant DME version. **(D)** Boxplot indicating overall
2 changes in beta values across different genomic locations for probes located in genes
3 containing both hyper- and hypo-methylated sites. Values are means for sites displaying
4 hyper-or hypomethylation in both DME 2 and DME 13 lines when compared to cells
5 expressing a mutant DME version. **(E)** Heatmap representing the methylation status
6 (beta-value) of the 3000 most variable probes (y-axis) for each of the different cell
7 samples (x-axis). Unsupervised clustering analysis grouped cell samples in 3 main
8 clusters (indicated by three major branches of the upper dendogram) and CpG sites in 4
9 main groups (indicated on the left by different colours, see text for details). **(F)** Genomic
10 location and CpG class distribution for each of the 4 groups of CpG sites identified by the
11 unsupervised clustering algorithm.

12 **Figure 5. DME sensitizes cells to oxaliplatin and 5-FU and alters the cell cycle**
13 **response to both anti-tumor drugs.** **(A)** Upper panels: non-transfected DLD-1 cells and
14 stable DME transfectants were treated with various concentrations of oxaliplatin or 5-FU
15 for 48 h, and cell viability was measured. Lower panels: comparison of 5-azaCdR and
16 DME on sensitivity to oxaliplatin or 5-FU. Cells were preincubated or not with 5-azaCdR
17 (2.5 μM) for 72 h, and then treated with anticancer drug for 48 h before measuring cell
18 viability. Data are the mean ± SE of three independent experiments. **(B)** Percentage of
19 cells in each cell cycle phase after cytostatic treatment. DLD-1 cells and different
20 transfectants were treated with oxaliplatin or 5-FU (25 mM) for 48 h, and analyzed by
21 flow cytometry analysis. Values are the mean ± SE of three independent experiments. **(C)**
22 Analysis of the phosphorylation status of Cdk2 at Tyr15 (Cdk2pTyr15) by
23 immunoblotting.

1 **Figure 6. Effect of DME expression on colonosphere formation *in vitro* and**
2 **tumor growth *in vivo*. (A)** Representative images of primary (left panel) and secondary
3 (right panel) colonospheres formed by DLD-1 cells and different transfectants. **(B)** Size
4 of primary and secondary colonospheres formed by the different cell lines. Data are the
5 mean ± SE of 20 (primary) or 40 (secondary) colonospheres. Asterisks indicate statistical
6 significance as determined by Student's *t*-test (**** $P<0.0001$). **(C)** Stable DLD-1
7 transfectants (2.5×10^6 cells) expressing either wild-type DME (DME 2) or a
8 catalytically-inactive mutant version (mut 7) were injected subcutaneously into the left or
9 right flank, respectively, of athymic nude mice (n = 10). **(D)** Tumor size was assessed at
10 various time points after injection. Data are the mean ± SE.
11
12

Supplemental material Legends

Supplemental Figure 1. DME expression in DLD-1 cells. (A) Detection of *DME* and *GAPDH* mRNA. RT-PCR was performed using specific primers from *DME* and *GAPDH* on total RNA from different stable transfectants. PCR products were analyzed on a 2% agarose gel. **(B)** Detection of DME protein by Western blotting using nuclear extracts and an antibody against DME. Anti-histone 3 antibody was used for loading control.

Supplemental Figure 2. DNA methylation analysis of *ROR2*, *p14* and *p16* genes in DLD-1 DME transfectants by bisulfite sequencing. Each vertical bar represents a CpG dinucleotide, and position of ATG codon is indicated as a red rectangle; blue arrows show the location of bisulfite sequencing primers. Lower panels show results of bisulfite sequencing of multiple clones, indicating methylated (black circles) or unmethylated cytosines (white circles).

Supplemental Figure 3. Comparison of active and passive DNA demethylation. Methylation levels analyzed by qMSP **(A)** and gene expression levels analyzed by qRT-PCR **(B)** in untreated cells, 5-azaCdR-treated cells (2.5 μM for 72 h), and untreated transfectants expressing DME. Values are shown relative to those detected in non-transfected cells. Data are the mean ± SE of three independent experiments.

Supplemental Figure 4. Stability of DME-induced DNA demethylation. Methylation levels at the *ROR2* locus were analyzed by qMSP at different time points in 5-azaCdR-treated cells (2.5 μM for 72 h), and untreated transfectants expressing DME. Values are shown relative to those detected in non-transfected, untreated cells. Data are the mean ± SE of three independent experiments.

1 **Supplemental Figure 5. Effect of DME expression on methylation levels at**
2 **repetitive DNA sequences.** CpG sites analyzed by bisulfite pyrosequencing are shown as
3 bars filled with black to represent percentage methylation.

4 **Supplemental Figure 6. Genomic distribution of CpG showing differential**
5 **methylation in both DME 2 and DME 13 transfectants when compared to cells**
6 **transfected with the empty vector**. Distribution of differentially-methylated CpG sites
7 according to their genomic location (inner ring) and CpG class (outer ring). The left plot
8 represents the distribution of all probes in the 450K methylation array. Center and right
9 plots depict sites displaying hyper- or hypomethylation in both DME 2 and DME 13 lines
10 when compared to cells transfected with the empty vector. Colors indicate the percentage
11 of sites at each location, as indicated at the bottom.

12 **Supplemental Figure 7. Genomic distribution of CpG sites displaying**
13 **differential methylation upon DME expression.** (**A**) Genes displaying differential
14 methylation upon DME expression. Left plot indicates the number of genes with hyper-,
15 hypo-, or both hyper- and hypomethylated sites in both DME 2 and DME 13 lines when
16 compared to cells expressing a mutant DME version. Right plot displays the number of
17 sites in each gene class. **(B)** and **(C)** Boxplots depicting overall methylation levels (beta-
18 values) across different genomic locations for probes located in genes containing only
19 hyper- or hypo-methylated sites. Values are means for sites displaying hyper-or
20 hypomethylation in both DME 2 and DME 13 lines when compared to cells expressing a
21 mutant DME version. **(D)** and **(E)** Percentage of differentially-methylated CpG sites
22 across different genic regions or according to their relative position to the TSS in genes
23 with hyper-, hypo-, or both hyper- and hypomethylated sites.

Supplemental Figure 8. Gene ontology analysis for genes displaying hyper-, hypo-, or both hyper- and hypomethylated sites upon DME expression. (A) KEGG pathway enrichment analysis. Gene ratios and statistical significance (p-values) are depicted by circle diameter and color, respectively, as indicated on the right. **(B-D)** Gene ontology functional categories enriched for genes displaying both hyper- and hypomethylated sites **(B)**, only hypomethylated sites **(C),** and only hypermethylated sites **(D)**. Colors indicate the degree of statistical significance (p-values) for each box.

Supplemental Figure 9. *In vitro* cell proliferation assay. For each cell line, 7.5 x 105 cells were plated and the number of viable cells was determined at different time points. Data are mean ± SE of three independent experiments.

Supplemental Table 1. Global DNA methylation levels in DLD-1 cells and stable transfectants.

Supplemental Table 2. Number of differentially-methylated CpG sites in DME-expressing cells.

Supplemental Table 3. p-values of pairwise comparisons across different locations in genes displaying both hyper- and hypomethylated sites, only hypomethylated sites, and only hypermethylated sited (see Figure 4D and Figure S6B-C).

Supplemental Table 4. List of primers used for qMSP, Bisulfite clone DNA sequencing and pyrosequencing, and qRT-PCR.