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DNMT1 modulates gene expression without its catalytic activity partially through its interactions with histone-modifying enzymes

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ABSTRACT

While DNA methyltransferase1 (DNMT1) is classically known for its functions as a maintenance methyltransferase enzyme, additional roles for DNMT1 in gene expression are not as clearly understood. Several groups have shown that deletion of the catalytic domain from DNMT1 does not abolish repressive activity of the protein against a reporter gene. In our studies, we examine the repressor function of catalytically inactive DNMT1 at endogenous genes. First, potential DNMT1 target genes were identified by searching for genes up-regulated in HCT116 colon cancer cells genetically disrupted for *DNMT1* (*DNMT1*$^{-/-}$ hypomorph cells). Next, the requirement for DNMT1 activity for repression of these genes was assessed by stably restoring expression of wild-type or catalytically inactive *DNMT1*. Both wild-type and mutant proteins are able to occupy the promoters and repress the expression of a set of target genes, and induce, at these promoters, both the depletion of active histone marks and the recruitment of a H3K4 demethylase, KDM1A/LSD1. Together, our findings show that there are genes for which DNMT1 acts as a transcriptional repressor independent from its methyltransferase function and that this repressive function may invoke a role for a scaffolding function of the protein at target genes.

INTRODUCTION

DNA methyltransferase1 (DNMT1) has been characterized as a maintenance DNA methyltransferase enzyme able to catalyze the addition of a methyl group to cytosines adjacent to guanines (1,2). The protein prefers hemimethylated substrates (2,3) although studies have shown it can catalyze methylation of unmethylated substrates as well (4).

Loss of function of DNMT1 in mice causes embryonic lethality with stunted development starting from Day 9.5 to 10.5 (5) and in xenopus, DNMT1 depletion by antisense RNA causes the embryos to die during gastrulation and neurulation (6). Additionally, depletion of DNMT1 in mouse fibroblasts causes cell apoptosis within 5–6 days of DNMT1 loss (7) and genetic disruption of *DNMT1* in HCT116 colon cancer cells causes mitotic catastrophe, G2/M cell-cycle arrest and eventually, apoptosis within 48 h (8).

Another variant of HCT116 cells that have been genetically disrupted for exons 3–5 of *DNMT1* retain a hypomorphic DNMT1 protein (9,10). In these cells, an alternative splice variant is transcribed and translated yielding a low level of truncated DNMT1 which lacks the binding sites for PCNA and the corepressor protein, DMAP, (11) but retains a low level of the active C-terminal catalytic domain (12). These hypomorphic cells show ~90–95% depletion of DNA methyltransferase activity, but minimal loss of global DNA methylation (9). The presence of this catalytically active hypomorph may partially explain why these knockout cell lines survive and proliferate (10,12). However, despite the presence of a truncated DNMT1, a number of genes have been found to be upregulated in these cell lines (13). This upregulation may be due to lower levels of truncated DNMT1 but may also hint at the importance of the deleted domain(s) in gene regulation.

The importance of the N-terminus of DNMT1 was illustrated, by our lab and others, in three reporter assays in which, despite the deletion of the C-terminal catalytic domain of DNMT1, repression of reporter genes was observed (11,14,15). In these studies, this

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region of DNMT1 was found to be associated with members of the HDAC family, HDAC1 and HDAC2, and the interaction with these deacetylases resulted in repression of gene transcription. These findings illustrated that the repressive activity of DNMT1 does not solely depend on the catalytic function of the protein.

Additional evidence suggests that DNMT1 mediated repression may not only be dependent on DNA methylation. Closer inspection of our previous performed microarray studies (13) reveals that approximately one-third of the genes upregulated in *DNMT1*$^{-/-}$ cells do not contain dense CpG islands. It has also been shown that treatment of some cells by a DNMT1 inhibitor, 5-aza-2′-deoxycytidine (DAC), causes increases in expression of some genes without evident changes in DNA methylation (16,17). Since DAC treatment is also known to cause DNMT1 degradation, (18,19) the repressive ability of the protein at specific silenced and unmethylated genes may be due to a non-catalytic function of DNMT1 potentially associated with additional proteins. To this end, a number of repressive proteins such as corepressor DMAP1 (11) as well as histone-modifying enzymes, HDAC1/2 (11,14,15), G9A (20), SUV39H1 (21), EZH2 (22), HP1α, β and γ (23) and KDM1A/LSD1 (24) have been found to interact with DNMT1.

In our current work, we show that, while the importance of the methyltransferase function of DNMT1 is undeniable, gene repression at some endogenous target genes can occur without need for catalytic activity of the protein. We provide evidence that a catalytically inactive DNMT1 can affect some histone modifications and that one histone demethylase, LSD1, associates with and is recruited by both wild-type (wt) and catalytically inactive DNMT1 to specific promoters and partially mediates repression of these genes. The results of this study suggest that DNMT1 has repressive functions other than its DNA methyltransferase activity suggesting scaffolding roles for the protein to recruit other transcriptional repressive complexes.

MATERIALS AND METHODS

Plasmids

wt*DNMT1* (NM_001130823.1) and catalytically inactive *DNMT1* (C1226W̄) with N-terminal FLAG tag inserts were cloned into a pEF1α IRES-puro vector.

Cell culture

HCT116 and *DNMT1*$^{-/-}$ subclone 5F, created previously (9), were cultured in 5A McCoy's modified media using 10% fetal bovine serum. The wt and mutant (mut) *DNMT1* vectors were transfected into *DNMT1*$^{-/-}$ cells using lipofectamine 2000 (invitrogen) and selected using 0.3–0.5 μg/ml of puromycin. Puromycin resistant clones were isolated and expanded and presence of DNMT1 was screened by western blots.

Western blot analysis

For whole-cell extraction of protein, cell pellets were resuspended in 4% SDS and processed through QIAshredder (Qiagen). Antibodies utilized in western blot analysis were as follows: αDNMT1 (Sigma D4567) 1:2000, αDNMT1 (epitomics 2788-1) 1:3000, αβactin (Sigma 5441) 1:10 000, αLSD1 (Millipore 09-058) 1:10 000, αCBP (Santa Cruz sc-369) 1:1000, αLaminB (Santa Cruz) 1:2000, α-tubulin (Sigma T6074) 1:10 000 and αGAPDH (Millipore) 1:10 000. Cytoplasmic and nuclear extracts were made by resuspending cell pellets with cytoplasmic extraction buffer: CEBN (10 mM HEPES 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.34 M Sucrose, 10% Glycerol, 0.2% NP40/IPEGAL), followed by incubation for 10 min on ice with vortexing every minute. Nuclear pellets were separated from cytoplasmic supernatant by centrifugation. The nuclear pellets were then washed once with CEB (10 mM HEPES 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.34 M Sucrose, 10% Glycerol) pelleted by centrifugation, resuspended in 4% SDS and sheared with QIAshredder (Qiagen).

DNMT1 and LSD1 knockdowns

HCT116 cells were transfected with either a non-targeting control (Dharmacon D-001810-01-05) or DNMT1 (Dharmacon J-004605-06-0005) targeting siRNA and using Lipofectamine 2000 (Invitrogen). Cells were transfected with 25 nM siRNA at 0, 24 h, and 48 h. Cells were harvested for analysis at 72 h post-transfection. LSD1 knockdowns in HCT116 parent cells and individual clones E1, wt1, wt2, mut1 and mut2 cells were performed as follows: The cells were plated to 20% confluency, infected the next day with lentiviral shRNA targeting LSD1 (Sigma TRCN0000046072) or non-targeting control (Sigma SHC002), split 48 h later and harvested 5 days post-infection for analysis.

Real-time–PCR

RNA was isolated using the RNeasy kit with on-column DNaseI treatment (Qiagen) and cDNA was made using Superscript3 and oligodT20 (Invitrogen). RT–PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen). βactin or GAPDH was used as the loading control. Primer sequences used for all studies can be found in Supplementary Table S1.

Methylation specific PCR and bisulfite sequencing

Genomic DNA was extracted with a lysis buffer containing 20 mmol/l Tris–HCl, 20 mmol/l EDTA, 2% SDS and 0.5 mg/ml proteinaseK. One microgram of genomic DNA was subjected to bisulfite treatment for subsequent PCR analyses (25). Methylation-specific PCR (MSP) was performed as previously described and the products were visualized on 2% agarose gels (26). Bisulfite sequencing was performed as previously described (27). MSP and Bisulfite sequencing primers are found in Supplementary Table S1.

Gene-expression microarrays

The mut and empty vector RNA were processed and quality controlled for microarray analysis as per the

Agilent protocol. Microarray data was loess normalized (limma package, version 3.6.9, in Bioconductor). Median Log_2 ratios of Cy5 (mut) to Cy3 (empty vector) were computed for all probes mapping to the same gene. Genes that had a Log_2 ratio of at least + 0.48 were called as upregulated while genes with −0.48 Log_2 ratios were called downregulated.

Chromatin Immunoprecipitation

Antibodies used for Chromatin Immunoprecipitation (ChIP) were as follows: αDNMT1 (Sigma), αFLAG (Sigma), αH3-K4me2(Millipore), αH3-K4me3 (Millipore), αH3-K9Ac (Millipore), αH3-K9me2 (Millipore), αH3-K9me3 (courtesy of Thomas Jenuwein), αH3-K27me3 (Millipore), αLSD1 (Abcam) and αH3 (Abcam). Cells were crosslinked as previously described (28). Nuclear extracts from $\sim 1 \times 10^6$ cells were used per IP. Crosslinked pellets were resuspended with CEBN. The solution was incubated on ice for 10 min with vortexing every minute and nuclear pellet was centrifuged at 6400 rpm in the cold for 5 min. The nuclear pellets were washed once with CEB and then resuspended with SDS lysis buffer (Millipore). Samples were sonicated to 500-bp to 1-kb fragments, 60–80 μg of chromatin was used per IP and appropriate amounts of antibodies, between 2 and 10 μg, were added to sonicated DNA. Dynal magnetic beads (Invitrogen) were added for 3 h and wash conditions were adjusted based on the antibodies. IP specific products were amplified using RT–PCR (primers are in Supplementary Table S1).

Co-immunoprecipitation

To prepare cell extracts, cells were washed with cold PBS, resuspended in cytoplasmic extract buffer [10 mM HEPES (pH 8), 10 mM KCl, 2 mM $CaCl_2$, 0.34 M sucrose, 10% Glycerol, 0.2% NP40 and protease inhibitors] and incubated on ice for 10 min. Samples were centrifuged at 4000 rpm for 5 min at 4°C. The pellet was resuspended in the cytoplasmic extract buffer once, centrifuged again at 4000 rpm for 5 min at 4°C, and then resuspended in a modified RIPA buffer 1 (50 mM Tris pH 7.5, 100 mM NaCl, 3 mM EDTA, 0.5% NP40 and protease inhibitors). The nuclear extract was sonicated for 20 pulses and then cleared by high speed centrifugation (30 min, 14 000 rpm, 4°C). Nuclear lysates were rotated with Sigma DNMT1 antibody (D4692) or Abcam LSD1 antibody (ab17721) overnight at 4°C, and we used a rabbit anti-CBP antibody (SC-583X, Santa Cruz) as the negative control of the immunoprecipitation. Protein A/G-agarose beads (Santa Cruz) were added to the samples and samples were rotated for 3 h at 4°C. Then, protein A/G beads were washed four times with modified RIPA buffer2 (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 0.5% TritonX-100 and protein inhibitors) by rotation for 5 min at 4°C. Proteins were extracted from Protein A/G beads by boiling in 1× LDS gel-loading buffer.

RESULTS

Defining DNMT1 target genes

Previous work has shown that DNMT1 lacking the catalytic domain represses transcription of a reporter gene. However, these studies do not resolve whether this protein can exert such activity on endogenous targets. Here, we investigate the gene repressive function of DNMT1 at such targets in the context of native chromatin.

We first identified DNMT1 gene targets using the previously discussed hypomorph *DNMT1*$^{-/-}$ HCT116 cells (9,10). By examining our previous expression microarray data for a clone of these cells, we identified over 1000 genes for which expression was increased in *DNMT1*$^{-/-}$ cells compared to HCT116 cells (13). We refined this list further by examining the expression profiles of three additional subclones of *DNMT1*$^{-/-}$ cells. By selecting genes that were upregulated in all four of these clones we identified 229 candidate genes (Figure 1A and Supplementary Table S2A). Additionally, to characterize the repressive effects of DNMT1 alone, we also eliminated genes that were upregulated in *DNMT3b*$^{-/-}$ in HCT116 since our previous studies strongly suggested a functional interaction or cross talk, between DNMT1 and DNMT3b (29). This reduced the list to 135 genes (Supplementary Table S2B). From these, we chose 11 genes to validate by RT–PCR which showed a varying upregulation on the arrays from high to slight in *DNMT1*$^{-/-}$ cells. All genes analyzed, except *POTEB* (Supplementary Figure S1), showed an increase in expression in *DNMT1*$^{-/-}$ relative to HCT116 cells (Figure 1B and Supplementary Figure S1) while a control gene, *cMYC*, was not further upregulated and even decreased slightly (Figure 1B). This slight decrease in *cMYC* levels may be due to modestly slower growth seen in *DNMT1*$^{-/-}$ cells (29).

To further validate that the above genes were specific targets and not altered due to secondary effects resulting from stable knockout of the protein, we employed a transient siRNA approach to knockdown DNMT1 (Figure 1C) in wt HCT116 cells. We observed increases in expression of all genes studied including *DSCR8*, *MAGEA10*, *TXNIP* and *DTX3,* but not the negative control *cMYC,* (Figure 1D) validating the specificity of the changes. However, these findings do not demonstrate if DNMT1 controls the expression of these genes directly by binding to the promoter or by secondary, indirect effects. To further address this issue, we used ChIP to investigate whether DNMT1 was recruited to these promoters. The specificity of the antibody for DNMT1 protein was validated by using the *DNMT1*$^{-/-}$ cells as the negative control. At all target genes studied, we observed localization of DNMT1 above background levels but not to *cMYC* (Figure 1E) demonstrating that these promoters were occupied by DNMT1 and further suggests that these are direct DNMT1 target genes.

DNMT1 catalytic activity is not required for gene repression at endogenous targets

We next assessed how the above repression effects of DNMT1 depend upon the DNA methylation catalytic

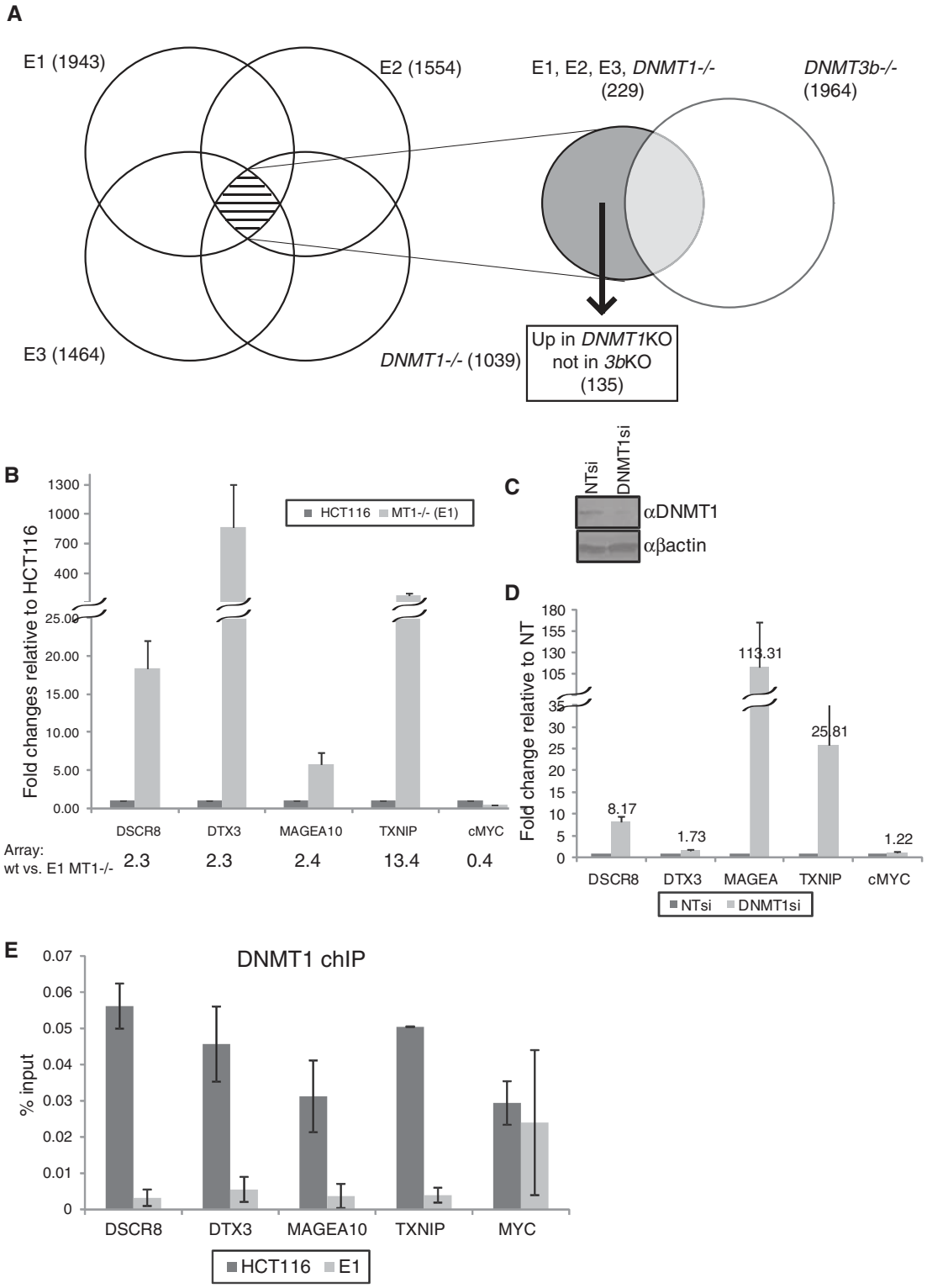

Figure 1. DNMT1 target genes. (**A**) Venn diagram depicts numbers of genes upregulated in clones containing empty vector pEF1αIRESpuro (E1, E2, E3) of HCT116 *DNMT1*$^{-/-}$ hypomorphs compared to parental HCT116. '*DNMT1*$^{-/-}$' is the parental *DNMT1*$^{-/-}$ hypomorph clone. A total of 229 genes were found upregulated 1.4-fold or greater in all four *DNMT1*$^{-/-}$ clones. From the 229 gene list, genes upregulated 1.4-fold also in *DNMT3b*$^{-/-}$ were eliminated and yielded 135 genes. (**B**) RT–PCR validation for upregulation of genes in *DNMT1*$^{-/-}$ (E1) cells. Fold change relative to HCT116 is calculated. *cMYC* was used as a negative control for a gene with unchanged expression in the *DNMT1*$^{-/-}$ cells. Microarray fold changes for individual genes are written below. (**C**) Western blot to assess transient knockdown of DNMT1 in HCT116 cells transfected with *DNMT1* siRNA (DNMT1si) or non-target siRNA (NTsi) for 72 h. αβactin serves as a loading control. (**D**) RT–PCR analysis of *DSCR8*, *DTX3*, *MAGEA10*, *TXNIP* and *cMYC* genes in HCT116 cells transfected with non-target control siRNA (dark grey bar) or *DNMT1* siRNA (light gray bar). Fold change relative to non-target control was calculated. Bars = standard error of three independent experiments. (**E**) ChIP at *DSCR8*, *DTX3*, *MAGEA10*, *TXNIP* and *cMYC* promoter regions for αDNMT1. Samples included HCT116 and *DNMT1*$^{-/-}$ (E1) cell lines. RT–PCR was conducted and the average levels of enrichment relative to input and standard errors were calculated for three independent ChIP experiments.

activity of the protein. To do this, wt or a catalytically inactive DNMT1 (mut), each containing a N-terminus FLAG tag, were stably expressed with puromycin selection in the *DNMT1*$^{-/-}$ hypomorph cells and compared to insertion of an empty vector (E) construct. The mut construct contains a single point mutation at amino acid position 1226 from a cysteine to tryptophan rendering the protein catalytically inactive (30) but otherwise preserving the full structure of the protein (Figure 2A). Individual transfected clones with exogenous wt or mut DNMT1 protein levels similar to levels of endogenous DNMT1 in HCT116 were selected for further analysis (Figure 2B). Additionally, in all clones, exogenous DNMT1 showed nuclear expression pattern similar to the parental HCT116 cells (Figure 2C).

We next studied the expression, by RT–PCR, of four DNMT1 target genes after re-introducing either wt or mut DNMT1. As expected, *DNMT1*$^{-/-}$ hypomorph vector only clone, E1, has increased expression of all tested genes (Figure 3A). Restoring wt or introducing mut DNMT1 reduces the expression of the DNMT1 specific genes below levels found in the control *DNMT1*$^{-/-}$ E1 clone, and for some genes similar to or below levels found in parental wt HCT116 (Figure 3A). No change in the expression of the control gene, *cMYC,* was observed with introduction of either wt or mut DNMT1. Similarly, in expression profiles of the 135 DNMT1 target genes, in the wt and mut *DNMT1* replacement clonal lines, we found reduced transcripts of some target genes in clones for both the catalytically inactive *DNMT1* or the wt *DNMT1* and no distinctions could be made between the overall expression patterns for these (Figure 3B). All of these data suggest that putting back a catalytically inactive *DNMT1* or a wt *DNMT1* have similar or indistinguishable repression effects on target genes. Together, these data are consistent with the hypothesis that there are genes for which the catalytic activity of DNMT1 is not required for specific gene repression.

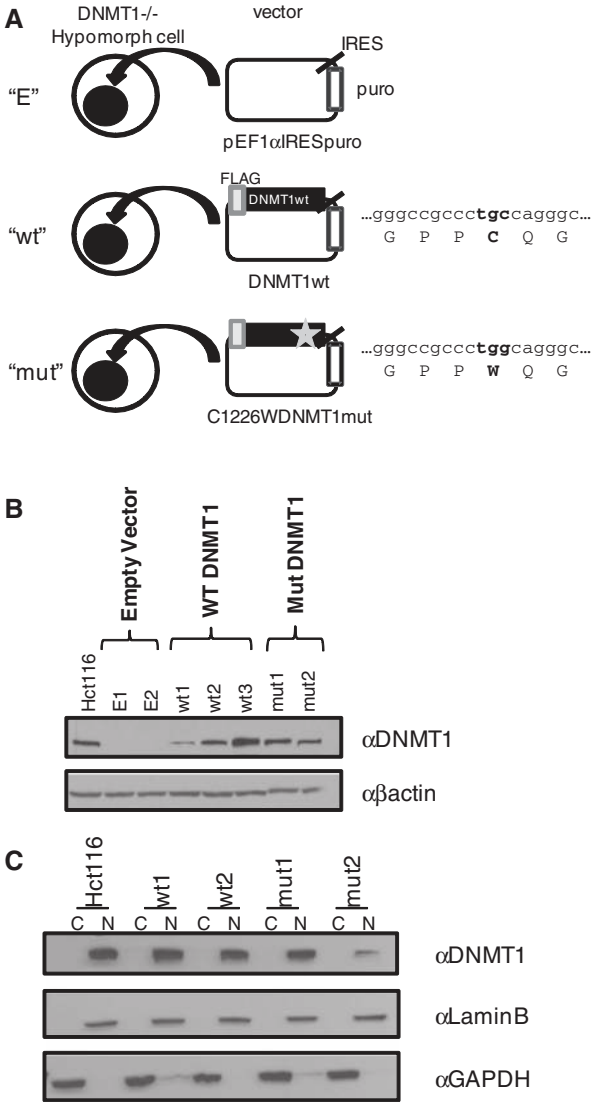

Figure 2. Stable insertion of wildtype (wt) FLAG-*DNMT1* and mut FLAG-*DNMT1* into *DNMT1*$^{-/-}$ hypomorphs. (**A**) *DNMT1*$^{-/-}$ hypomorph cell lines were stably transfected with vector alone (E), wt FLAG - *DNMT1* (wt) and FLAG - *DNMT1* containing a point mutation, C1226W, in the catalytic domain (mut). The star represents the position of the point mutation introduced into the *DNMT1* vector. (**B**) Whole-cell western blot analysis of DNMT1 levels in Hct116, *DNMT1*$^{-/-}$ clones (E), wt and mut clones. βactin was used as a loading control. (**C**) Cytoplasmic 'C' and nuclear 'N' proteins were isolated. Western blot analysis of DNMT1 in HCT116, 2 wt clones and 2 mut clones. LaminB was used as nuclear loading control and GAPDH as a cytoplasmic loading control.

DNA methylation is not required for gene repression

We next examined the DNA methylation status at the promoters of four DNMT1 target genes to determine whether this modification was required for gene repression. All four genes have CpG islands within their promoter region from weak to strong CpG islands (*DSCR8*: CpGobs/CpGexp = 0.71 to *DTX3*: CpGobs/CpGexp = 0.98) as characterized by the criteria of Gardiner–Garden and Frommer and Takai *et al*. (31,32). In *DNMT1*$^{-/-}$ E1 clones, as analyzed by MSP, there is a partial loss of DNA methylation at both the *DTX3* and *TXNIP* promoters (Figure 4A) while in most of the *DNMT1* wt restoration clones, the methylation is restored. Interestingly, both *DTX3* and *TXNIP* gene promoters remained partially unmethylated when catalytically inactive DNMT1 was reintroduced (Figure 4A) suggesting that DNA methylation was not required for the mut protein to restore repression of these genes.

To further quantitate the DNA methylation of the DNMT1 target genes, we performed bisulfite sequencing for *DSCR8*, *MAGEA10* and *TXNIP* by analyzing the CpGs near the transcription start site. Similar to the above MSP data, depletion of *DNMT1* caused loss of DNA methylation at all the genes studied but the degree of loss varied from clone to clone (Figure 4B–G) ranging from the genes being almost completely unmethylated in E2 to retaining high levels of DNA methylation in E3. In addition, this variability in methylation (Figure 4D–G) correlated with the variability in expression (Supplementary Figures S2A and B) seen in the empty vector clones, thereby giving us confidence that this was

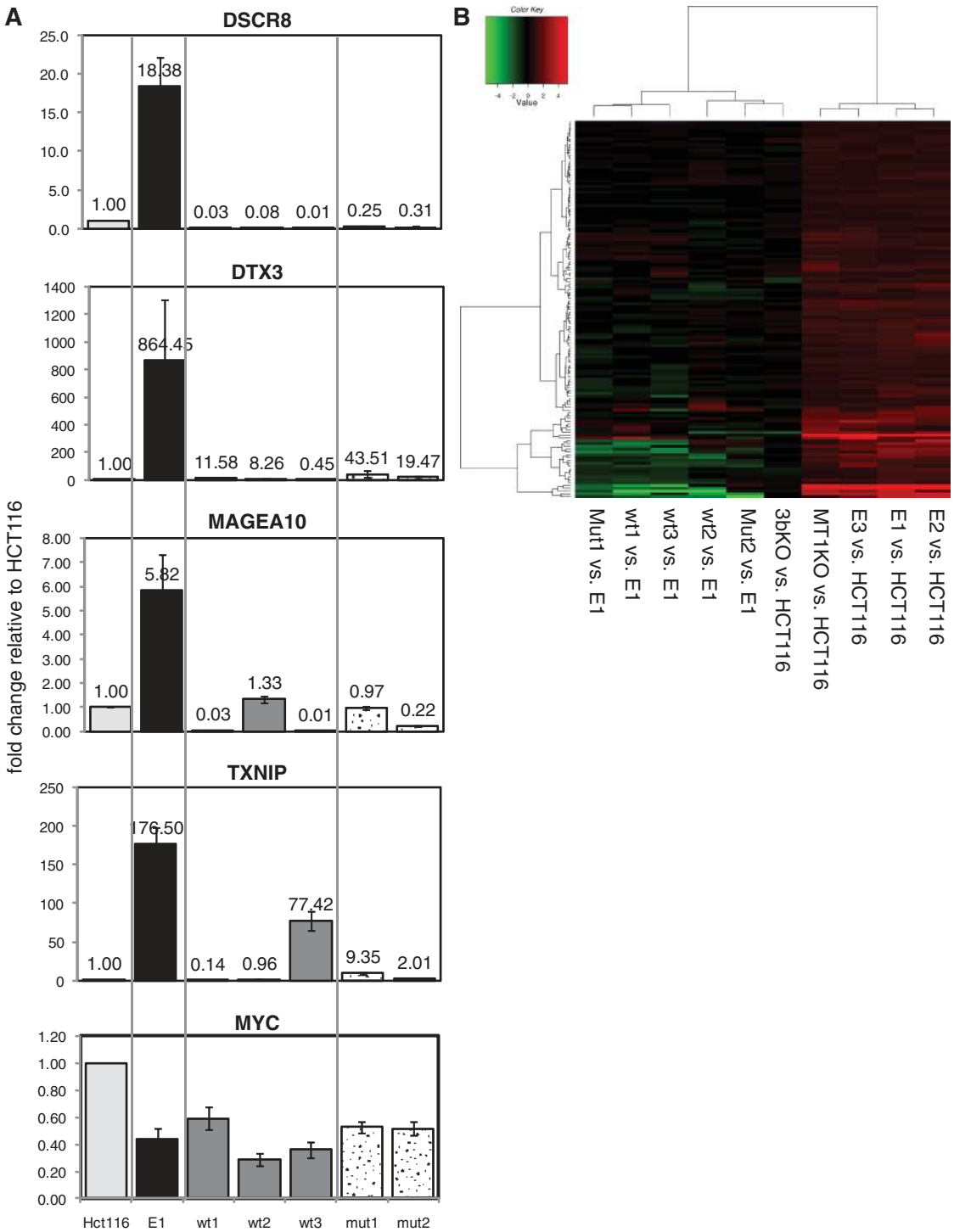

Figure 3. DNMT1 catalytic activity is not required for gene repression of some genes. (**A**) RT–PCR analyses were performed in parental Hct116, E1 (*DNMT1*$^{-/-}$), wt*DNMT1* (wt1, wt2, wt3) and mut*DNMT1* (mut1 and mut2) replacement cells. Fold change relative to HCT116 was calculated. *cMYC* was used as a negative control. Numbers above bars are the average fold values relative to HCT116. Bars depict standard error from three independent experiments. (**B**) Agilent Microarray. The 135 genes that were upregulated 1.4-fold in DNMT1KOs and not DNMT3bKOs were mapped. The right four columns are parental *DNMT1*$^{-/-}$, E1, E2 and E3 clones versus wt HCT116 where E represents clones with empty vector pEF1αIRES puro. The middle column is the expression changes of DNMT3bKO cell lines compared to HCT116. The left five columns are E1 versus wt1, wt2, wt3, mut1 and mut2. Red depicts increased expression, green depicts decreased expression.

the right region for analysis. Restoration of wt *DNMT1* caused remethylation of *TXNIP* (Figure 4F and G) and some spotty remethylation of *MAGEA10* (Supplementary Figure S3) and *DSCR8* (Figure 4B and C) providing evidence that the wt DNMT1 construct has catalytic activity. However, *MAGEA10* near the transcription start site and most of the residues in *DSCR8* remained mostly unmethylated even in the presence of wt

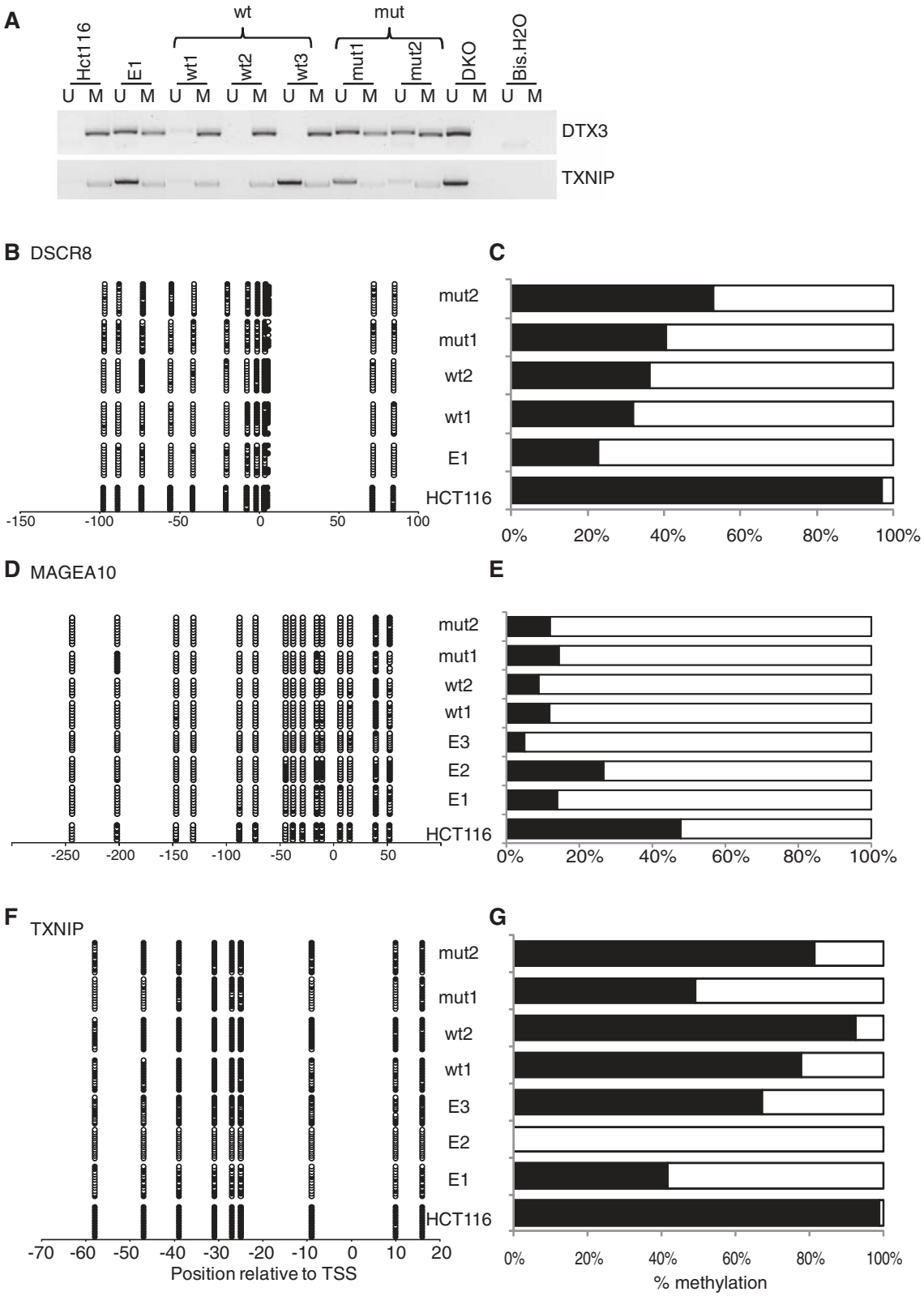

Figure 4. DNA methylation analysis after DNMT1 wt or mut replacements. (**A**) MSP of *DTX3* and *TXNIP* in parental HCT116 cells, *DNMT1*$^{-/-}$ hypomorph clone E1, 3 wt*DNMT1* restoration clones (wt1, wt2, wt3), 2 mut*DNMT1* replacement clones (mut1, mut2) and DKO, a cell line with both *DNMT1* and *DNMT3b* genetically disrupted in HCT116 cells. Unmethylated sequences are represented by an amplification signal in the U lanes and presence of methylation by amplification in the M lanes. Bisulfite sequencing of (**B**) *DSCR8*, (**D**) *MAGEA10* and (**F**) *TXNIP*. E2 and E3 are two subclones of *DNMT1*$^{-/-}$ hypomorph cells containing the control empty vector. Each horizontal line is an individually sequenced TA cloned allele with each circle representing a CpG dinucleotide as distributed in the promoter region. Base pairs upstream and downstream relative to transcription start site (TSS at 0) are numbered along the ×-axis. Black circles are methylated cytosine residues, white circles are unmethylated cytosine residues. (**C, E, G**) Quantitation of results as total % methylated cytosine (black bars) and % non- methylated (white bars) relative to the total number of CpGs in the sequences.

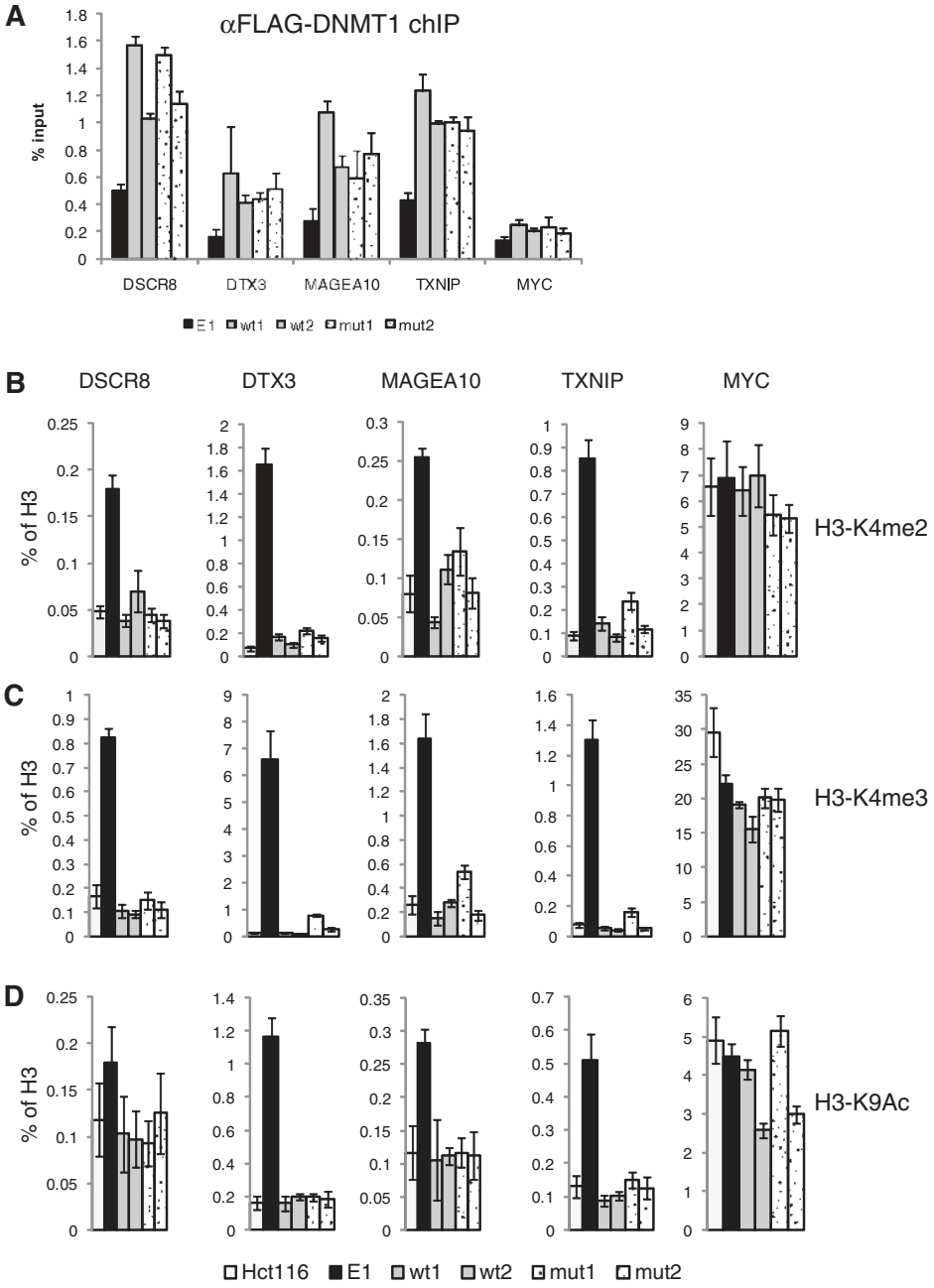

Figure 5. DNMT1 recruitment to promoters correlates with loss of active histone marks. ChIP at *DSCR8*, *DTX3*, *MAGEA10*, *TXNIP* and *MYC* promoter regions for (**A**) αFLAG vector (**B**) H3-K4me2, (**C**) H3-K4me3 and (**D**) H3-K9Ac. Samples included HCT116, *DNMT1*$^{-/-}$ empty vector (E1), wt*DNMT1* clones (wt1 and wt2) or mut*DNMT1* clones (mut1, mut2). RT–PCR was conducted and the average levels of enrichment relative to input for αFLAG and histone 3 (H3) IP for histone marks were calculated for three independent ChIP experiments.

DNMT1 (Figure 4B–4E) suggesting that, at these genes and at these particular regions, DNA methylation is not required for gene repression. At *TXNIP* (Figure 4F) and some sites of *MAGEA10* (Supplementary Figure S3) promoters, the mut1 and mut2 clones containing the catalytically inactive mut showed varying levels of DNA methylation similar to the above control empty vector clones. Despite varying levels of DNA methylation, we observed that mut DNMT1 repressed the expression of both target genes below levels found in any *DNMT1*$^{-/-}$ hypomorph clones (Supplementary Figure S2A and B). We, therefore, conclude that DNA methylation is not required for gene repression of target genes demonstrating the non-catalytic activity of the DNMT1 protein.

DNMT1 recruitment to promoters alters histone modifications

To learn more about how the insertion of exogenous *DNMT1* into *DNMT1*$^{-/-}$ cells may be working to

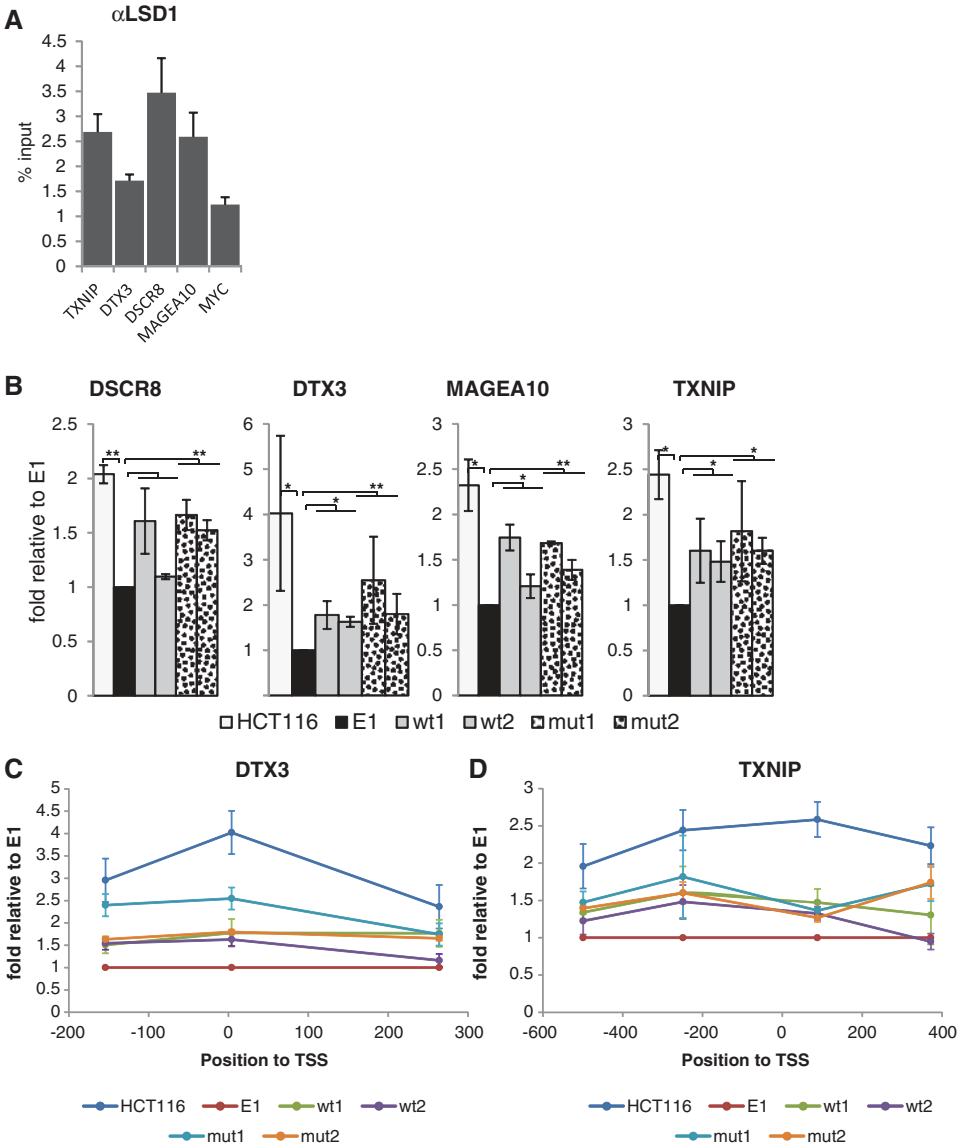

Figure 6. DNMT1 recruits LSD1 to target promoters. (**A**) ChIP of LSD1 at *DSCR8*, *DTX3*, *MAGEA10*, *TXNIP* and *MYC* in HCT116 cells. Average % input was calculated. ChIP of a single locus (**B**) at DNMT1 target genes for αLSD1 in HCT116, *DNMT1*$^{-/-}$ empty vector (E1) clones, wt*DNMT1* clones (wt1 and wt2) or mut*DNMT1* clones (mut1 and mut2) or multiple loci at *DTX3* (**C**) and *TXNIP* (**D**). RT–PCR was conducted and the average levels of enrichment relative to input and then fold change relative to E1 were calculated for three independent ChIP experiments. *P*-value was calculated using a one-tail *t*-test analysis comparing HCT116 versus E1, E1 versus wt1 + wt2 and E1 versus mut1 + mut2. $*P < 0.05$, $**P < 0.01$.

repress target gene promoters, ChIP for the FLAG tagged wt and mut DNMT1 was performed. The promoter regions of all DNMT1 responsive genes, but not *cMYC*, showed an enrichment of both FLAG tagged proteins (Figure 5A) illustrating that both the wt and mut proteins were being recruited specifically to these genes.

In order to better understand the mechanism of DNMT1 repression, especially in the mut replacement clones, we mapped the chromatin changes coupled to recruitment of the protein. H3-K4me2 (Figure 5B), H3-K4me3 (Figure 5C) and H3-AcK9 (Figure 5D), all histone modification marks correlated with active transcription, were enriched at promoters of target genes in the *DNMT1*$^{-/-}$ E1 clones. *CMYC*, however, showed a slight decrease of H3-K4me3 mark in E1 (Figure 5C) corresponding to slight decrease in expression as seen in RT–PCR (Figure 3A). With reinsertion of either wt or mut DNMT1, the active marks were subsequently depleted at these same promoters. Repressive marks, H3-K9me2 (Supplementary Figure S4A), H3-K9me3 (Supplementary Figure S4B) and H3-K27me3 (Supplementary Figure S4C), however, did not consistently change with loss of DNMT1. Our data indicate that, while loss of DNMT1 at the promoters of the genes studied greatly influences the active histone marks with concomitant increased expression of target genes, repressive histone marks are not dramatically affected. We also illustrate that the catalytic activity of DNMT1 is not

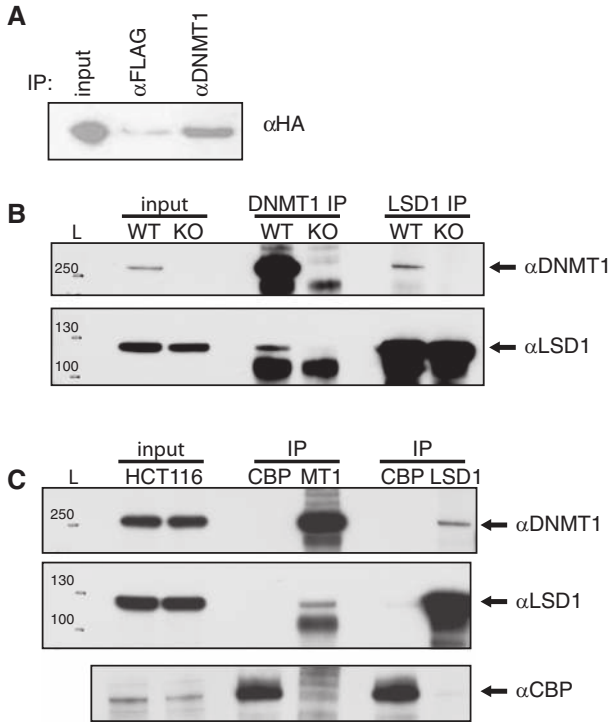

Figure 7. LSD1 interacts with DNMT1. **(A)** Co-immunoprecipitation and western blot analyses were performed in HCT116 cells over-expressing HA-LSD1 and immunoprecipitation of anti-DNMT1 or anti-FLAG as a negative control and western blot for anti-HA. **(B)** Endogenous co-IP and western blot analyses were performed in HCT116 (WT) and DNMT1$^{-/-}$ 5F (KO) using anti-DNMT1 or anti-LSD1 antibodies. (**C**) Endogenous co-IP using anti-LSD1 (LSD1), anti-DNMT1 (MT1) and negative control, anti-CBP (CBP) antibodies were performed in HCT116 cells. Western blots for all three proteins were performed.

required for depletion of active histone modifications at the promoters of its target genes suggesting further, that the protein, DNMT1, has inherent capacity for repressing the expression of the target genes in a manner distinct from the enzymatic activity associated with DNA methylation.

DNMT1 recruits LSD1 to target promoters

We next assessed localization of enzymes which modulate some of the dynamics observed with regard to changes in histone modifications associated with gene expression. One of these proteins, LSD1/KDM1A, responsible for demethylating H3-K4me2 and H3-K4me1 (33), is known to interact with DNMT1, but with unknown specificity for targeting gene promoters (24). We used ChIP to examine the localization of LSD1 to DNMT1 target genes. First, we found that LSD1 was associated to all the promoters in HCT116 cells above background (Figure 6A) with *cMYC*, a non-DNMT1 target, having the lowest amount of LSD1. Next, we observed that LSD1 levels at the gene promoters were significantly lower in the *DNMT*$^{-/-}$ clones than in the wt, parent HCT116 cells (Figure 6B). With the insertion of wt DNMT1, there is a distinct and significant increase of LSD1 at the promoter regions of *DTX3, MAGEA10* and *TXNIP* (Figure 6B) and insertion of mut DNMT1 shows a significant increase of LSD1 localization at all the gene promoters studied (Figure 6B). We further mapped LSD1 localization at several sites near the transcription start sites for two genes, *DTX3* (Figure 6C) and *TXNIP* (Figure 6D). Here, we observed HCT116 cells as well as both wt and mut reinsertion clones had higher levels of LSD1 localization compared to the *DNMT1*$^{-/-}$ cells, especially near the transcriptional start site (Figure 6C and D).

Next, we assessed whether DNMT1 interacted with LSD1. We found that overexpression of HA tagged LSD1 immunoprecipitated with endogenous DNMT1 (Figure 7A). Additionally, the two endogenous proteins could be bi-directionally immunoprecipitated by antibodies against both DNMT1 and LSD1 (Figure 7B and C) but neither protein interacted with nuclear protein CBP (Figure 7C). In addition, LSD1 did not show any interactions in the DNMT1 KO clones (Figure 7B). These studies suggest that LSD1 is at least one protein being recruited by DNMT1 to specific sites and, therefore, may be responsible for the loss of H3-K4me2 which accompanies subsequent repression of genes by the protein.

To that end, we next examined the importance of LSD1 on gene expression at our target genes by using shRNA to knock down the protein in wt or mut DNMT1 replacement cells (Figure 8A). A general trend of increased gene expression of some of our DNMT1 target genes after LSD1 knockdown was observed with some variability in the mut replacement clones (Figure 8B). This suggests that LSD1, recruited by DNMT1, has some role in regulating the expression of DNMT1 target genes.

DISCUSSION

Our studies show that at some endogenous gene targets, DNMT1 can repress gene expression independent of its catalytic function. Our work markedly extends studies which have suggested such function but were performed only with exogenous reporter constructs (11,14,15). In contrast, the present work identifies target genes in their native chromatin configuration. Our detailed studies of selected target genes, coupled with our expression arrays suggest there may be hundreds of DNMT1 target genes. The precise number is difficult to discern in our system given the clonal variation in gene upregulation we observed for HCT116 *DNMT1*$^{-/-}$ cells. The small overlap of common genes found regulated by DNMT1 may be due to the presence of a limiting amount of a truncated DNMT1 leading to inefficient and spotty basal methylation (10). Nevertheless, the 229 genes identified from four overlapping *DNMT1*$^{-/-}$ subclones provided confidence that these genes were bona fide DNMT1 target genes.

Previous work has shown that DNMT1 homodimerizes via the TS domain although the requirement for the dimerization for DNMT1 activity has not been elucidated (34). It may be possible that some of the effects seen at the target genes are due to the truncated DNMT1 dimerizing with the exogenous DNMT1. However, since we also

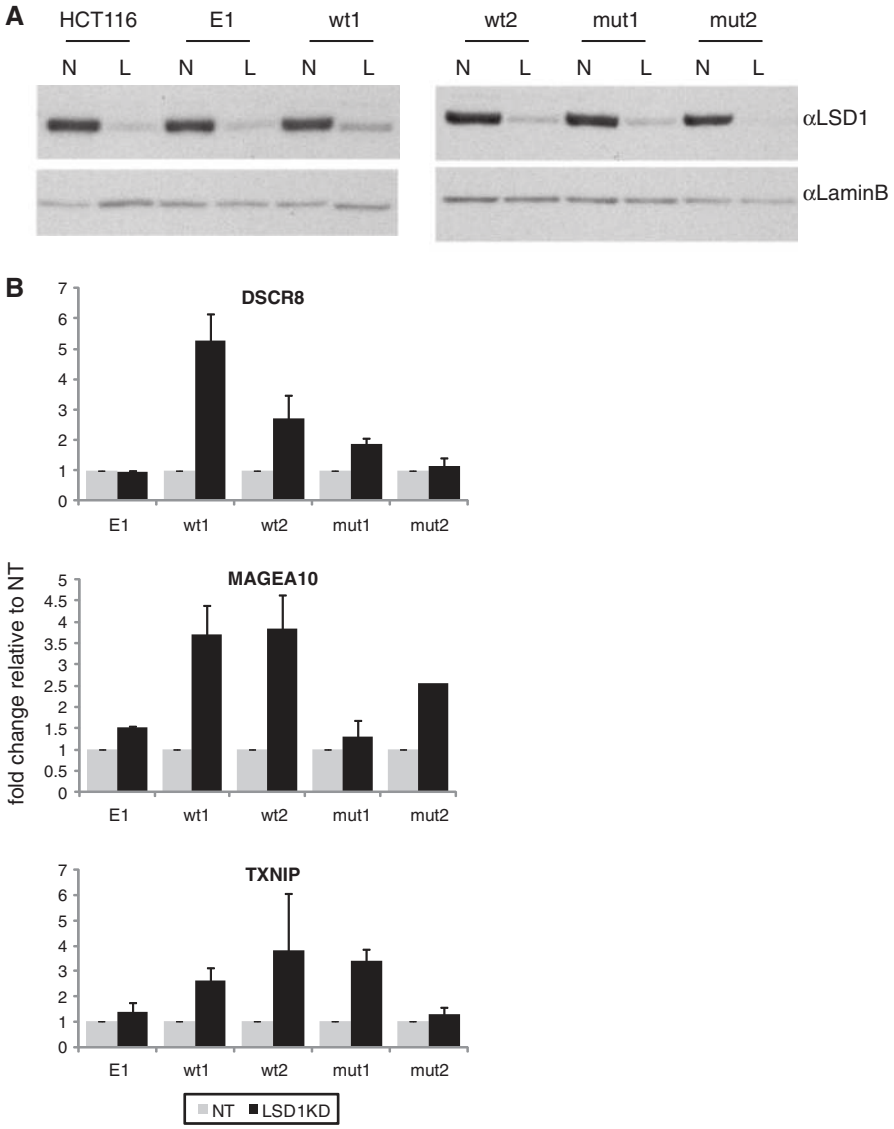

Figure 8. Knocking down LSD1 induces variable increases in gene expression of some DNMT1 target genes. (**A**) LSD1 was transiently knocked down, using lentiviral infection delivery in E1, wt1, wt2, mut1 and mut2 cells with LSD1 (L) versus non-target (N) shRNA for 5 days. Successful knockdown was verified by western blot of αLSD1 with αLaminB used as a loading control. (**B**) RT–PCR analysis of DSCR8, MAGEA10 and TXNIP genes in each cells transfected with non-target control (light grey bar) or LSD1 (black bar) shRNA were measured. Fold change relative to NT was calculated. Bars = standard error of three independent experiments.

observed that expression of *DSCR8*, *MAGEA10*, *TXNIP* and *DTX3* were repressed without DNA methylation, this supported our hypothesis that the catalytic activity is not required for gene repression.

While our current data show that DNA methylation is not required for the repressive action of DNMT1 upon some of its target genes, the degree of gene repression in the mut *DNMT1* reinsertion clones was often a bit weaker than for that produced by the exogenous wt protein. This was also observed in previous studies mentioned above wherein full length DNMT1 reduced transcription of the reporter gene better than did the protein when the N-terminus was truncated (14). Thus, both catalysis of DNA methylation and possible scaffolding effects of DNMT1 may be variably important for control of target genes. This is consistent with all of our studies where, despite the varying DNA methylation levels observed in our mut DNMT1 clones, we still observed repression of target genes compared to any of the $DNMT1^{-/-}$ hypomorph subclones.

Our present studies, strongly suggest that DNMT1 may have a multi-faceted function which combines both a transcriptional repression mechanism and DNA methyltransferase activity to provide multiple layers of gene silencing. This is an important concept for understanding the full role of this protein in the basic biology of gene expression control. There may be a wide-ranging participation of this protein, as a corepressor, in such regulation, even for genes that do not normally utilize

promoter DNA methylation for control of their expression. This concept invites further investigations of the sites at which DNMT1 functions and the types of protein complexes in which it participates to perform these. Those protein complexes that control a range of histone modifications such as H3-K4me2, H3-K4me3 and H3-K9Ac, are suggested by our studies. In addition to interaction with H3-K4 demethylases (KDMs), interactions with HDAC1 and 2 have been previously shown (11,14,15) and these deacetylases can be components of many repressive complexes (35,36).

In addition to the importance of complex protein interactions for normal gene control, our findings have translational implications. For example, there is much interest in DNMT1, and other DNA methyltransferases, as targets for cancer therapy in terms of reversing abnormal gene silencing associated with DNA hypermethylation of promoter CpG islands (37,38). In this regard, clinically approved DNA demethylating drugs, such as 5-azacytidine and 5-aza-2′deoxycytidine not only block the catalytic function of DNMT1 but also induce its degradation and depletion from the nucleus (18,19). Our present work, then, emphasizes that alterations in expression induced by these drugs could span beyond genes controlled by DNA methylation alone. Investigating this possibility could broaden our concepts of how DNMT1 might be involved with, and targeted for therapy in, diseases like cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–2, Supplementary Figures 1–4.

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