Epigenetics 6:9, 1138-1148; September 2011; © 2011 Landes Bioscience

Dynamics of bivalent chromatin domains upon drug induced reactivation and resilencing in cancer cells

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Key words: chromatin remodeling, DNA methylation, epigenetic drugs, colorectal cancer, epigenetic silencing

Epigenetic deregulation revealed by altered profiles of DNA methylation and histone modifications is a frequent event in cancer cells and results in abnormal patterns of gene expression. Cancer silenced genes constitute prime therapeutic targets and considerable progress has been made in the epigenetic characterization of the chromatin scenarios associated with their inactivation and drug induced reactivation. Despite these advances, the mechanisms involved in the maintenance or resetting of epigenetic states in both physiological and pharmacological situations are poorly known. To get insights into the dynamics of chromatin regulation upon drug-induced reactivation, we have investigated the epigenetic profiles of two chromosomal regions undergoing long range epigenetic silencing in colon cancer cells in time-course settings after exposure of cells to chromatin reactivating agents. The DNA methylation states and the balance between histone H3K4 methylation and H3K27 methylation marks clearly define groups of genes with alternative responses to therapy. We show that the expected epigenetic remodeling induced by the reactivating drugs, just achieves a transient disruption of the bivalent states, which overcome the treatment and restore the transcriptional silencing approximately four weeks after drug exposure. The interplay between DNA methylation and bivalent histone marks appears to configure a plastic but stable chromatin scenario that is fully restored in silenced genes after drug withdrawal. These data suggest that improvement of epigenetic therapies may be achieved by designing strategies with long lasting effects.

Introduction

The combination of genetic and epigenetic lesions in cancer cells results in altered gene expression profiles. DNA methylation and different modifications on histone tails are the two principal forms of epigenetic regulation and both are largely disturbed in cancer cells.[1,2] Many unmethylated CpG islands often become hypermethylated during cancer progression resulting in epigenetic inactivation of the associated gene. This appears to be a principal mechanism of tumor suppressor inactivation, but not all silenced genes are considered to have antitumor activities and heterogeneous profiles have been identified for different tumor types.[1,2] Most CpG island hypermethylations appear as isolated and independent events, but the concurrent hypermethylation of neighboring CpG islands accompanied by global remodeling of the chromatin in large chromosomal regions may also occur.[3,4] This is a phenomenon known as Long Range Epigenetic Silencing (LRES) and has been reported to affect multiple chromosomal regions in different tumor types, including colorectal, prostate and breast cancer.[3-10]

Histone modifications can lead to either activation or repression depending upon which residues are modified and the type of modification.[11] For instance, in histone 3, dimethylaton and trimethylation of lysine 4 (H3K4me3) are associated with transcriptional activity, while trimethylation of lysine 27 (H3K27me3) is characteristic of silenced promoters. This later mark is driven by the presence of the polycomb repressor complex and is believed to direct de novo methylation in cancer-related silenced genes.[12,13] Different studies have reported that in genes that become silenced in cancer cells, the repressive mark H3K27me3 co-exists with active marks (H3K4me3 and H3K4me2).[6,14,15] These two opposite modifications participate in the mitotic inheritance of lineage-specific gene expression programs and have key developmental functions. Its co-localization is considered characteristic of stem cells and is believed to keep developmental regulator genes poised for induction.[16-21] More recently, it has been shown that bivalent chromatin domains are also prone to DNA hypermethylation

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Submitted: 04/18/11; Accepted: 07/15/11
DOI: 10.4161/epi.6.9.16066

in aging, providing an epigenetic link between the processes of aging and cancer.[22,23]

Stem-cell like signatures are mimicked by cancer cells and contribute to define their properties.[24-26] Resetting of epigenetic profiles appears as a novel and promising therapeutic strategy in cancer,[1,27] but a better understanding of the mechanisms setting epigenetic memory and how the chromatin landscapes are either maintained or modified is required to make it a broad reality. This is especially important, since most of the critical genes in cell programming and targets of epigenetic therapies show bivalent chromatin signatures in both stem and cancer cells.

Genome-scale approaches just illustrate the ubiquitous and complex reorganization of epigenomic profiles upon treatment with gene-reactivating drugs, as shown in a recent study.[28] Due to the heterogeneity of chromatin landscapes before and after the treatment, insights into the regulatory mechanisms can be only obtained from a detailed analysis of specific loci. An interesting example is a recent investigation in which a clear picture of chromatin and gene expression dynamics has been achieved by analysis of green fluorescent protein expression under the control of a methylated cytomegalovirus promoter after treatment with 5-AzadC.[29] This artificial setting illustrates the different epigenetic events associated with changes in gene activity, but it remains unsolved which mechanism or driving signal retains the "switch-off" memento and is able to reset the original state of the chromatin. Si et al. suggest that residual DNA methylation near the CMV-GFP locus could play a role. It is also unknown if the dynamics of chromatin remodeling and DNA methylation observed in this system apply to endogenous genes.[29]

Although important insights into the mechanisms of epigenetic control have been made, the interaction between different coexisting epigenetic marks and the dynamics of the events responsible for the repression/activation of chromatin are still poorly known.[11] Here we have characterized the epigenetic profiles of two chromosomal regions undergoing long range epigenetic silencing in most colorectal cancers, how these profiles are affected by epigenetic drugs and how they are reset upon drug withdrawal and maintenance in in vitro culture. Our results illustrate the dominant nature of DNA methylation and bivalent histone marks, which endure drug-induced changes in transcriptional activity.

Results

DNA methylation, expression and histone patterns define three types of genes in long range epigenetic silencing (LRES) regions. In previous studies we have shown that LRES affects chromosomal regions 2q14.2 and 5q35.2 in most colorectal cancers.[3,6,30] Although genetic activity is downregulated all along the LRES region, uneven epigenetic profiles are likely to define different chromatin domains. To better characterize the epigenetic regulation of the diverse chromatin domains in LRES we have investigated gene expression and epigenetic profiles in nine and six genes embedded in chromosomal regions 2q14.2 and 5q35.2, respectively, in HCT116 colon cancer cells. An overview of the chromosome maps and the molecular profiles of both regions are depicted in **Figure 1 and Supplemental Figure 1**.

According to the expression levels and the epigenetic state of their promoter, the genes could be classified into three groups (**Fig. 2**). First group consists of silenced genes exhibiting methylated CpG island-promoter; this group includes EN1, SCTR and INHBB (2q14.2) and HRH2, CPLX2 and SNCB (5q35.2) that express at low levels in normal colon cells.[3,6] A second group consisted of genes with unmethylated CpG island-promoter, and includes DDX18, INSIG2, PTPN4, RALB, TSN (2q14.2), SFXN1 and THOC3 (5q35.2). These genes tend to be downregulated in colorectal tumors (as compared with the normal colon cells) but still retain high expression levels.[3,6] Finally, a third group includes MARCO (2q14.2) and PCLKC (5q35.2) genes that are low expressed and do not contain a CpG island in the promoter region (**Fig. 1 and Sup. Fig. 1**).

Histone modification profiles were also analyzed by chromatin immunoprecipitation (ChIP) and each one of the groups exhibited characteristic profiles (**Fig. 2**). A good agreement between H3K4me2 and H3K4me3 profiles was observed (**Sup. Fig. 2**) and for simplification, H3K4me2 data have been represented in figures and Tables throughout the manuscript.

The concurrent presence of repressive H3K27me3 and active H3K4me2/me3 chromatin marks was observed in genes of the first group containing a DNA hypermethylated promoter (**Fig. 2**). This is consistent with a bivalent state, as it has been reported previously for some of these genes[6] and in other genes that become hypermethylated in cancer.[14,15,31] Genes with these features (N1, SCTR and INHBB, HRH2, CPLX2 and SNCB) are referred as MBV (methylated and bivalent). Noteworthy, the same genes that showed higher levels of H3K4me2/me3 within this group, presented the highest levels of H3K27me3, as it is the case of CPLX2 and INHBB (**Fig. 2**). H3K9Ac was absent from MBV genes and some of them (i.e., EN1, SCTR) exhibited low levels of H3K9me2 (**Sup. Fig. 3**). The H3K27me3/H3K4me2 ratio was above 0.5 in all MBV genes.

The activated state of genes of the second group was associated with high levels of H3K4me2/me3 (**Fig. 2**) and H3K9Ac marks (**Sup. Fig. 3**). However, there was no correlation between the gene expression levels and the amount of active marks when comparing genes among them. As expected, H3K27me3 mark was absent on the promoters of these genes. The H3K27me3/H3K4me2 ratio was at least ten fold lower than the minimum value observed in MBV genes. Genes of this group are referred as ACTIVE, because they were expressed in all experimental situations, although the levels were not maintained.

MARCO and PcLKC (also known as PCDH24) genes displayed histone modification profiles compatible with bivalent chromatin and accordingly their gene expression levels were very low. MARCO exhibited the highest levels of repressive marks H3K27me3 and H3K9me2 among all analyzed genes (**Fig. 2 and Sup. Fig. 3**). Genes of this group do not contain a CpG island in the promoter and are referred as NoCpGi.

Dynamics of epigenetic profiles in LRES regions upon 5-AzadC/TSA treatment and drug withdrawal. It has been repeatedly shown that epigenetically silenced genes can be

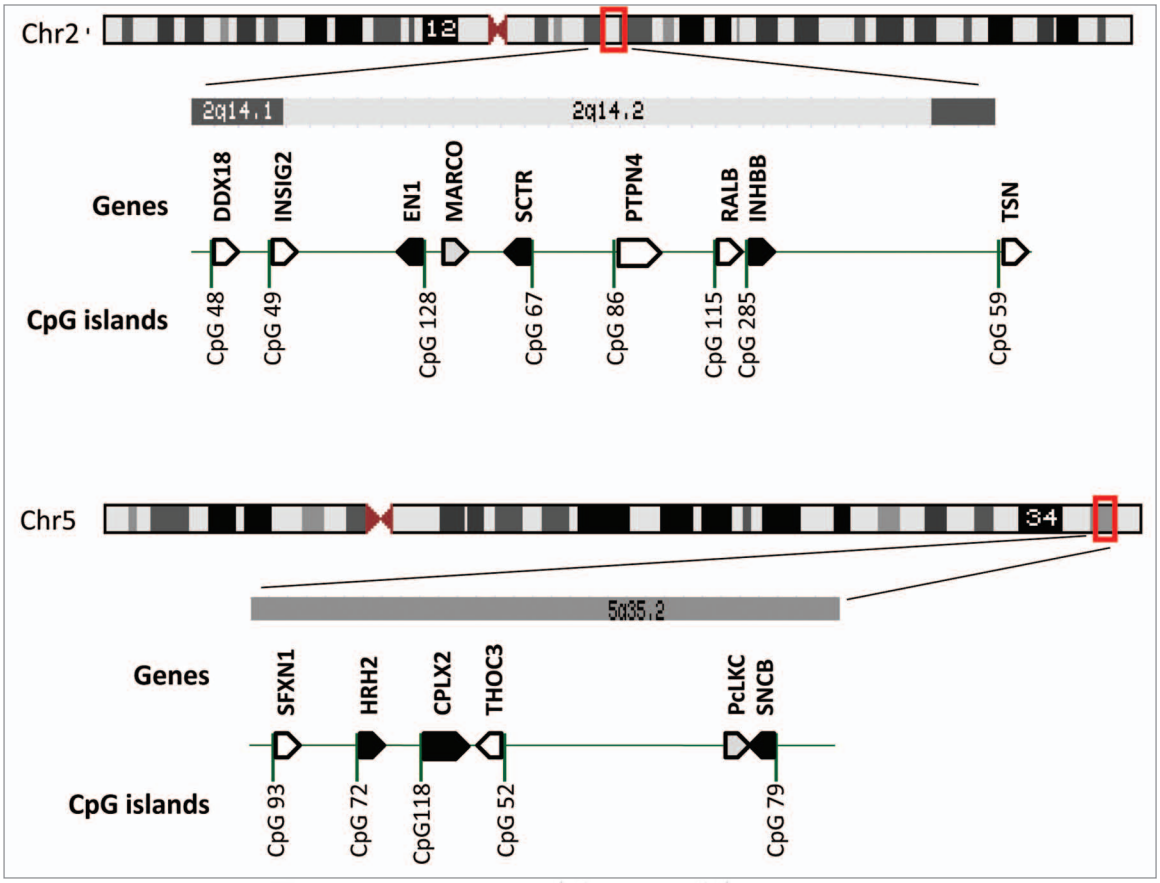

Figure 1. Scheme of the chromosomal regions analyzed at epigenetic level. Genes are depicted as arrowheads (indicating the transcription direction) and coded according to its epigenetic signature in HCT116 cells. Closed black arrowhead: DNA methylated and bivalent chromatin marks (MBV), Open arrowhead: Active genes, Grey arrowhead: No CpG island.

reactivated by treating the cells with the demethylating agent 5-Aza-2'-deoxycytidine (5AzadC) and the inhibitor of histone deacetylases Trichostatin A (TSA).[27,32] More recently it has also been shown that bivalent domains in silenced genes are not resolved in spite of the reactivation upon drug treatment.[6] To get a better picture of the events associated with gene activation and silencing in these chromosomal regions we performed a time course experiment after treating the cells separately with 5AzadC or TSA, and with a combination of both. Cells were analyzed at days 1 (the same day the drugs were removed), 16, 22 and 29 after treatment. As expected, 5AzadC alone or in combination with TSA resulted in global demethylation of the seven hypermethylated genes as determined by direct bisulfite sequencing and melting curve analysis (data not shown). For a subset of genes (EN1, SCTR and INHBB), a quantitative determination was performed using the MassCLEAVE™ DNA methylation assay system. A 60% of loss of methylation was observed just after the treatment (day 1) in 5AzadC treatment alone (data not shown) and after the co-treatment that affected most CpG sites (**Fig. 3**). Some CpG sites (SCTR 6_7 and SCTR 40) exhibiting lower methylation levels (about 50%) in untreated cells did not display changes upon treatment.

Upon drug withdrawal, a remethylation to >90% was reached in 5AzadC treated cells after 16 days (data not shown), while the co-treatment appeared to delay the full remethylation, that was achieved two weeks later (day 29) (**Fig. 3**). TSA treatment alone did not affect DNA methylation (data not shown).

5AzadC treatment restored the expression of genes with a hypermethylated promoter (MBV group) (data not shown), although the co-treatment with TSA resulted in a more substantial reactivation, that was maintained for about two weeks but returned to original levels 4 weeks later (**Fig. 4**), consistent with the DNA methylation profiles. H3K4me2/3 and H3K27me3 marks were also increased in response to the drugs, which is suggestive of balanced dynamics. After drug withdrawal, the active chromatin mark H3K4me2/me3 exhibited a time dependent decrease that, after four weeks, reached levels similar to the untreated cells for genes of the chromosomal band 5q35.2 and even lower for those of the 2q14.2 region. On the other hand, the repressed chromatin mark H3K27me3 displayed a heterogeneous profile and in some genes retained higher levels all along the time course study (**Fig. 4**). This result could be interpreted as a partial disruption of the balance between active and inactive marks during the resilencing process. Nevertheless, the alternation of different cell populations with distinct chromatin signatures might also contribute

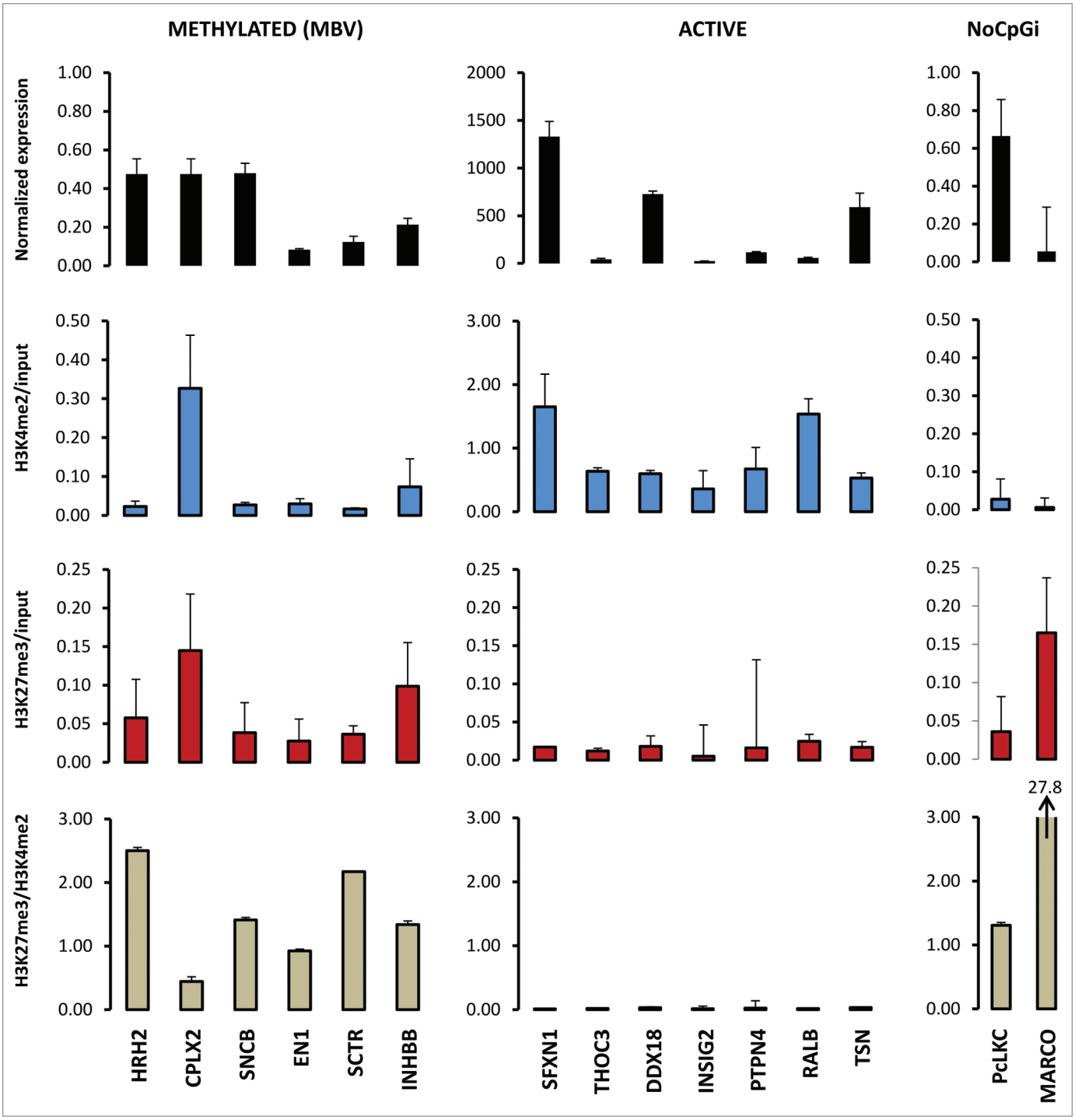

Figure 2. Gene expression and chromatin modification patterns of genes classified into three groups according to their epigenetic signatures in HCT116 cells. MBV corresponds to silenced genes exhibiting a DNA methylated promoter embedded in bivalent chromatin (presence of methylation in H3K27 and H3K4); ACTIVE corresponds to unmethylated and expressed genes; NoCpGi corresponds to genes without CpG island. Gene expression levels were normalized to 18S. H3K4me2 and H3K27me3 ChIP levels were normalized to input and the ratios between both marks are shown. All quantifications were performed by real-time PCR in triplicate. Error bars indicate standard deviation. Note that gene expression and H3K4me2 values are represented in a different scale for ACTIVE genes due to their high values as compared with the other two groups. The regional profiles of genes arranged by chromosomal position in shown in Supplemental Figure 1.

to the observed changes. H3K4me2 and H3K4me3 active marks showed parallel changes in most of the experiments in which both were analyzed, although H3K4me2 changes appeared to occur at a slower pace than H3K4me3 (**Sup. Fig. 2**).

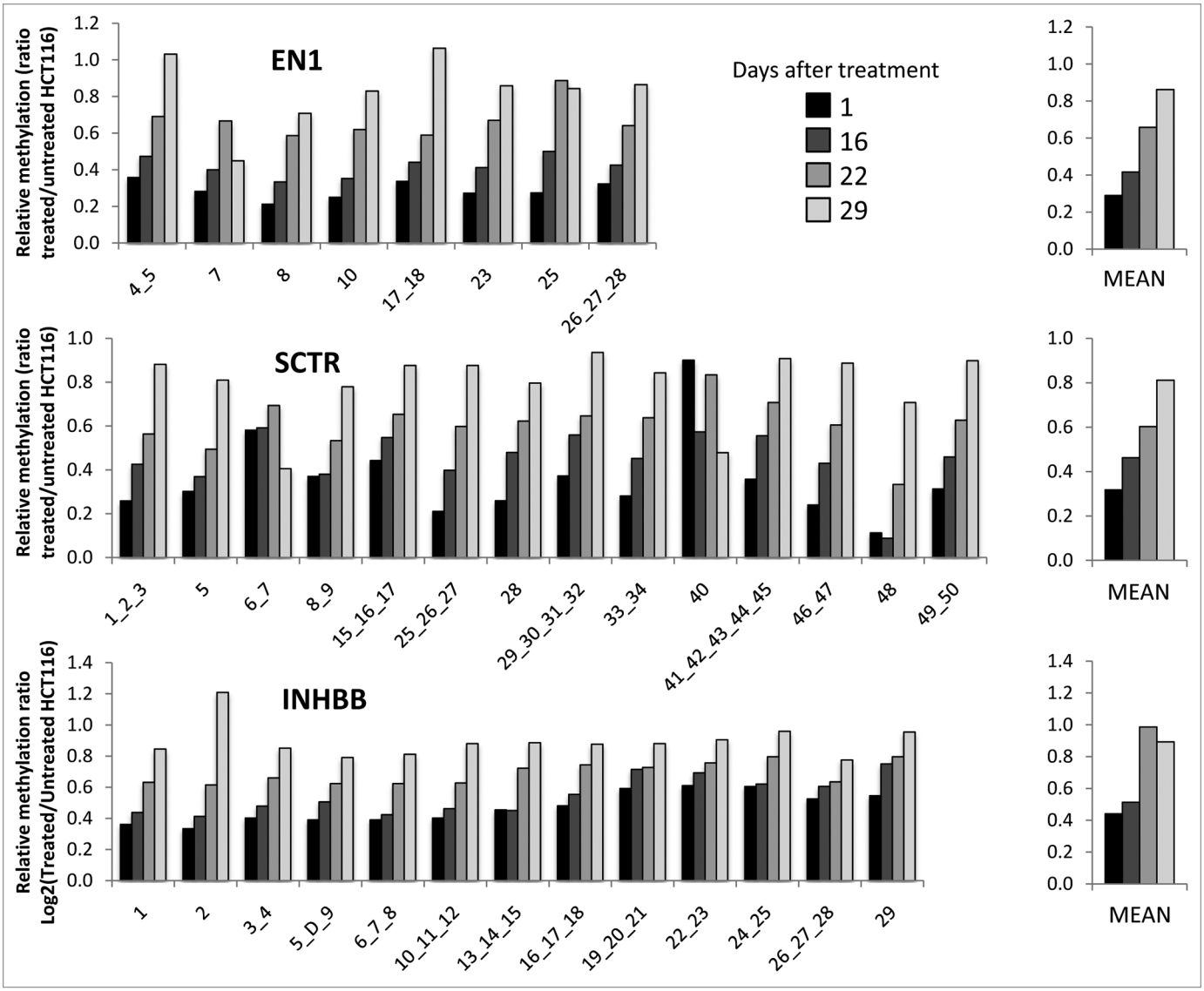

Figure 3. Relative methylation levels of the named CpG sites contained in the EN1, SCTR and INHBB amplicons as analyzed by MassCLEAVE™ after 5AzadC/TSA co-treatment. A pool of three direct bisulfite PCR products was used for analysis. Represented values correspond to the ratio of treated versus untreated HCT116 cells (Y axis). The gradient scale represents the different time points corresponding to days 1, 16, 22 and 29 after treatment (from dark to light). Mean methylation values of all the CpG sites are represented in the right part.

On the other hand, ACTIVE genes exhibited a completely different behavior in response to the various treatments. Most of them suffered a clear decrease in the transcription rates by day 1, either with 5AzadC or TSA alone (data not shown), or with the combination of both (**Fig.** 4). Reduction of expression was paralleled by an initial decrease in H3K4me2/me3 mark. The initial expression levels were recovered 16 days after the drug withdrawal as the H3K4me2/me3 mark did. H3K27me3 mark remained low or undetectable in these genes along the time course, although its sporadic presence could be detected in some genes (**Fig.** 4).

Finally, MARCO and PcLKC gene expression was slightly induced by the treatment. This reactivated status was maintained over 16 days. Afterwards, transcription rates started to decrease to the low or undetectable levels of untreated cells (**Fig.** 4). Changes in chromatin modifications were also minor, with slight increases of H3K4me2/3 and H3K27me3 in PcLKC along the time course (**Fig.** 4).

Notably, for all three groups of genes, an enrichment of H3K4me2/me3 levels was found after treating the cells with TSA alone (**Sup. Fig. 2B** and data not shown), but this effect had disappeared by day 16 for most of the genes, and was not reflected on the expression patterns. Regarding other histone modifications analyzed, H3K9Ac was increased in most genes after TSA or the combined treatment but not when treated with 5dAzaC alone as expected (data not shown). H3K9me2 and total H3 levels displayed small variations without a consistent pattern.

Maintenance of chromatin states in DNA methylation deficient cells. Since DNA demethylation appeared to reactivate silenced genes but was not able to resolve bivalent chromatin,

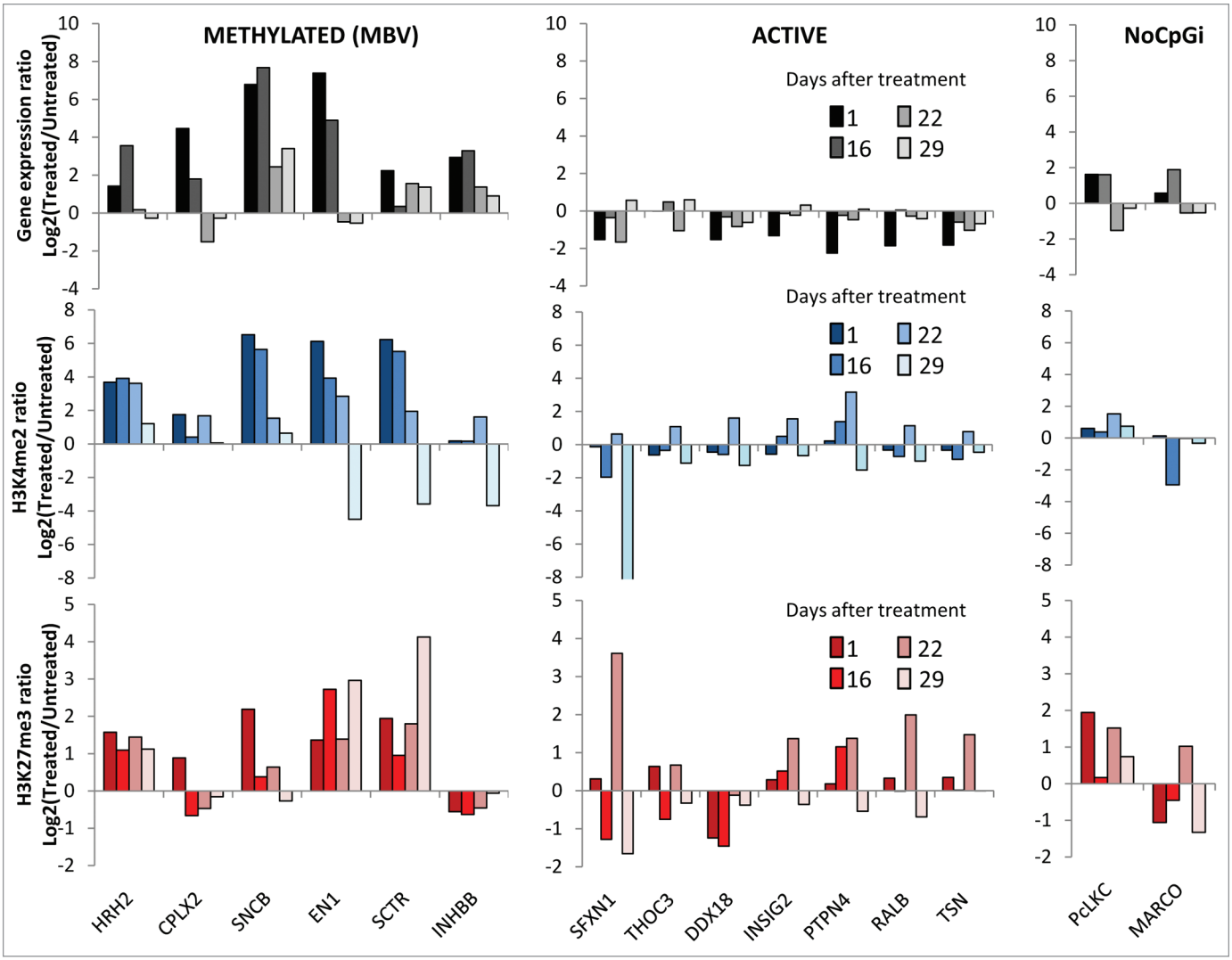

Figure 4. Relative expression and histone mark (H3K4me2 and H3K27me3) levels in the three classes of genes after 5-AzadC and TSA co-treatment. Relative values were calculated as the log2 of the treated/untreated ratio. The gradient scale indicates the different time points corresponding to days 1, 16, 22 and 29 after treatment (from dark to light).

we wondered if stable demethylation will be sufficient to override the epigenetic memory. We analyzed the DKO cells, a variant of HCT116 colon cancer cells which is deficient for DNA methyltransferases,[33] although retaining some residual DNMT1 activity.[34] As expected, hypermethylated genes (MBV) in the wild-type HCT116 were fully demethylated in DKO cells (data not shown), and their expression was restored to higher levels than in the parental cells, although two of the genes, EN1 and CPLX2, exhibited a limited reactivation (**Fig. 5A**). As in drug induced gene reactivation, DKO cells exhibited an overall increase of the H3K4me2/me3 and H3K27me3 marks in the demethylated MBV genes (**Fig. 5A**), which suggests a dynamic rebalancing of these two histone modifications enduring the retention of the bivalent states. These results are in agreement with previous observations in other genes.[15] Genes with unmethylated CpG islands (ACTIVE group) in the HCT116 cells and those without CpG island (noCpGi group) exhibited null or minimal changes in gene expression and histone modification marks (**Fig. 5A** and data not shown).

The treatment of DKO cells with TSA resulted in an increased expression of all the MBV genes (**Fig. 5B**) that was paralleled by a partial disruption of the balance between H3K4me2

Figure 5 (See opposite page). (A) Relative gene expression and histone modification profiles in DKO cells in regard to parental HCT116. Relative values were calculated as log2 of DKO/HCT116 ratio. Gene expression was normalized to 18S. ChIP results were normalized to the respective input fractions. Major differences are seen in the methylated bivalent genes (MBV) that exhibit DNA demethylation, higher expression and increased levels of histone marks H3K4me2 and H3K27me3. (B) Relative gene expression and modification profiles in DKO cells treated with TSA in regard to untreated DKO cells. Relative values were calculated as log2 of treated/untreated cells ratio. Gene expression was normalized to 18S. ChIP results were normalized to the respective input fractions.

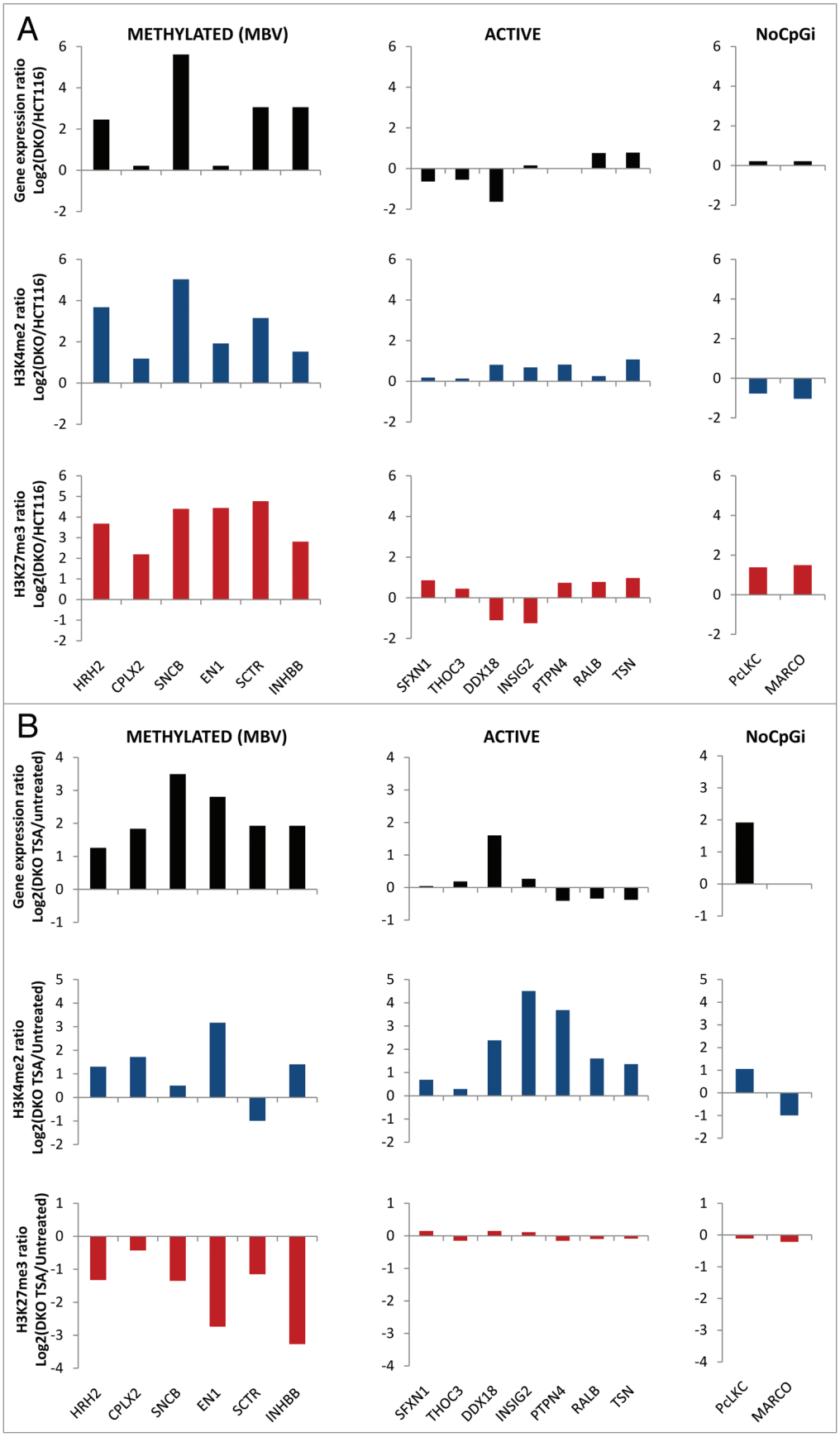

Figure 5. For figure legend, see page 6.

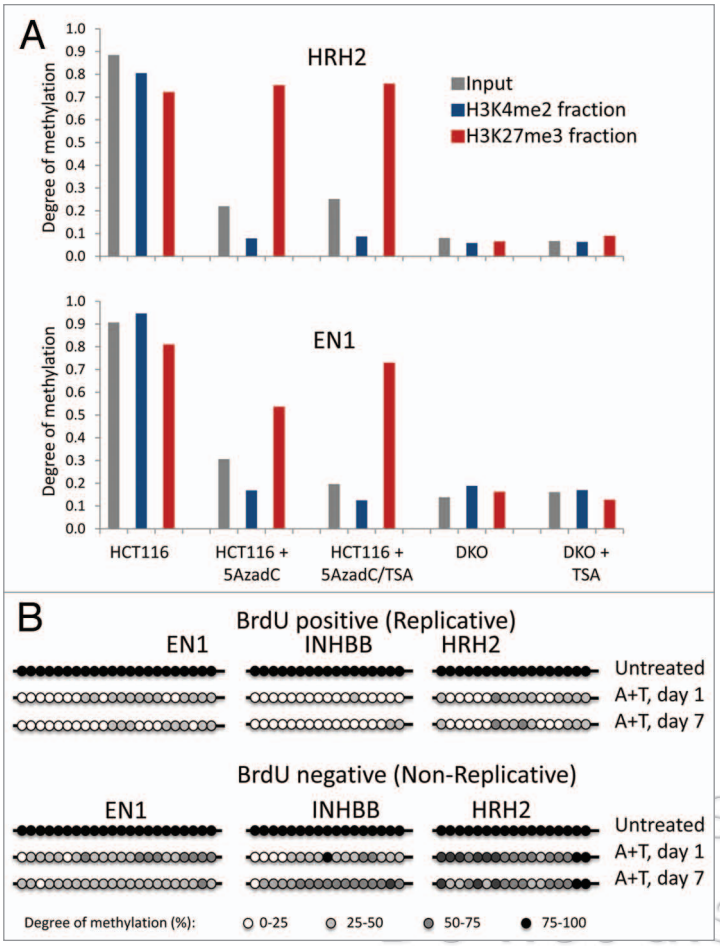

Figure 6. DNA methylation analysis of cell fractions after 5AzadC/TSA treatment. (A) MassCLEAVE™ DNA methylation analysis of input, H3K4me2 and H3K27me3 immunoprecipitated DNA fractions in EN1 and HRH2 gene CpG islands. Each bar represents the mean methylation (1.0 is full methylation) of all CpG sites contained in the amplicon analyzed in untreated HCT116, 5AzadC and 5AzadC/TSA treated cells and DKO and TSA DKO treated cells. (B) Direct bisulfite sequencing of CpG island regions corresponding to three MBV genes in HCT116 cells after BrdU labeling and FACS.

and H3K27me3 marks (higher H3K4me2 levels but lower H3K27me3) (**Fig. 5B**). The rest of genes were not affected by TSA, with the exception of DDX18 and PcLKC, which were also upregulated. A global increase of H3K4me2 levels was also seen in most genes (**Fig. 5B**).

These results suggest that retention of the bivalent nature of chromatin in the MBV genes is preserved even in the absence of an efficient DNA methylation system, although the balance between H3K4me2/me3 and H3K27me3 marks is partially disrupted after inhibition of histone deacetylases with TSA.

Epigenetic profiles of different chromatin and cell fractions. In vitro treatments with epigenetic drugs clearly show that silenced genes can be reactivated but do not illustrate the direct associations among the different components of the epigenetic code and gene activity in response to the drug. This is because changes observed in response to drugs may be driven by only a fraction of the cells and the fraction of cells exhibiting changes may be different for every assessed parameter. To determine whether drug induced DNA demethylation is directly associated with the changes detected in active and repressive histone modifications, we analyzed DNA methylation in H3K4me2 and H3K27me3 chromatin fractions in untreated HCT116 and DKO cells, 5-AzadC and 5-AzadC/TSA treated HCT116 cells and TSA treated DKO cells. DNA methylation profiles of five MBV genes (EN1, SCTR, INHBB, CPLX2 and HRH2) were analyzed in the Input, H3K4me2 and H3K27me3 ChIP fractions by bisulfite sequencing and MassCLEAVE™.

High levels of DNA methylation were observed in all chromatin fractions analyzed in HCT116 cells, suggesting that DNA methylation was homogeneous and ubiquitous in the five genes analyzed. Illustrative results of HRH2 and EN1 genes have been represented (**Fig. 6A**). Upon treatment (5AzadC alone or in combination with TSA), marked differences between H3K4me2/me3 and H3K27me3 fractions were appreciated. A dramatic decrease in DNA methylation was observed in the input and the H3K4me2/me3 fraction of treated cells (20–30% and 10–30% of that found in untreated cells), but the H3K27me3 fraction retained a higher level of DNA methylation (40 to 100% of that found in untreated cells). This discordance suggests that drug treatment ensues in at least two different cell populations. The responsive cell population would be characterized by the loss of DNA methylation, gene re-expression, increased levels of H3K4me2, and probably by loss of the H3K27me3 mark. A second population of resistant cells would retain DNA methylation and probably increased levels of H3K27me3.

Next we wondered if cells retaining DNA methylation underwent replication. We analyzed the DNA methylation and histone marks in replicating and non-replicating cells after fluorescence assisted cell sorting (FACS) of cells cultured in medium containing bromodeoxyuridine (BrdU). All fractions exhibited DNA demethylation, although it was deeper in cells that have incorporated high levels of BrdU. After 1 week, the demethylation was maintained and the differences between BrdU positive and negative cells were still apparent (**Fig. 6B**). Combined analysis of histone modifications and DNA methylation was performed in cells labeled with propidium iodide. Cells were fractioned by FACS into two groups (G_0/G_1 and S/G_2). Both groups retained balanced levels of H3K27me3 and H3K4me3, although DNA demethylation tended to be deeper in H3K4me3 fractions and cells in S/G_2 phases. Equivalent results were obtained 1 day and 7 days after treatment (data not shown).

We do not know if gene reactivation of silenced genes takes place in all cells, but a deeper DNA demethylation is consistently associated with enrichment of the H3K4me2/me3 marks and, as expected, DNA replication. H3K27me3 mark was present in all cell fractions. DKO cells exhibited low methylation levels similar to drug treated cells and no differences among treatments and chromatin fractions.

Discussion

Reactivation of tumor suppressor genes that have undergone epigenetic silencing appears as a promising therapeutic strategy.[32,35] It has been shown that reactivated promoters are often slowly remethylated and the gene is resilenced after withdrawal of the DNA methylation inhibitor.[36-40] Recent discoveries have revealed chromatin scenarios linking stem cell and cancer biology [6,12,14,15,25] and have underscored the impact of neighboring epigenetic states on the regulation of chromatin stability.[3,6,9] To get insights into the dynamics of chromatin reactivation and resilencing in cancer cells submitted to epigenetic treatments, we have investigated the epigenetic profiles of two of these chromosome regions, 2q14.2 and 5q35.2, containing genes silenced in colorectal cancer. We have defined three different genetic compartments based on the epigenetic signatures of the genes.

The first group is represented by six genes undergoing epigenetic silencing in cancer cells (as denoted by DNA hypermethylation) and the retention of bivalent chromatin epigenetic marks (MBV genes). It is of note the bivalent nature of these genes in murine colon cells, that has been demonstrated by co-immunoprecipitation of H3K4me3 and H3K27me3 (unpublished data). When reactivated, the expression of these genes is restored but at low levels, similar to those detected in non-tumorigenic cells,[3,6] and histone modifications profiles are also changed to a more pronounced bivalent state (elevated H3K4me2/3 and H3K27me3 signals) as it has been previously reported in reference 6 and 40. Upon drug withdrawal, the bivalent marks are maintained, but the balance is partially disrupted by a predominance of the repressive mark H3K27me3, suggesting its contribution to the recovery of the silenced state. The overall data are consistent with a coordinated dynamics of bivalent chromatin signatures among the MBV genes Nevertheless, it should be noted that our analysis is limited to specific regions inside the CpG island. Regional profiles along and outside the promoter region may be also affected by the treatments and may exhibit different dynamics during the resilencing process. Future studies a genome scale should address this issue. The effects of the treatment are reversed at mid-term and a complete resilencing of the reactivated genes is accomplished three weeks after drug withdrawal, while the DNA remethylation is fully restored one week later for most of the CpG islands analyzed. The combined treatment resulted in an extended recovery period as compared with 5-AzadC alone. These results are consistent with a secondary nature of DNA methylation in regard to gene activity.[39,41] The percentages of demethylation and the rate of remethylation were of the same order as previously described for other genes.[36,38,39] While treatment with the histone deacetylases inhibitor agent TSA alone did neither induce DNA demethylation nor gene reactivation, it had a synergistic effect with 5-AzadC, in agreement with other studies.[42,43]

The use of cells deficient in DNA methyltransferase activity (DKO) has allowed us to contrast the results presented above in a more stable system. As it has been described before, most hypermethylated tumor suppressor genes in HCT116 cells, such as p16INK4a or TIMP3, are found demethylated and re-expressed in DKO cells as compared with the parental HCT116 cells.[33,44] Most HCT116 hypermethylated genes are reexpressed and exhibit higher levels of H3K4me2 and H3K27me3 marks in the DKO cells (**Fig. 5A**). These results are similar to what we obtain when HCT116 cells are treated with 5-AzadC alone (our data and ref. 40). As expected, DKO cells treated with TSA exhibited increased expression of genes hypermethylated in HCT116. Interestingly, TSA had deep effects on DKO cells by disrupting the balance between bivalent marks (**Fig. 5B**). While H3K4me2 is increased in most genes, the repressive H3K27me3 mark is lost from most of the genes in which promoter hypermethylation was present (MBV). Our results suggest that DNA methylation also contributes to maintain the repressive histone code in genes poised for silencing and confirm the role of DNA methylation as the foremost player in preserving the inactivation of silenced genes.[38] Besides that, retention of bivalent signatures probably acts as a memento and is likely to participate in the restoration of the silencing.[6]

Another interesting finding arises from the analysis of DNA methylation in different chromatin fractions and in various experimental conditions. At global level, all the MBV genes analyzed in HCT116 untreated cells show coexistence of histone marks H3K4me2 and H3K27me3 with fully methylated DNA (**Fig. 6A**). Nevertheless, upon DNA demethylation by treatment with 5AzaC, H3K27me3 chromatin fraction retains higher levels of DNA methylation, while the H3K4me2/me3 fractions are extensively DNA demethylated. This result could be interpreted as a partial disruption of bivalent domains that become active in a fraction of the cells (unmethylated DNA and H3K4me2), while reaching a new balance in "drug-resistant cells" (methylated DNA and bivalent marks).

It remains to be elucidated if the recovery of the silenced state after drug removal is due to chromatin re-remodeling or to a cell population renewal in which a reservoir of cells resistant to the drug (denoted by the presence of high levels of methylation associated with the H3K27me3 chromatin fraction) become prevalent in the cell population. While it seems clear the homogeneity of the parental cells, it could be hypothesized that the treatment induces the presence of mixed cell populations with different chromatin landscapes (active, silenced, bivalent). We tried to resolve this conundrum by coimmunoprecipitation of both histone marks H3K4me2 and H3K27me3, but it did not work in our hands, although we have been able to do it in murine cells (data not shown). Our data indicate that the retention of repressive marks is unlikely to be driven by a resting cell population, since epigenetic profiles of bivalent chromatin domains are similarly affected in replicating and non-replicating cells. In agreement with previous studies,[6,15] H3K27me3 is retained and even increased in the reactivated loci. Hence, this repressing histone modification could trigger the resilencing and the DNA remethylation in the affected regions. Additional studies using inhibitors of H3K27 methyltransferase, as the DZNep,[45] may contribute to better understand the role of H3K27me3 mark in this process. Residual DNA methylation in the locus or near the locus is also likely to play a role.[29]

As we have shown, treated cells recover the original chromatin states after a few weeks of drug withdrawal. Moreover, they show

similar sensitivity and response to the drugs upon retreatment as never-treated cells (data not shown), suggesting that selection of drug-resistant cells is not sufficient to explain our results. Epigenetic drugs used here appear to induce plastic effects on chromatin. The transient disruption of the silenced states achieved by these treatments is overcome by a dominant repressive chromatin landscape determined by DNA methylation and bivalent chromatin marks. Design of new drugs with long lasting effects should improve the efficiency of epigenetic therapies.

In summary, our work illustrates the complexity of the epigenetic changes occurring upon drug-induced activation of silenced genes. While DNA methylation appears to play a dominant role in long lasting silencing, other players, characterized here as bivalent histone marks, contribute to maintain the epigenetic memory of silenced genes. The histone modification H3K27me3 is likely to play an important role in maintaining the memory of silenced genes that are in a bivalent context. Novel epigenetic strategies in cancer should target this repressive mark in order to get a more efficient effect of current DNA demethylating therapies.

Materials and Methods

Cell culture and 5-Aza-2'-deoxycytidine and trichostatin A treatments. The HCT116 colorectal carcinoma cell line was obtained from the American Type Culture Collection (ATCC). Cell culture and treatment with 5-Aza-2'-deoxycytidine (5AzadC) and Trichostatin A (TSA) were performed as described in reference 3, with minor modifications. Briefly, 0.65 x 10^6 cells were seeded in 10-cm cell culture dishes and 24 h later treated with 0.5 μM 5-Aza-2'-deoxycytidine (5AzadC; Sigma) for 48 h. Cells were treated with Trichostatin A (TSA) (Sigma) at 0.3 μM for 16 h. For co-treatment of cells with 5AzadC and TSA, 0.8 x 10^6 cells were seeded and treated initially with 5AzadC for 48 h, afterwards the medium was removed and cells were treated with TSA for an additional 16 h; then, the medium was changed and cells were harvested at the indicated time points. For control samples, half a million cells were seeded and cultured at the same time in which the drugs were omitted. DKO cells (HCT116 deficient in both DNA methyltransferases),[46] were cultured under the same conditions as wild type HCT116 cells. A complete new treatment of HCT116 cells with 5AzadC and TSA was done in parallel as control of drug efficiency.

Fluorescence-activated cell sorting (FACS). Cell sorting of replicating and non-replicating cells was performed using a modification of the method described previously in reference 47. Briefly, untreated and treated (5AzadC + TSA) cells were incubated for 24 h with 30 μM BrdU (Sigma) immediately and 7 days after the treatment. Cells were fixed with 3.7% formaldehyde at room temperature for 10 min, washed 3 times with cold PBS. The blocking solution (15% goat serum in 0.1% TritonX100) was added to the cells and incubated for 30 minutes at room temperature. After removal of blocking solution, fixed cells were incubated for 1 h at room temperature with anti-BrdU mouse monoclonal antibody 1:250 (Becton Dickinson, Ref. 555627), washed 3 times with PBS, and incubated again with anti-mouse FITC-conjugated antibody 1:250 (Invitrogen, Ref. A10543) for 45 minutes at room temperature in a dark chamber. Cells were washed twice with PBS and Tween (0.02%) and 2 more times with PBS. Flow cytometry and sorting was performed on an Influx flow cytometer (BD FACSAria™ II). Standard negative controls were used to set up the threshold and to fractionate cells positive and negative for BrdU.

Cell cycle analysis. Cell cycle analysis using PI was performed in 5dAzadC+TSA treated cells 1 and 7 days after the treatment. Cells were resuspended in 0.9 ml PBS, permeabilized with 2.1 ml 100% ethanol and kept at -20°C for 30 min. Then, cells were washed twice to eliminate the ethanol and 1 ml of the analysis solution compound (Propidium iodide 500 μg/ml (Sigma), sodium citrate 38 mM and Ribonuclease A (Sigma) was added. Flow cytometry and sorting was performed as described above. Fractions for G_0/G_1 and S/G_2 were collected.

Bisulfite sequencing and massCLEAVE™ analysis. Genomic DNA was obtained using standard protocols. Bisulfite treatment was performed as previously described in reference 41, or using the EZ DNA methylation kit™ (Zymo Research). Three independent PCR reactions were carried out and products were pooled to ensure a representative methylation profile. The primers used for the bisulfite PCR amplifications are listed in **Supplemental Table 1**. MassCLEAVE™ methylation analyses were performed as previously described in reference 48. Data was analyzed using the MassCLEAVE™ technology as previously reported in reference 48.

RNA extraction and quantitative real-time RT-PCR. RNA and the corresponding cDNA were obtained using standard protocols. Expression was quantified using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems) or the Light Cycler 2.0 real time PCR system (Roche Diagnostics). The primers used for RT-PCR amplification are listed in **Supplemental Table 2**. The reactions were performed in triplicate. Gene expression levels were normalized using 18S determinations.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were carried out using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions. The complexes were immunoprecipitated with antibodies specific for total histone H3 from Abcam (ab1791) and acetylation of histone H3 (Lys 9) (no. 07-352), dimethyl-histone H3 (Lys 4) (no. 07-030), dimethyl-histone H3 (Lys 9) (no. 07-441), trimethyl-histone H3 (Lys 27) (no. 07-449) from Upstate Biotechnology. As negative control we used rabbit IgG serum (Jackson Immunoresearch). The amount of immunoprecipitated target was measured by real-time PCR as described above. Positive and negative controls for each histone modification were used to set the lower limits.[3,6,49] Amplification primers for gene promoters are listed in **Supplemental Table 3**. PCRs were performed in triplicate.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

R.M. was supported by a FPI fellowship from the Spanish Ministry of Science and Innovation. This work was supported by grants from the Spanish Ministry of Science and Innovation

(SAF2008/1409, CSD2006/49), National Health and Medical Research Council (NHMRC) and Cancer Institute NSW, Australia. M.W.C. is supported by the Netherlands Organization for Scientific Research NWO/ZonMW (916-10-108) and a Marie Curie grant (IRG: 248397).

Note

Supplemental materials can be found at: www.landesbioscience.com/journals/epigenetics/article/16066

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