Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer

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Densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones[1,2]. Recently, these two epigenetic processes have been dynamically linked. The methyl-CpG-binding protein MeCP2 appears to reside in a complex with histone deacetylase activity[3,4]. MeCP2 can mediate formation of transcriptionally repressive chromatin on methylated promoter templates *in vitro*, and this process can be reversed by trichostatin A (TSA), a specific inhibitor of histone deacetylase[3–5]. Little is known, however, about the relative roles of methylation and histone deacetylase activity in the stable inhibition of transcription on densely methylated endogenous promoters, such as those for silenced alleles of imprinted genes[6], genes on the female inactive X chromosome[7] and tumour-suppressor genes inactivated in cancer cells[8,9]. We show here that the hypermethylated genes *MLH1*, *TIMP3* (*TIMP-3*), *CDKN2B* (*INK4B*, *p15*) and *CDKN2A* (*INK4*, *p16*) cannot be transcriptionally reactivated with TSA alone in tumour cells in which we have shown that TSA alone can upregulate the expression of non-methylated genes. Following minimal demethylation and slight gene reactivation in the presence of low dose 5-aza-2′deoxycytidine (5Aza-dC), however, TSA treatment results in robust re-expression of each gene. TSA does not contribute to demethylation of the genes, and none of the treatments alter the chromatin structure associated with the hypermethylated promoters. Thus, although DNA methylation and histone deacetylation appear to act as synergistic layers for the silencing of genes in cancer, dense CpG island methylation is dominant for the stable maintenance of a silent state at these loci.

Aberrant promoter region CpG island hypermethylation is associated with transcriptional silencing of *MLH1* (ref. 10), *TIMP3* (K.E.B., manuscript submitted) and *CDKN2A* (ref. 9) in the colorectal carcinoma cell line RKO. This allowed us to study the effects of histone deacetylase inhibition on these three genes in the same cells. We also examined *CDKN2B*, which is aberrantly hypermethylated and transcriptionally silenced in the leukaemia cell line KG1a (ref. 11).

We first examined whether histone deacetylase activity is required for the maintenance of a transcriptionally silent state at these loci (Fig. 1*a*). The histone deacetylase inhibitor TSA failed to reactivate expression of *MLH1* or *TIMP3* to a level detectable by RT-PCR (Fig. 1*a*). Further treatment with 200–300 nM TSA (up to 2 weeks for *MLH1)* or with higher doses (1–5 μM) of TSA also failed to reactivate either gene (data not shown). Similarly, *CDKN2B* (in KG1a) and *CDKN2A* (in RKO) were not reactivated with any tested dose of TSA (Fig. 2*b*,*c*, and data not shown).

TSA increased the expression of transcribed genes in the same cells in which the silent, hypermethylated genes *MLH1*, *TIMP3* and *CDKN2A* were not affected. TSA (300 nM) elevated the expression of *CDKN2B* and *CDKN1A* (*p21*), two genes basally expressed in RKO cells, by up to twofold following a 12-hour treatment with TSA (Fig. 1*b*). Thus, *CDKN2B*, which is not methylated and is transcribed in RKO, can be upregulated by TSA; however, TSA cannot reactivate *CDKN2B* in KG1a, in which the gene is silenced in association with promoter region hypermethylation (Fig. 2*b*). Our results also confirm the TSA induction of *CDKN1A* in colorectal carcinoma[12], and should be

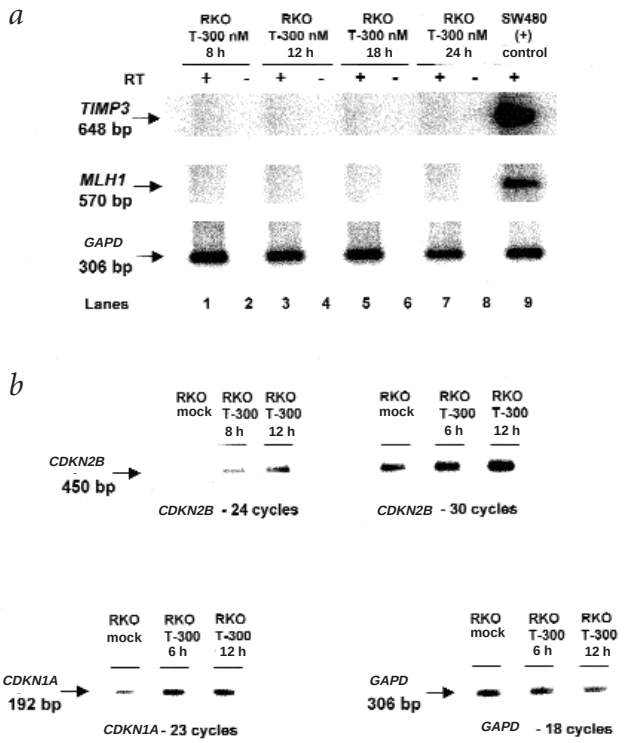

Fig. 1 Histone deacetylase inhibition by TSA is not sufficient to reactivate genes silenced in association with promoter region hypermethylation, but can upregulate expression of transcribed genes in the same cells. ***a***, Blotted and hybridized RT-PCR products for *TIMP3*, *MLH1* and *GAPD*. *MLH1* and *TIMP3* are not reactivated by addition of TSA (300 nM) over 6–24 h in the colorectal carcinoma cell line RKO, in which they are hypermethylated (lanes 1, 3, 5, 7). Control SW480 colorectal carcinoma cells are not methylated at *MLH1* and *TIMP3* and express both genes (lane 9). *GAPD* is shown below as a control for the amount of RNA amplified. ***b***, Blotted and hybridized RT-PCR products for *CDKN2B*, *CDKN1A* and *GAPD*. TSA (300 nM) upregulates the expression of *CDKN2B*, a gene whose promoter region is not hypermethylated in RKO cells, and *CDKN1A*, a gene known to be expressed in RKO cells. Increased expression of both *CDKN2B* and *CDKN1A* after 12 h of treatment reveals an approximately twofold increase over mock-treated cells relative to *GAPD*.

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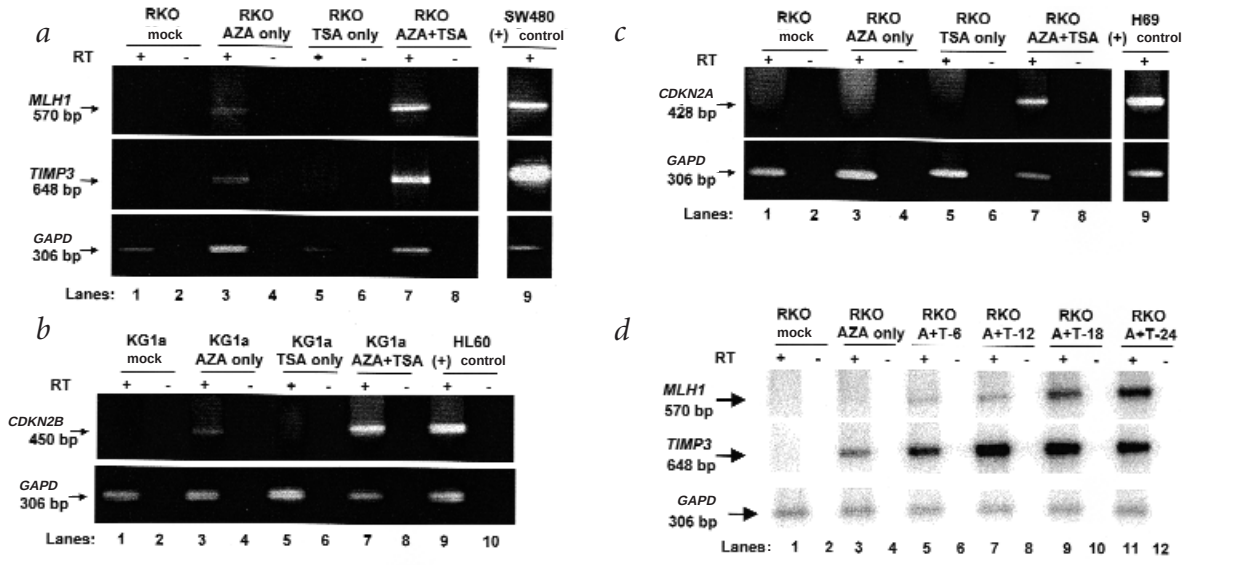

Fig. 2 Histone deacetylase inhibition by TSA facilitates transcriptional reactivation induced by the demethylating agent 5Aza-dC and is effective within 6 h of treatment. *GAPD* RT-PCR provides a control for initial RNA amounts (bottom of each panel). ***a***, RT-PCR analysis of *MLH1*, *TIMP3* and *GAPD* expression. RKO cells do not basally express *MLH1* or *TIMP3* (lane 1). Treatment with 5Aza-dC alone induces a low level of reactivation (lane 3); however, co-incubation with TSA induces robust reactivation of both genes (lane 7). Both genes are unmethylated and basally expressed in control SW480 cells (lane 9). ***b***, RT-PCR analysis of *CDKN2B* and *GAPD* expression. KG1a mock-treated cells do not basally express *CDKN2B* (lane 1). Treatment with 5Aza-dC alone showed minimal reactivation of *CDKN2B* expression (lane 3). Coincubation with TSA produces robust expression (lane 7) within the range for basal expression by control HL60 cells that are unmethylated for *CDKN2B* (lane 9). ***c***, RT-PCR analysis of *CDKN2A* and *GAPD* expression. RKO cells do not basally express *CDKN2A* (lane 1). Treatment with 5Aza-dC alone did not induce detectable reactivation of *CDKN2A* (lane 3), however, co-incubation with TSA results in *CDKN2A* transcription (lane 7). NCI-H69, a small-cell lung carcinoma cell line that is unmethylated at *CDKN2A*, serves as a positive control for *CDKN2A* expression (lane 9). ***d***, Blotted and hybridized time course of TSA-mediated reactivation of *MLH1* and *TIMP3* following 5Aza-dC treatment. Cells incubated for as little as 6 h in the presence of TSA following 42 h of initial 5Aza-dC treatment show reactivation of both *MLH1* and *TIMP3* (lane 5), with more expression with increasing time of co-incubation (lanes 7, 9, 11). Again, mock-treated RKO cells do not express either gene (lane 1). Phosphorimager quantitation showed the reactivation of *MLH1* to be maximal at 24 h after TSA treatment, whereas *TIMP3* reactivation reached a maximum at 12 h of TSA treatment.

considered in light of the report that the TGFβ inducibility of both *CDKN2B* and *CDKN1A* in human keratinocytes is dependent on intact p300 (ref. 13), a known histone acetyltransferase. Our data suggest that dense CpG island methylation in gene promoter regions is dominant over active histone deacetylase activity in maintaining transcriptional repression.

To further investigate the roles of histone deacetylation and CpG island methylation in transcriptional silencing, we induced partial demethylation of the four genes in the presence or absence of histone deacetylase inhibition (Fig. 2). We have previously shown that a 1-μM dose of the demethylating agent 5Aza-dC results in substantial reactivation of these genes within 3–5 days[9–11]. A lower dose of 5Aza-dC (100 nM) administered over 48 hours produced little or no reactivation of transcription for *MLH1* and *TIMP3* (Fig. 2*a*). In four separate experiments, however, we observed robust expression of both genes after addition of 300-nM TSA for 24 hours following 24 hours of 5Aza-dC treatment (Fig. 2*a*). Phosphorimager analysis of hybridized RT-PCR products revealed a fourfold induction by the combined drug treatment in an experiment in which low-level reactivation was seen with 5Aza-dC treatment alone. Combination 5Aza-dC/TSA treatment similarly increases reactivation for both *CDKN2B* (Fig. 2*b*) and *CDKN2A* (Fig. 2*c*). TSA treatment for as little as 6 hours was sufficient for increased expression of *MLH1* and *TIMP3* following 100-nM 5Aza-dC treatment (Fig. 2*d*). These results indicate that although active histone deacetylation may not be needed to maintain a silenced transcriptional state at the four loci studied, this activity has a role in silencing when levels of DNA methylation are reduced.

To examine the mechanism of the observed gene reactivation, we investigated the methylation status of *MLH1* and *TIMP3* following treatment of cells with 5Aza-dC and TSA alone and in combination. Bisulfite genomic sequencing revealed that the CpG islands of *MLH1* and *TIMP3* were unmethylated at each CpG site in SW480 that expressed these genes (Fig. 2), but most CpG sites were methylated in each allele in RKO cells in which these genes are transcriptionally silent (Fig. 3). Low-dose 5Aza-dC alone significantly reduced the number of methylated CpG sites per allele of the two genes (*MLH1*, $P<0.01$; *TIMP3*, $P=0.05$, Student's paired *t*-test; Fig. 3*a*,*b*), whereas TSA alone resulted in no methylation change. Two points are critical concerning this demethylation. First, although the demethylation following 5Aza-dC is significant, most CpG sites per allele remain methylated. Second, the combination of TSA subsequent to 5Aza-dC treatment did not extend the degree of demethylation seen with 5Aza-dC alone (*MLH1*, $P=0.28$; *TIMP3*, $P=0.42$). Thus, the increase in gene expression caused by the combination of the two drugs occurs with retention of extensive methylation in both *MLH1* and *TIMP3*. It is possible that the TSA-mediated effects we have observed could emanate from only a small number of alleles not detected by our bisulfite sequencing analysis, which become extensively demethylated following 5Aza-dC treatment. If this is the case, our studies still suggest that decreased methylation is a prerequisite for effective transcription following histone deacetylase inhibition.

We next investigated whether the increase in expression in response to combination of 5Aza-dC and TSA is associated with gross changes in chromatin structure in these promoters. We analysed the restriction endonuclease accessibility of the intact chromatin surrounding the promoter region of *MLH1* (Fig. 4). SW480 cells, which are unmethylated at and express *MLH1*, show chromatin accessibility, indicating the presence of open, transcriptionally active chromatin in this region (Fig. 4*c*). In contrast, the same region in untreated RKO cells is refractory to

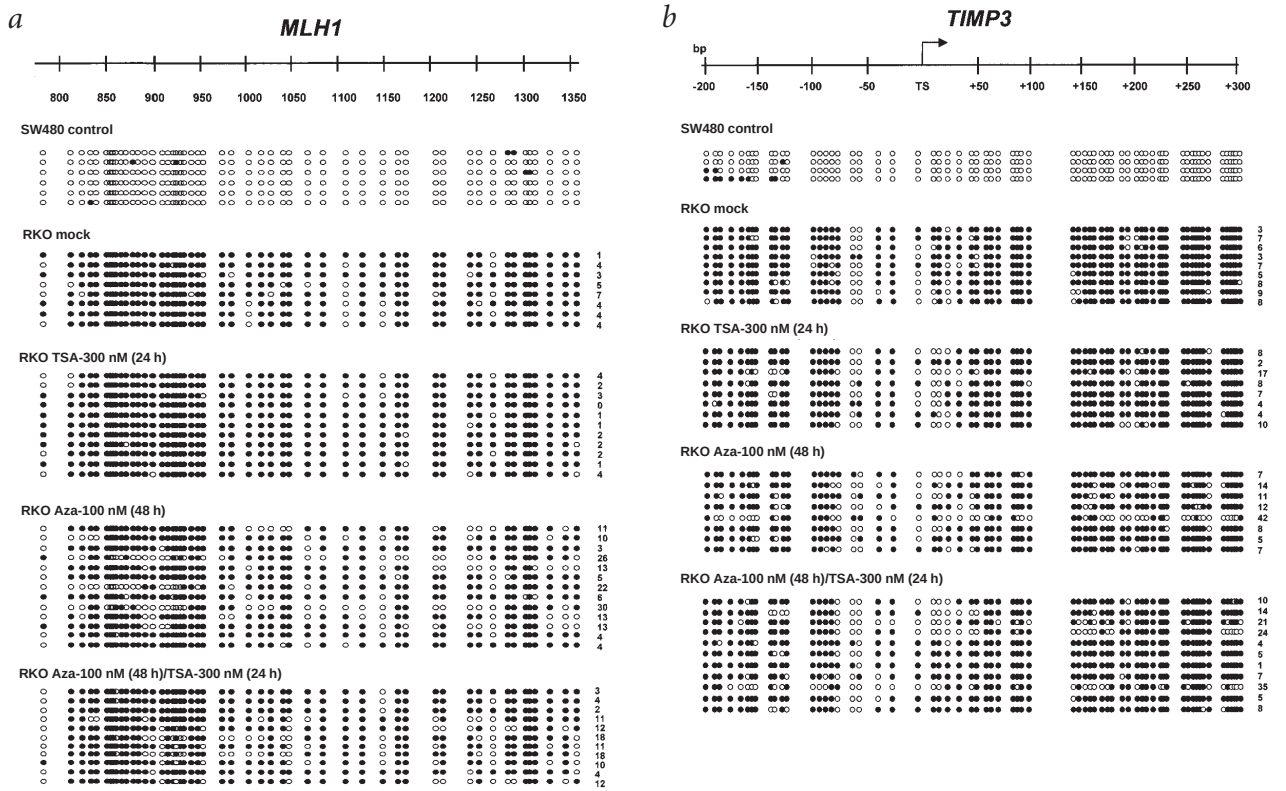

Fig. 3 TSA treatment does not further extend demethylation induced by 5Aza-dC. The CpG island promoter regions of *MLH1* (***a***) and *TIMP3* (***b***) were analysed for methylation status by bisulfite sequencing analysis. Each row of circles represents a single cloned allele, and each circle represents a single CpG site (open circle, non-methylated; filled circle, methylated cytosines). For *MLH1*, the numbering in the schematic at the top of the diagram represents position with regard to published sequence data[29]. For *TIMP3*, the numbering in this schematic corresponds to position relative to published transcription start[30] (arrow). SW480 cells that express both genes (Fig. 2*a*) have no methylated cytosines in the central regions of either CpG island. In contrast, RKO cells that were mock-treated show methylation of most CpG sites in the CpG island for both genes. TSA alone (300 nM) does not significantly change the methylation pattern of either gene (see numbers of unmethylated CpG sites listed to the right of each allele). Treatment with 5Aza-dC (100 nM) alone resulted in demethylation of both *MLH1* and *TIMP3* (*MLH1*, $P<0.01$; *TIMP3*, $P=0.05$); combined treatment with TSA (300 nM) did not extend this effect (*MLH1*, $P=0.28$; *TIMP3*, $P=0.42$).

digestion, suggesting, as found for other densely methylated promoters[14,15], a closed chromatin conformation (Fig. 4*b*). RKO cells that had been treated with low-dose 5Aza-dC, TSA or a combination of both drugs showed no significant accessibility to any of the enzymes tested (Fig. 4*b*,*c*). We similarly analysed *Msp*I accessibility for the promoter region of *TIMP3*. As for *MLH1*, DNA from SW480 cells showed digestion, whereas DNA from RKO cells showed no accessibility for all treatments tested (data not shown). Thus, minimal demethylation and/or enhancement of histone acetylation of these CpG islands does not cause major changes in chromatin structure, and the accompanying increased expression of both genes observed with the combination of 5Aza-dC and TSA occurs in the setting of largely closed chromatin. Alternatively, it is possible that a small number of demethylated alleles affected by the addition of TSA may be undergoing complete conversion to an open chromatin conformation and were not detected. Previous studies have suggested, however, that inhibition of histone deacetylase activity can induce gene expression without a large-scale change from repressive to accessible chromatin[16–19].

Recent reports have suggested that chromatin structure is the dominant force in methylation-associated gene silencing[3,4,20]. MeCP2 has been shown to recruit histone deacetylase activity to *in vitro*-methylated promoters, and this deacetylase activity appears to be essential for the establishment of gene silencing[3,4]. Our results suggest that, after establishment of transcriptional silencing at these loci, dense DNA methylation may be the dominant component for maintenance of silencing at CpG island promoters.

Thus, methylation and histone deacetylation appear to act as layers for epigenetic gene silencing. This lends support for the concept that one function of DNA methylation may be to 'lock' genes into a silenced chromatin state[21]. This final locking appears to be very firm for the genes we have studied, and may be involved in transcriptional repression of methylated inactive X chromosomal genes and imprinted alleles. Consistent with our findings, it has been recently reported that TSA alone cannot reactivate silenced alleles on the inactive X chromosome[22]. This is in contrast to reports in *Neurospora crassa* and *Brassica napus*[23,24], in which TSA alone was found to reactivate methylated genes; our results may suggest a more dominant role for methylation over histone deacetylase activity in mammals for the maintenance of gene silencing in association with CpG methylation.

Further investigation of the relationship between DNA methylation and histone deacetylase activity in the silencing of mammalian genes will increase understanding of basic aspects of transcriptional regulation and may also have applied implications. Reactivation of hypermethylated genes such as those we have studied might impede tumour progression. To maximally achieve gene reactivation it may be necessary to simultaneously block the processes essential to both the formation and maintenance of the transcriptionally repressive chromatin associated with such genes—that is, DNA methylation and histone deacetylation.

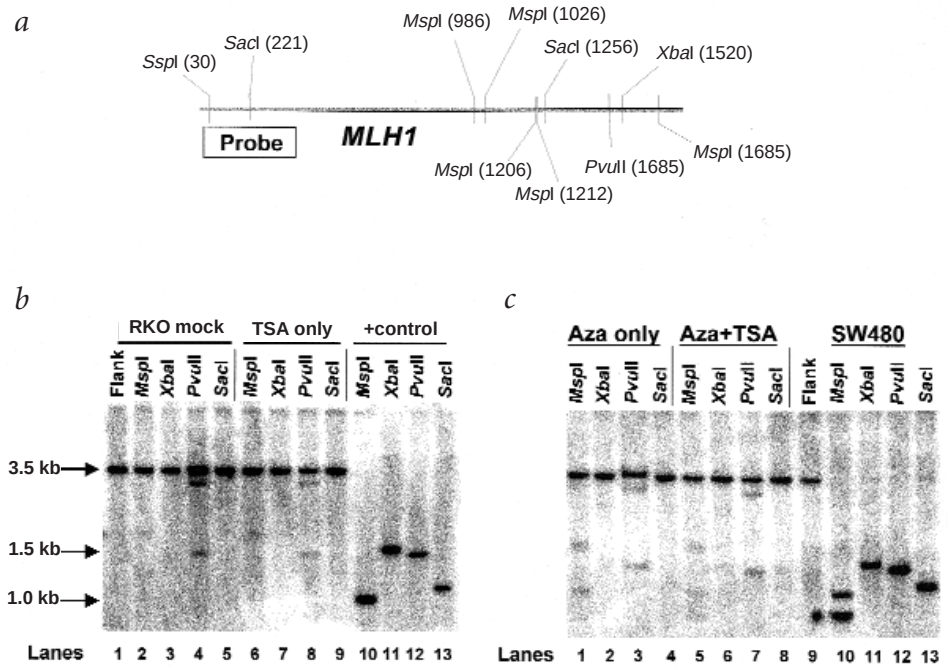

Fig. 4 Neither TSA, 5Aza-dC nor the combination of both agents results in gross chromatin change at the *MLH1* CpG island region as monitored by accessibility to the restriction endonucleases *Msp*I, *Xba*I, *Pvu*II and *Sac*I. ***a***, Schematic showing location of the probe, *Msp*I, *Xba*I, *Pvu*II and *Sac*I sites and a flanking *Ssp*I site within the promoter region of *MLH1* with numbering relative to published sequence[29]. ***b***, RKO cells methylated and not expressing *MLH1* maintain chromatin structure inaccessible to restriction with the endonucleases *Msp*I, *Xba*I, *Pvu*II and *Sac*I (lanes 2–5), with predominant band size of approximately 3.5 kb as seen for digestion with the flanking endonuclease *Ssp*I alone (lane 1). Treatment with TSA alone (lanes 6–9) does not result in any increase in chromatin accessibility. ***c***, Treatment with 5Aza-dC alone (lanes 1–4) also does not result in any increase in chromatin accessibility. Similarly, combination 5Aza-dC/TSA treatment does not result in increased accessibility to restriction endonucleases (lanes 5–8). SW480 cells that express *MLH1* and are unmethylated at this locus show digestion indicating an open chromatin conformation relative to RKO cells (lanes 10–13). Digestion of naked genomic DNA was included to show the positions of completely digested fragments (*b*, lanes 10–13). As a control, these blots were re-probed with the promoter region of *CDKN2B*, which is not methylated in RKO or SW480 cells, and which also has a flanking *Ssp*I site as well as sites for *Msp*I, *Pvu*II and *Sac*I. These endonucleases were capable of digesting the DNA of the promoter region of *CDKN2B* in the chromatin from both cell lines (data not shown).

Methods

Cell culture. RKO cells were grown in RPMI 1640 (Paragon) media supplemented with 10% fetal bovine serum (Paragon); SW480 cells in McCoy's media (Gibco/BRL) supplemented with 10% fetal bovine serum; KG1a cells in IMDM (Gibco/BRL) media supplemented with 20% fetal bovine serum; and HL60 cells in RPMI 1640 media supplemented with 5% fetal bovine serum.

TSA and 5Aza-dC treatment of cells. Cells were split to low density 12–24 h before treatment. Cells were then treated with 5Aza-dC (Sigma) or were mock-treated with the same volume of 1×PBS. Following this initial incubation (24–48 h), TSA (Wako) was added to the media from a 5-mM ethanol dissolved stock or cells were mock-treated with an identical volume of ethanol. Cells were then incubated an additional 6–24 h at 37 °C. For the combination 5Aza-dC/TSA treatment analysis of *MLH1* and *TIMP3* in RKO cells, 5Aza-dC (100 nM) was added for an initial incubation of 24 h, after which TSA (300 nM) was added for an additional 24 h. For analysis of *CDKN2B* in KG1a cells, 5Aza-dC (500 nM) was added for an initial incubation of 24 h, after which TSA (1 μM) was added for an additional 24 h. For analysis of *CDKN2A* in RKO cells, 5Aza-dC (1 μM) was added for an initial incubation of 48 h, after which TSA (500 nM) was added for an additional 24 h. For time course analysis of *MLH1* and *TIMP3* re-expression, RKO cells were incubated in the presence of 5Aza-dC (100 nM) for a total of 48 h and were co-incubated with TSA (300 nM) for the final 6, 12, 18, or 24 of those 48 h. For *CDKN2B* and *CDKN1A* analysis in RKO cells, cells were treated with TSA (300 nM) for 6 or 12 h.

RNA and RT-PCR. Cells were washed with 1×PBS (Quality Biologic), trypsinized, pelleted and resuspended in guanidium isothiocyanate with 2-mercaptoethanol[25]. Cells were frozen at –70 °C, thawed and total cellular RNA was extracted via standard methods[25]. RNA was resuspended in DEPC (Sigma)-treated water and was quantitated at OD 260/280. Quantitation was also verified by agarose gel ethidium staining. Reverse transcription reactions were performed using total RNA (3 μg). Reactions were done in a 15.75 μl total volume and conditions were as follows: mix (10.4 μl) containing 5×first strand buffer (6.0 μl; Gibco BRL), DTT (3.0 μl, 0.1 M; Gibco BRL), dNTPS mix (0.6 μl, 25 mM; Pharmacia), random hexamers mix (0.5 μl; Gibco BRL) and RNasin (0.3 μl, 12 U; Promega) was added to RNA (18.1 μl) in DEPC-treated distilled water. Reactions were mixed, incubated 5 min at 65 °C and placed on ice. 14.25 μl were then moved to a new tube (the remaining was used for the control minus RT reaction), and MMLV RT enzyme (1.5 μl, 300 U; Gibco BRL) was added to RT+ tubes. All reactions were then mixed, spun and incubated approximately 1 h at 37 °C. Reactions were then heated approximately 5 min at 95 °C and were frozen at –20 °C. RT reactions were then diluted either 5- or 10-fold (consistent within PCR experiments, but varying between experiments) for *MLH1*, *TIMP3*, *CDKN2B* and *CDKN1A*. RT reactions were not diluted for *CDKN2A* PCR reactions. When RT reactions were diluted, *GAPD* PCR reactions were done with the same dilutions used in PCR for the genes of interest to ensure accurate relative quantitation. All PCR reactions, except reactions shown in Fig. 2*c*, were performed using diluted cDNA (10 μl) in a 50 μL total reaction volume with *Taq* polymerase (1.25 U; Sigma) and were hot started. PCR reactions (Fig. 2*c*) were performed using undiluted cDNA (2 μl) in a 50 μL volume with *Taq* polymerase (1 U) and no hot start. PCR cycle numbers were as follows: *MLH1*, 35 cycles; *CDKN2B*, 24, 30 (expressing cells) or 35 (non-expressing cells) cycles; *TIMP3*, 35 cycles; *CDKN2A*, 39 cycles; *CDKN1A*, 23 cycles; and *GAPD*, 18 or 20 cycles. More detailed PCR conditions and primer sequences are available upon request.

Blotting and hybridization of RT-PCR products. RT-PCR products (7 μl) or *GAPD* RT-PCR products (3 μl; diluted with distilled water to 10 μl total loading volume for loading accuracy) were loaded on 1.5% agarose gels. After running, gels were pre-soaked in NaOH (0.4 N) and were then blot-

ted in NaOH (0.4 N) to Zetaprobe (Bio-Rad). Filters were pre-hybridized in Blotto for approximately 1 h. Probes were created via gel purification of RT-PCR products of the specific gene of interest and were ^{32}P-labelled using a Prime-It II labelling kit (Stratagene). Probes were diluted in Blotto, and blots were then hybridized, washed and exposed.

Bisulfite genomic sequencing. DNA was extracted from the same flasks as used above for mRNA expression analysis. DNA was treated with sodium bisulfite as described[26]. Treated DNA was resuspended in either 15 µL (for *MLH1*) or 25 µL (for *TIMP3*) of distilled water and was amplified by PCR using primers specific for the treated DNA. All PCR reactions were done in 50 µL final volumes. For *MLH1*, bisulfite-treated genomic DNA (4 µl) was used for PCR, reactions were hot-started and 42 cycles of PCR were used. In some cases, nested PCR for *MLH1* was performed; these reactions were cycled 42 times, diluted (1:50) and subjected to a second PCR with nested primers. Diluted PCR reaction (15 µl) was used for nested reactions, and the reactions were then subjected to hot start and 32 cycles of PCR. For *TIMP3*, bisulfite-treated genomic DNA (3 µl) was used for PCR, reactions were hot started and 37 cycles of PCR were used. More detailed PCR conditions and primer sequences are available on request. PCR products were cloned into the vector pCR2.1-TOPO (Invitrogen) according to the manufacturer's protocol. Individual clones were grown and prepared using Wizard mini-prep kits (Promega) and were sequenced via automated sequencing (Johns Hopkins University School of Medicine Biosynthesis and Sequencing Facility, Department of Biological Chemistry).

Nuclease accessibility assays. RKO cells were either mock-treated or treated with 5Aza-dC (100 nM) alone for 48 h, TSA (300 nM) alone for 24 h, or TSA (300 nm) for 24 h following an initial 24 h incubation with 5Aza-dC. Treated cells were washed once with 1×PBS, trypsinized and resuspended in 1×PBS (10 ml). Nuclei were extracted according to published methods[27]. Briefly, cells were counted, pelleted and resuspended in pre-chilled RSB buffer at 2×10^6 cells/ml. Cells were then aliquoted per reaction to pre-chilled eppendorf tubes and pelleted, then resuspended in cell lysis buffer and incubated on ice for 10 min. Nuclei were collected by spinning 5 min at 2,000*g*, and were washed once more with lysis buffer (500 µl). Nuclei were then resuspended in 1×restriction buffer (500 µl; NEB buffer diluted from 10× with distilled water). Appropriate units of *Msp*I, *Xba*I, *Pvu*II, or *Sac*I restriction endonucleases were added and reactions incubated for 30 min at 37 °C. Stop buffer with 0.4 mg/ml proteinase K (500 µl) were added, tubes were mixed and incubated overnight at 37 °C. Genomic DNA was extracted using standard phenol/chloroform extraction. DNA (7 µg) was restricted with 50 units of appropriate flanking enzymes (*Ssp*I for *MLH1* or *Sty*I for *TIMP3*; New England Biolabs). Control DNA (7 µg), which was also digested with *Msp*I, *Xba*I, *Pvu*II or *Sac*I, was included to show fully cut product size. Reactions were incubated at least 12–16 h at 37 °C. Digestions were ethanol precipitated, resuspended in distilled water and run on a 1.5% agarose gel. Gels were then acid-nicked and blotted overnight to Zetaprobe (Bio-Rad) in NaOH (0.4 N) solution. Filters were pre-hybridized in Blotto solution hybridized overnight in Blotto plus specific probe radiolabelled with ^{32}PdCTP. Specific genomic probes for *MLH1* and *TIMP3* were generated by PCR and are available on request. *MLH1* blots were subsequently re-probed with the *CDKN2B* promoter region as a control for restriction endonuclease activity[28].

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