

Synergism between DNA methylation and macroH2A1 occupancy in epigenetic silencing of the tumor suppressor gene p16(CDKN2A)

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ABSTRACT

Promoter hypermethylation and heterochromatinization is a frequent event leading to gene inactivation and tumorigenesis. At the molecular level, inactivation of tumor suppressor genes in cancer has many similarities to the inactive X chromosome in female cells and is defined and maintained by DNA methylation and characteristic histone modifications. In addition, the inactive-X is marked by the histone macroH2A, a variant of H2A with a large non-histone region of unknown function. Studying tumor suppressor genes (TSGs) silenced in cancer cell lines, we find that when active, these promoters are associated with H2A.Z but become enriched for macroH2A1 once silenced. Knockdown of macroH2A1 was not sufficient for reactivation of silenced genes. However, when combined with DNA demethylation, macroH2A1 deficiency significantly enhanced reactivation of the tumor suppressor genes p16, MLH1 and Timp3 and inhibited cell proliferation. Our findings link macroH2A1 to heterochromatin of epigenetically silenced cancer genes and indicate synergism between macroH2A1 and DNA methylation in maintenance of the silenced state.

INTRODUCTION

Abnormal epigenetic silencing of gene transcription is recognized as a frequent event in many types of cancer. Similar to mutation, epigenetic silencing causes gene loss of function, thereby contributing to tumorigenesis (1,2). Although little is known about the events leading to silencing, the molecular features of chromatin associated with the silenced promoters are well characterized (3).

There is considerable similarity between heterochromatin of epigenetically inactivated genes in cancer, and that of genes residing on the inactive X chromosome (4). Both are characterized by DNA methylation of CpG islands at promoter regions and by histone H3 and H4 deacetylation (5–9). Indeed, DNA demethylating agents and histone deacetylase inhibitors are effective in reactivating transcription from such loci (10,11). Both the inactive X and silenced tumor suppressor genes (TSGs) in cancer cells are characterized by histone H3 lysine 9 dimethylation (H3K9me2) and HP1 enrichment (12–14). Another prominent feature of the inactive X is enrichment for polycomb complex proteins and for H3K27 trimethylation (15–17). These marks are often associated with silenced loci in cancer and have been proposed to precede, and possibly direct, DNA methylation (18–20).

Another, less-studied, characteristic of the inactive X chromosome is enrichment for the histone variant macroH2A. MacroH2A was originally reported to associate with the inactive X, based on immunofluorescence experiments (21). This association was later confirmed by chromatin immunoprecipitation (ChIP) experiments that revealed specific enrichment for macroH2A on the inactive X of female cells (22–24). There are three isoforms of macroH2A encoded by two genes, with one gene coding for two splice variants: macroH2A1.1 and 1.2 (25–27). Mice lacking the macroH2A1 variants develop normally but display some alteration in gene expression in adulthood (28,29). The fact that macroH2A1 KO female mice are fully viable suggests that the protein is not essential for establishment of the inactive state (28). However, a role for macroH2A in maintenance of the inactive X was demonstrated when depletion of macroH2A1 from cells was found to augment reactivation of a silenced reporter residing on the inactive X, following application of DNA demethylating drugs and histone deacetylase inhibitors (30).

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MacroH2A is not restricted to the inactive X and is detected at significant levels on autosomal chromosomes. Genome-wide profiling of macroH2A distribution demonstrates that macroH2A is associated primarily with transcriptionally repressed regions and that loss of macroH2A modulates gene expression during differentiation (31,32). Other reports suggest a role for macroH2A in regulation of transcription of dynamic/inducible genes, such as heat-shock response (33), response to viral infection (34), PARP1-mediated transcription (35) and transcription requiring adenosine triphosphate (ATP)-dependent chromatin remodeling (36). The large non-histone portion at the C-terminus of the protein is comprised of a hinge domain and a macro domain for which the crystallographic structure has been determined. Macro-domains are present in many proteins and can bind ADP-ribose or related molecules (37); nonetheless, this ability is conserved only in the less abundant macroH2A1.1 isoform.

Two recent studies link macroH2A1 to cancer. In a computational analysis of gene expression in murine and human tumors, expression of macroH2A1 was included in a small gene expression signature which correlated with the ability of c-Myc to maintain tumorigenesis (38). Studying macroH2A1 isoforms in a panel of lung cancer samples (39), macroH2A1.1 levels were found to be elevated in quiescent cells and decreased in proliferating cells. Reduced levels of macroH2A1.1 correlated with high proliferative index and increased risk of recurrence. The functional significance of these observations is currently unknown. Here, we report that macroH2A1 is associated with silenced TSG promoters and takes part in maintaining the silent state of such genes.

MATERIALS AND METHODS

Cell lines

HCT116 cells were cultured in McCoy's 5A medium, SW480 in RPMI medium, Tert-immortalized WI-38 cells in MEM medium and RKO in DMEM. All media were supplemented with 10% fetal calf serum (FCS), penicillin (50 µg/ml), streptomycin (50 µg/ml) and 2 mM (L-Glutamine). In addition, WI-38 medium contained 0.1 nM NEAA. 5-aza-dC (Sigma) was applied for 6 days to concentrations of 50–1000 nM.

Antibodies and western blot analysis

Anti-macroH2A1 is described in ref. (40). Anti-MacroH2A2 was kindly provided by A. Ladurner (EMBL); anti-β-actin: Abcam (ab6276); anti-p16: Santa Cruz (sc-468). Acetylated H3K9,K14 (Millipore 06-599), H2A.Z (Millipore 07-594). Secondary antibodies coupled to horseradish peroxidase (Jackson Immunoresearch Laboratories).

ChIP

ChIP assays were performed according to the standard Upstate protocol (41), using Bioruptor bath sonicator (Diagenode) to shear DNA to an average size of 500 bp.

Chromatin from 1.5×10^6 cells was precleared with 40 µl salmon sperm DNA–protein A–agarose beads (Upstate Biotechnology) for 30 min, followed by an overnight incubation with antibody. Eluted DNA was isolated using QIAGEN PCR purification kit.

For polymerase chain reaction (PCR) analysis, the bound fraction was compared with a 1:100 dilution of the input DNA. When different samples were compared, the bound/input values were normalized according to a positive control to correct for differences in overall IP efficiency between samples (results before normalization are shown in Supplementary Figures S7–S9). At least three biological replicates were performed to confirm ChIP results.

PCR and real-time PCR

PCR reactions were carried out using Red Load Taq Mastermix (Larova). Primers are listed in Supplementary Table S1. Quantitative real-time PCR (qPCR) assays were carried out on ABI Prism 7900 system using SYBR Green PCR Master Mix (Applied Biosystems). Efficiencies of all primer pairs were greater than 1.89 per cycle.

SNapShot assay

We adapted the SNapShot procedure (Applied Biosystem) to identify and quantitate an SNP caused by frameshift mutation in one allele of the CDKN2A locus in HCT116 cells, taking a similar approach to that described by (42). Briefly, a PCR product carrying the polymorphic site was generated using the primers CDKN2A SNaPshot F+R (Supplementary Table 1). Excess primers and dNTPs were removed using ExoI/SAP. The purified fragment was then used as template for SNaPshot with the primer CDKN2A SNaPshot (this primer ends one base before the CDKN2A mutation). Thermocycling: 30×(10 s 96°C, 5 s 50°C, 30 s 60°C) was used for single base extension reaction and DNA was treated with 1 U of CIAP (Fermentas) to remove unincorporated ddNTPs. ABI PRISM 3700 DNA Analyzer (Applied Biosystem) was used for visualization and quantitation was done using the Genotyper 2.1 software (ABI PRISM).

shRNA lentiviral vectors

Construction of lentiviral vectors pLKO1.puro/hygro carrying shRNA directed against macroH2A1 was as described (43). The macroH2A1 target sequence used was 5'-CCAGTTACTTCGTGCTACAA-3'. The macroH2A2 target sequence used was 5'-ACAGCGATAAAGAAGGAAGCTT-3'. Scrambled sequence was 5'-GTTGCCCTAGACTTAGAACT-3'. Viral particles were produced in 293T cells as described in 43 and target cells were exposed to two rounds of infection followed by the relevant selection (2 µg/ml puromycin or 140 µg/ml hygromycin).

RNA isolation and reverse transcription-PCR

RNA was isolated using Tri[®]-Reagent (Sigma) followed by treatment with 2 U of RQ RNase free DNase

(Promega) according to the manufacturer's instructions. Reverse transcription (RT) was performed on 1 µg DNase treated RNA using 0.5 µg random hexamer primers in a reaction volume of 20 µl according to the manufacturer (ImpromIItm RT promega).

Methylation analysis

According to the manufacturer's instructions, 1.2 µg genomic DNA was bisulfite-modified using EZ DNA Methylation Kit (Zymo Research). PCR reactions were performed using Faststart taq DNA polymerase (Roche).

RESULTS

Silencing of p16 (CDKN2A) is accompanied by enrichment for MacroH2A1

Considering the similarity between the heterochromatin of the inactive X chromosome and that of epigenetically silenced TSGs, we hypothesized that macroH2A1 may also be associated with promoters of TSGs, specifically when they are epigenetically silenced. To test this possibility, we used ChIP to assess macroH2A1 enrichment at active and silenced promoters of known TSGs.

We used WI-38 human embryonic lung fibroblasts, in which p16^{Ink4a} (CDKN2A) was found to undergo epigenetic silencing through DNA methylation of its promoter. Silencing occurs spontaneously during passage of these cells in culture, while treatment of the non-expressing cells with 5-aza-dC restores p16 expression (44). We compared cells from a late passage (p32) in which CDKN2A is methylated and silenced, to cells from an early passage (p18) which are unmethylated and express p16 (Figure 1A). Using ChIP with anti-macroH2A1 we observed enrichment for macroH2A1 at the CDKN2A promoter only in the late passage cells that have silenced the gene (Figure 1B), demonstrating specific association of macroH2A1 with the inactive state. To validate the efficacy of our ChIP assay, we compared enrichment for macroH2A1 at several non-expressed genes to that of housekeeping genes. MacroH2A1 levels were significantly elevated in the non-expressed tissue-specific genes (Supplementary Figure S1A and B). We also performed ChIP on macroH2A1-deficient ES cells, as negative control confirming that the bound fraction is dependent on macroH2A1 presence (Supplementary Figure 1C).

MacroH2A1 is enriched on the epigenetically silenced, but not the active allele of p16 (CDKN2A)

To further confirm the specific association of macroH2A1 with the silent state we took advantage of the unique configuration of the p16(CDKN2A) locus found in the HCT116 colon cancer cell line, in which one allele is epigenetically silenced, while the other allele is transcriptionally active, but is non-functional due to a frame-shift mutation in the first exon (45). This combination allows a comparison between an active and silent allele within the same ChIP experiment. We used the point mutation (a single base insertion on the active allele) to discriminate

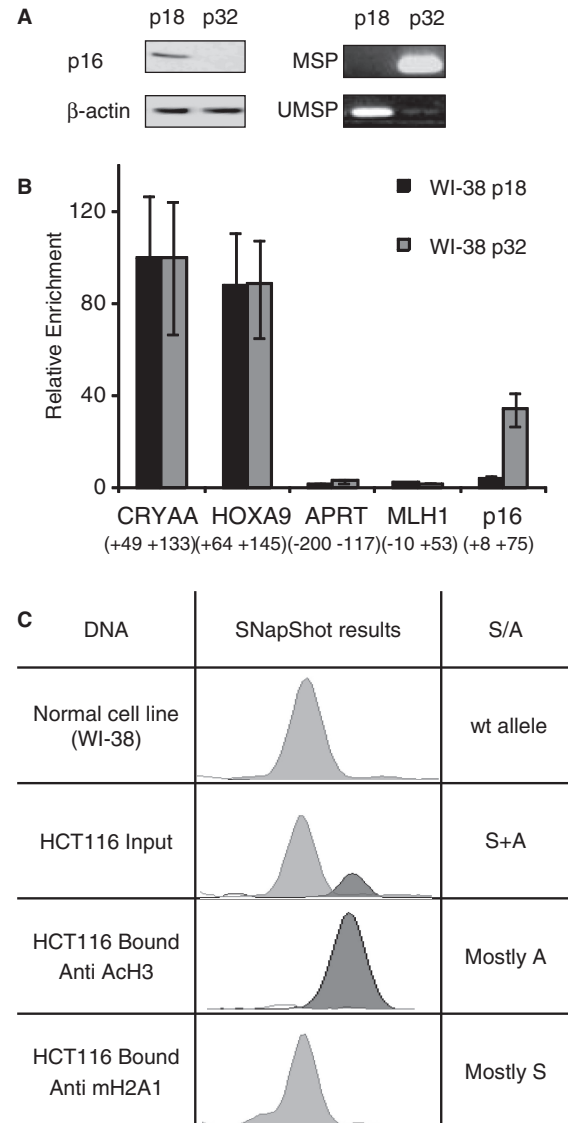


Figure 1. Silencing of CDKN2A is accompanied by macroH2A1 enrichment. (A) Expression and methylation status of CDKN2A in early passage (p18) and late passage (p32) WI-38 cell: western blot for p16 and β-actin showing loss of expression in p32 cells (left panel). Methylation of the CDKN2A promoter in p32 cell confirmed by methylation-specific PCR (MSP) on bisulfite-modified DNA (right panel). (B) ChIP with macroH2A1 antibody. The bound/input ratio for each cell line was calculated using quantitative real-time PCR. To compare between cell lines, enrichment of the bound fraction was normalized according to the positive control α-Crystallin, which is inactive in both cultures (Supplementary Figure S6). An 8.5-fold enrichment for macroH2A1 at the CDKN2A promoter is observed in p32 cells versus p18 cells. Similar results were observed in three biological replicates (see also Figure 5). HoxA9 is another positive control and is not expressed. Aprt and MLH1 are expressed in both samples. The numbers under the gene names indicate position of the interrogated region relative to TSS. Error bars represent standard deviation. (C) ChIP analysis using anti-macroH2A1 or anti-acetylated H3 antibody on HCT116 cells. Input and bound fractions were analyzed by SNaPshot assay to discriminate between the silenced (wild-type) allele of CDKN2A (S) and the active mutant allele (A). The ratio between the two alleles (S/A ratio) was determined based on peak area using Genotyper 2.1 software (ABI PRISM).

between the alleles in a single base extension ‘SNapShot’ assay.

Following ChIP with an antibody against acetylated H3, the bound fraction was greatly enriched for the active allele as expected (Figure 1C). By contrast, the macroH2A1-enriched fraction contained almost exclusively the epigenetically silenced allele of CDKN2A and not the mutated, transcriptionally active, allele. In two repeated ChIP experiments, we consistently observed a >5-fold enrichment in macroH2A1 on the silenced allele in the bound fraction. This result strongly supports specific association of macroH2A1 with the inactive state.

MacroH2A1 is enriched on epigenetically silenced TSGs in colon cancer cell lines

To determine whether macroH2A1 enrichment might be a characteristic of epigenetic silencing in cancer, we analyzed distribution of macroH2A1 at active and silenced promoters in colon cancer derived cell lines. We examined two colon cancer cell lines, RKO and SW480, as the epigenetic status (expression, DNA methylation and chromatin modifications) of many loci has been extensively described in these lines (46,47).

We verified the epigenetic status of selected loci in these lines by comparing expression levels in the absence or presence of the DNA demethylating drug 5-aza-dC and by sequencing bisulfite converted DNA (Supplementary Figure S3). In agreement with previously published data (46), p16 was epigenetically silenced in both RKO and SW480 and was reactivated upon demethylation, while other genes were silenced in only one of the cell lines (Figure 2A). We then focused our analysis on CpG islands of genes that are silenced in one cell line but not the other. In agreement with our prediction, we always found higher macroH2A1 levels at the silenced promoters. For MLH1, TIMP3 and CRBP1, which are silenced in RKO cells, macroH2A1 levels were found to be higher in the RKO cells. This pattern was reversed for GATA4, which is silenced specifically in SW480 cells (Figure 2B).

Knockdown of macroH2A1 contributes to the re-expression of silenced p16

Next, we asked whether macroH2A1 is functionally important for silencing. Using a lentiviral vector we introduced stable shRNA against macroH2A1 into WI-38 (p32) cells with the silenced p16. Although macroH2A1 protein was effectively depleted from the cells (Figure 3A), this had no effect on transcription of p16 (Figure 3B). However, studying the role of macroH2A1 in silencing of the inactive X, Hernandez-Munoz *et al.* (30) reported that combining knockdown of macroH2A1 with DNA demethylation and HDAC inhibitors had a synergistic effect on reactivation of a silenced reporter gene. We first repeated these experiments and observed a strong synergistic effect in X-inactivation, even in the absence of HDAC inhibitors (Supplementary Figure S2). Next, we analyzed p16 expression in WI-38 (p32) cells that were treated with increasing concentrations of the DNA demethylating agent

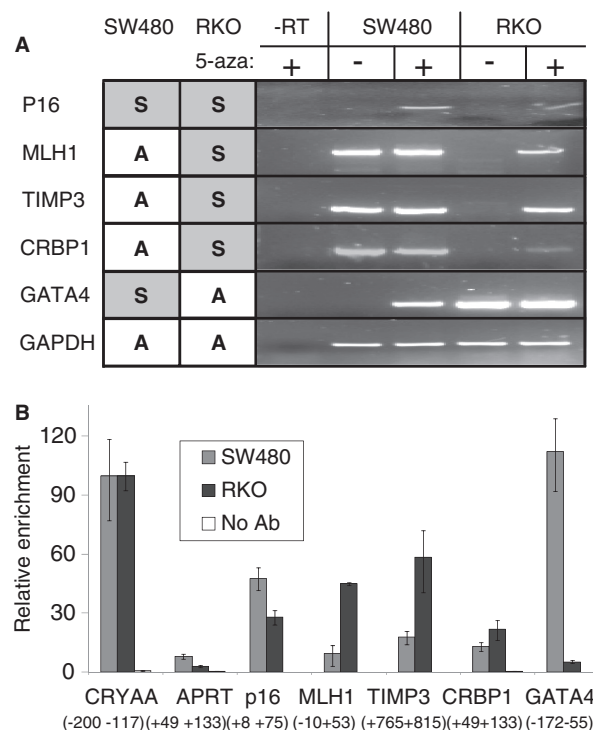


Figure 2. MacroH2A1 is associated with epigenetically silenced TSG in colon cancer cell lines. (A) Epigenetic status of selected genes in RKO and SW480 cells was confirmed by assaying for reactivation upon demethylation. Cells were cultured with 1 μ M 5-aza-dC for 5 days and transcription was evaluated by RT-PCR. Genes that were expressed before treatment were considered active (A). Genes that became transcribed only upon demethylation were considered to be epigenetically silenced (S). Results were consistent with previously published reports on these cell lines (10,46). (B) Chromatin immunoprecipitation using anti-macroH2A1 antibody. Enrichment for macroH2A1 at promoter regions was measured by real-time PCR analysis of the bound fraction relative to input. To compare between RKO and SW480 cells the bound/input values were normalized according to the positive control α -Crystallin (CRYAA, which is inactive in both cultures, Supplementary Figure S6). The numbers under the gene names indicate position of the interrogated region relative to TSS. Error bars correspond to standard deviation of the $\Delta\Delta C_t$. IP with no antibody gave very low background signal. Similar results were observed in three repeated experiments (see also Figure 5).

5-aza-dC. We observed significantly higher expression levels of p16 in macroH2A1 knockdown cells compared with control cells (Figure 3B).

Reactivation of TSGs in RKO colon cancer cell line following KD of macroH2A1

To determine whether macroH2A1 depletion can promote reactivation of silenced genes in cancer cells, we knocked down macroH2A1 in RKO cells (Figure 3A) and evaluated the reactivation of four silenced genes (MLH1, TIMP3, CRBP1 and CDKN2A). MacroH2A1 deficiency was not sufficient for reactivation of silenced genes, but similar to WI-38 cells, combining knockdown of macroH2A1 with low levels of 5-aza-dC resulted in enhanced reactivation of p16, MLH1 and TIMP3 as compared to demethylation alone (Figure 3C). Three additional biological replicates done at 200 nM 5-aza-dC repeatedly indicated enhanced expression in

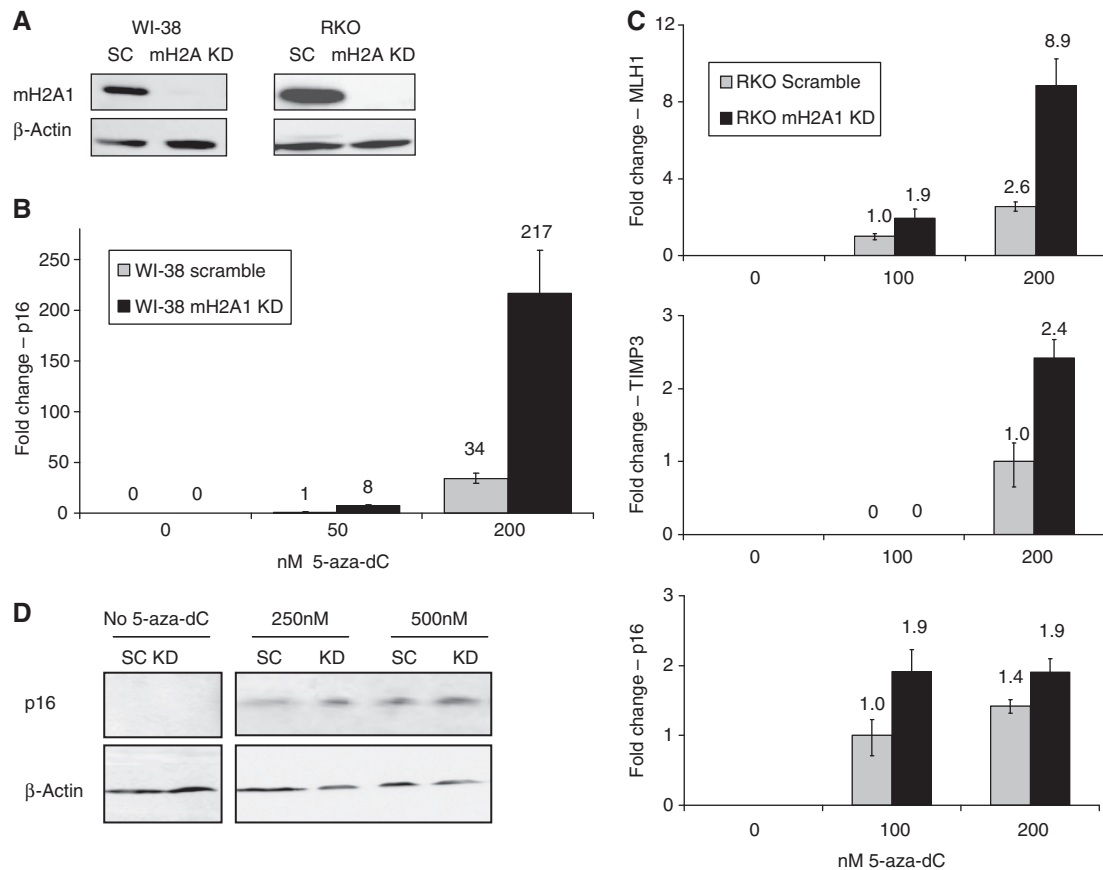


Figure 3. KD of macroH2A1 facilitates TSGs reactivation. (A) Western blot showing levels of macroH2A1 in WI-38 (passage 32) and RKO cells infected with Lentiviral macroH2A1 specific shRNA (mH2A KD) or scrambled shRNA (SC). (B) Real-time RT-PCR analysis of p16 expression in WI-38 (passage 32) cells with macroH2A1 KD or scrambled KD following treatment with 5-aza-dC. cDNA was analyzed using real-time PCR and the p16 expression level was normalized to GAPDH. Graph shows fold change in expression level relative to control (scrambled KD) treated with 50 nM 5-aza-dC. KD of macroH2A1 resulted in an 8-fold increase in p16 levels following treatment with 50 nM 5-aza-dC. (C) Quantitative real-time RT-PCR analysis of MLH1, TIMP3 and p16 expression in RKO cells. In three repeated experiments, macroH2A1-deficient cells consistently show better reactivation following treatment with low concentrations of 5-aza-dC. (D) Western blot analysis of p16 levels in HCT116 colon cancer cell line carrying macroH2A1 KD or scrambled shRNA. Because HCT116 cells express a mutated transcript for one allele, the analysis was done using western blot analysis and detects only expression from the silenced allele. p16 expression following treatment with 5-aza-dC is enhanced in macroH2A1-deficient cells.

macroH2A1-deficient cells over controls. 2.54 ± 1.04 (SD) fold increase for p16, 3.1 ± 1.1 for MLH1 and 2.58 ± 0.23 for TIMP3. Similarly, knockdown of macroH2A1 appears to enhance the effect of demethylation in reactivation of the silenced copy of p16 in HCT116 colon cancer cells (Figure 3D). Reactivation of CRBP1 in RKO cells was evident only following treatment with at least $1 \mu\text{M}$ 5-aza-dC and simultaneous knockdown of macroH2A1 did not promote further reactivation (data not shown). This is consistent with the relatively low level of macroH2A2 detected at the CRBP1 promoter in RKO cells (Figure 2B). The ability of 5-aza-dC to induce dose-dependent demethylation of the examined loci was confirmed by bisulfite sequencing (Supplementary Figure S3).

Human cells carry two macroH2A genes (26). Knockdown of macroH2A2 in RKO cells did not promote reactivation of silenced genes and did not enhance the reactivation obtained by DNA demethylation. Knockdown of both macroH2A1 and

macroH2A2 resulted in the enhancement of reactivation to a level similar to that of macroH2A1 knockdown alone (Supplementary Figure S4). RKO cells appear to have relatively low levels of macroH2A2 (data not shown) and it does not seem to significantly contribute to maintenance of silenced state in the loci we examined.

We also measured the effect of macroH2A1 knockdown on growth of RKO cultures: As shown in Figure 4, knockdown of macroH2A1 had a mild growth inhibitory effect even without 5-aza-dC treatment. In the presence of the drug, macroH2A1 knockdown had a significant effect on growth compared with controls (Figure 4).

In conclusion, we find synergism between DNA demethylation and macroH2A1 deficiency in reactivation of three important TSGs and in inhibiting cell culture growth.

Inverse relationships between macroH2A1 and H2A.Z

Recent genome-wide mapping studies link the histone variant H2A.Z to promoter regions of transcriptionally

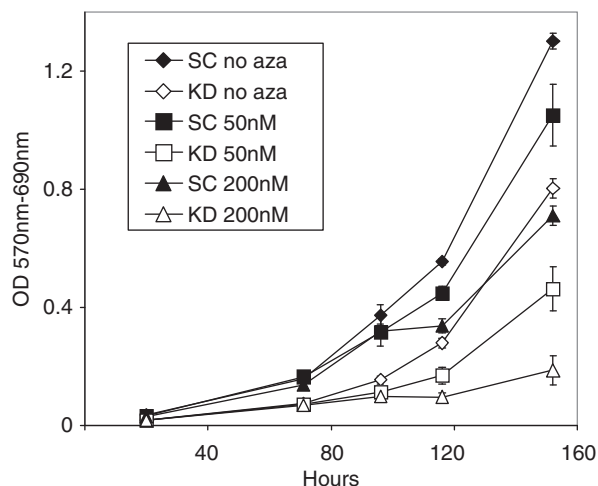


Figure 4. KD of macroH2A1 inhibits proliferation of RKO cells. Growth curves of RKO cells deficient for macroH2A1. Cells were cultured in 96 well plates (3000 cells/well) with 0, 50 or 200 nM 5-aza-dC for 6 days. Proliferation of cells was determined using MTT assay.

active genes (48,49). Other recent studies describe the inverse relations between H2A.Z and DNA methylation (50). We speculated that in silenced loci, H2A.Z might be replaced by macroH2A1. We therefore examined the occupancy of H2A.Z at the promoters of active and silenced TSGs (Figure 5). In marked contrast to macroH2A1, H2A.Z was consistently enriched at promoters of transcriptionally active genes and was absent in cases where the same gene was silenced both in WI-38 cells and in the colon cancer cell lines (Figure 5A and B). In most of the promoters we studied, the TSS is located within a CpG island. An exception is the TIMP3 promoter, where the CpG island is positioned 579–1465 bp downstream of the TSS. Interestingly, we find that the switch from H2A.Z in the active, unmethylated state to macroH2A1 in the silenced and methylated state is clearly observed in the CpG island but not in the vicinity of the TSS (Figure 5B).

To further define the function of H2A.Z and macroH2A in expression, we examined the presence of the two variants during reactivation of transcription. Demethylation with 5-aza-dC did facilitate some gene expression (Supplementary Figure S5) but macroH2A and not H2A.Z remained associated with the reactivated loci (Figure 5C and D). Following knockdown of MacroH2A1, gene expression was further enhanced (Supplementary Figure S5) but even under these conditions, H2A.Z levels were not significantly elevated (Figure 5D). This result is consistent with the genome-wide analysis, which indicates that only highly expressed promoters are associated with H2A.Z (49). It also suggests that it is MacroH2A1 presence rather than the H2A.Z absence that contributes to silencing.

MacroH2A1 and *de novo* DNA methylation

In cancer, H3K27 methylation was suggested to pre-mark sites of aberrant methylation (18). During X-inactivation macroH2A1 enrichment of the inactive-X is observed after methylation of H3K27 but prior to DNA methylation (4).

We considered the possibility that like in X-inactivation, macroH2A presence may precede DNA methylation during silencing of TSGs. Silencing and methylation of p16 is known to occur during culturing of WI-38 cells (44). However, we concluded that the gradual silencing of p16 during culturing of WI-38 cells is the result of a rare silencing and methylation event in a minor population of cells, which, due to the growth advantage of p16-deficient cells, quickly take over the population. We were therefore not able to directly determine the order events using this system.

Using genome-scale DNA methylation analysis we have recently analyzed methylation status of ~27 000 CpG sites and identified nearly 1000 sites that like p16 (CDKN2A) becomes methylated in late passage WI-38 lung fibroblasts (51). To examine if macroH2A presence may precede DNA methylation in the context of cancer associated DNA methylation, we took advantage of recently published data on macroH2A1 genomic distribution in lung fibroblasts (52) and determined the frequency of *de novo* methylation as a function of macroH2A1 enrichment. As shown in Figure 6, we find that macroH2A1 enriched regions show higher propensity to undergo *de novo* methylation. Importantly, genomic analyses show that macroH2A1 enrichment often correlates with H3K27 methylation (52,53). It was therefore of interest to determine whether H3K27 methylation directs both macroH2A1 association and DNA methylation or whether macroH2A1 has an independent contribution to recruitment of aberrant methylation.

To address this question in an indirect way, we took advantage of available data on H3K27 methylation in human primary lung fibroblasts and calculated the frequency of aberrant methylation as a function of both H3K27me3 and macroH2A1. Figure 6b shows that as previously reported (18), aberrant methylation is higher in H3K27me3 enriched sites. However, we find that for any given level of H3K27me3 the frequency of aberrant methylation increases with macroH2A1 levels, supporting the possibility that macroH2A1 presence may promote DNA methylation at CpG islands independent of H3K27 methylation.

DISCUSSION

Several complementary mechanisms are involved in maintenance of the inactive X (4). The same mechanisms were shown to take part in abnormal silencing of genes in cancer cells (2). Here, we demonstrate that macroH2A1, an established feature of the inactive X, is also associated with epigenetically silenced TSGs and takes part in maintenance of the inactive state. As in the case of the inactive X (30), we found that macroH2A1 contributes to gene silencing in synergy with DNA methylation.

Studying the p16(CDKN2A) locus in three different cell lines, we observed enrichment for macroH2A1 specifically when the gene is epigenetically inactivated. In this locus, the removal of macroH2A1 improved the effectiveness of DNA demethylation in reactivation of transcription,

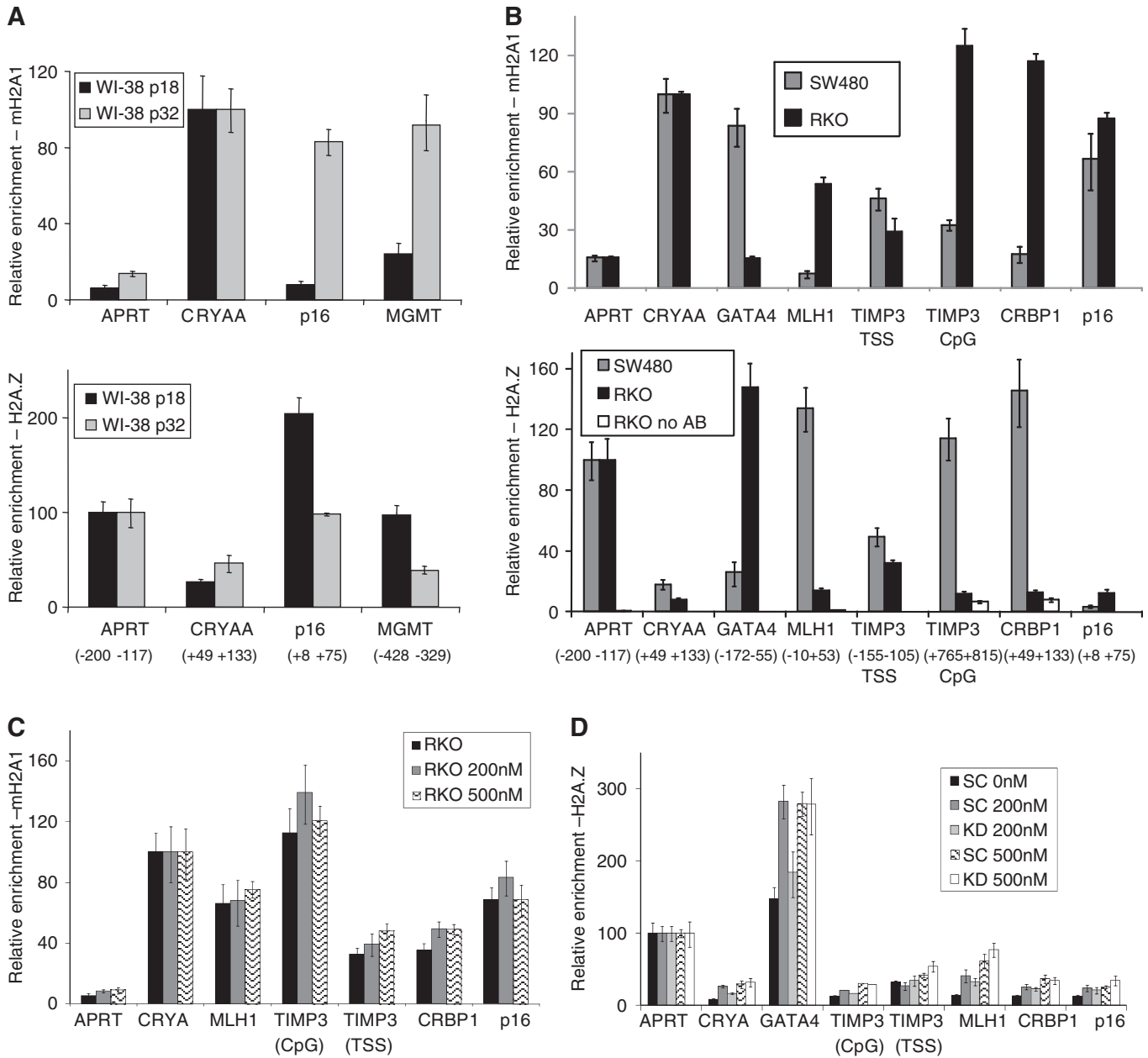


Figure 5. Inverse relations between macroH2A1 and H2A.Z in active and silenced TSGs promoters. Chromatin immunoprecipitation using anti-macroH2A1 antibody and anti-H2A.Z antibody on WI-38 early and late passage (A) or RKO and SW480 cells (B). Enrichment for macroH2A1 was normalized to the positive control α -crystallin (CRYAA). H2A.Z enrichment was normalized to APRT, and α -Crystallin served as the negative control. Error bars correspond to standard deviation of the $\Delta\Delta C_t$. IP with no antibody gave very low background signal. MGMT promoter was found to be silenced and methylated in late passage WI-38 cells but was not reactivated even following treatment with 1 μ M 5-aza-dC (data not shown). For TIMP3 promoter, enrichment was measured both near the transcription start site (TSS) and at the adjacent CpG island (CpG). (C) MacroH2A enrichment before and after 5-aza-dC treatment. High levels of macroH2A enrichment are observed in silenced TSGs, before and after treatment with 200 or 500 nM 5-aza-dC. (D) H2A.Z enrichment before and after 5-aza-dC treatment and macroH2A1 KD. H2A.Z is depleted from silenced loci and no change in H2A.Z levels is observed after treatment which facilitated gene reactivation.

indicating a functional role for macroH2A1 in maintenance of the inactive state. Similarly, we observed specific enrichment of macroH2A1 in the silenced state and functional contribution of macroH2A1 to silencing of the TSGs MLH1 and TIMP3.

Synergism between DNA demethylation and macroH2A1 deficiency is evident also when measuring cell proliferation. Although this effect is consistent with the role of p16 as a major regulator of cell proliferation,

there is no evidence that the effect on growth is directly related to TSG reactivation.

Our results demonstrate that in addition to changes in DNA methylation and histone modifications in the promoter region, aberrant silencing results in a change in nucleosome composition: While H2A.Z is present in the active state, it is replaced by macroH2A1 in the silenced state (Figure 5). We find that following demethylation and macroH2A1 knockdown, transcription

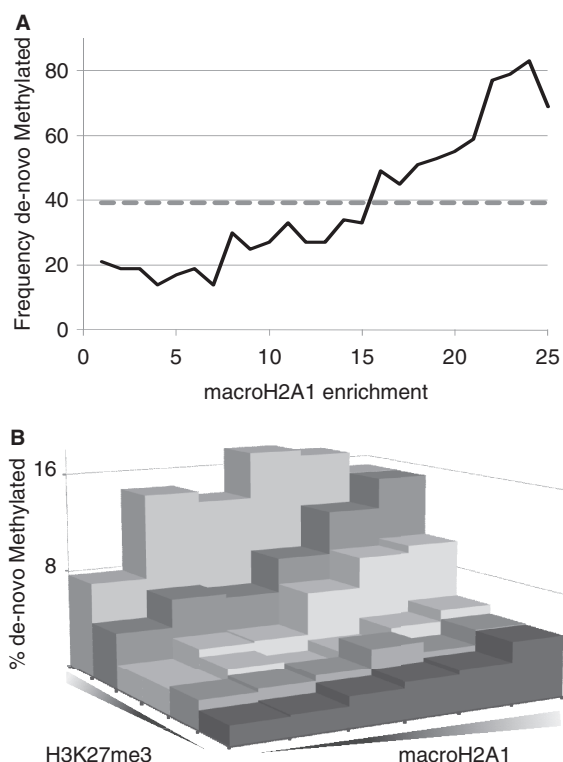


Figure 6. Probability of de-novo methylation as a function of macroH2A1 enrichment. (A) x-Axis describes macroH2A1 enrichment at 18976 CpG sites in IMR90 lung fibroblasts (all probes are within CpG islands). Sites were grouped into 25 equal size groups, from the least enriched (group 1) to the most enriched sites (group 25). For each group we calculated the fraction of probes that were *de-novo* DNA methylated in late passage WI-38 cells compared to primary WI-38 culture (see Supplementary Data for additional details). The dashed line represents the expected result assuming uniform distribution. The results show that macroH2A1 enriched regions undergo *de-novo* methylation more frequently. (B) Same data as in A divided into 36 groups according to both macroH2A1 and H3K27me3 levels in primary lung fibroblasts. For each group we determined the percentage of probes showing *de novo* DNA methylation in late passage.

can be restored even without H2A.Z association, indicating that H2A.Z presence is not required for transcription from these promoters and suggesting that the repressive effect of macroH2A1 is not achieved through exclusion of H2A.Z. A recent study identified a special nucleosome, assembled with H2A.Z and the histone H3 variant H3.3, located at the TSS of highly expressed genes (49). The study further shows that the association of this nucleosome with DNA is very loose and that preparative methods used previously resulted in the loss of these unstable nucleosomes, and an apparent ‘nucleosome free region’ (NFR). In contrast to the labile nature of H2A.Z nucleosomes, *in vitro* experiments suggest that nucleosomes containing macroH2A have stronger affinity to DNA (54) and are refractory to Pol II transcription initiation and to ATP-dependent chromatin remodeling (36,55) or reduce the recruitment of SWI/SNF chromatin remodeling complexes (56).

In silenced TSGs DNA methylation is the major mechanism responsible for maintenance of the inactive state. However, while DNA demethylation using 5-aza-dC

removes critical repressive marks and facilitates reactivation of transcription, it does not restore a fully euchromatic state: demethylation is often partial (57), histone H3K27 methylation remains higher than in the active state (47) and H2A.Z is not restored (Figure 5). The contribution of HDAC inhibitors to reactivation of TSGs is observed only if preceded by DNA demethylation (10). Similarly, the repressive effect of macroH2A1 presence is evident only under these conditions.

The means by which macroH2A1 promotes silencing are not fully understood. In addition to the mechanisms proposed above, macroH2A variants were shown to affect the activity of PARP1 and its association with chromatin (33,35). Interaction of macroH2A1.2 with the E3 ubiquitin ligase Spop has also been proposed to promote silencing (30). It will be important to determine which of these mechanisms is relevant in the case of silenced TSGs as all these pathways involve enzymes which can serve as drug targets with potential of enhancing the effects of DNA demethylating agents.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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