Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene

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Summary

We attempted to answer two central questions about epigenetic silencing of the tumor suppressor gene *p16*^{INK4a} in this study: (1) whether the maintenance of associated histone modifications is dependent on DNA methylation and (2) whether such histone modifications can occur prior to DNA methylation. By coupling chromatin immunoprecipitation with gene targeting and the analysis of specific alleles, we found that elimination of DNA methylation from a *p16*^{INK4a} allele resulted in profound changes in surrounding histones. After continued passage of such cells, methylation of histone H3 lysine-9 occurred in conjunction with re-silencing in the absence of DNA methylation. These results have important implications for understanding the biochemical events underlying the silencing of tumor suppressor genes and the resultant growth suppression.

Introduction

A variety of tumor suppressor genes have been shown to be silenced in human cancers (Jones and Baylin, 2002). In virtually all cases, the silencing is accompanied by DNA hypermethylation of the promoters of these genes, and DNA methylation has often been interpreted to be causally involved in the silencing. DNA methylation is also associated with silencing of genes on the inactive X chromosome. However, studies have shown that DNA methylation occurs only after X chromosome inactivation, excluding DNA methylation as the proximate cause (Heard et al., 1997; Migeon, 1994). Analogous results have not been described for tumor suppressor genes on the autosomes.

A recently developed experimental system has given us the opportunity to evaluate the biochemical events underlying silencing in a temporal fashion. When both the *DNA Methyltransferase 1* (*DNMT1*) and *DNA Methyltransferase 3B* (*DNMT3B*) genes are disrupted through gene targeting, virtually all DNA methyltransferase activity and DNA methylation are eliminated (Rhee et al., 2002). This situation is akin to that occurring in developing germ cells, wherein DNA methylation is erased, permitting parental imprinting (Razin et al., 1984; Reik et al., 2001). This system has given us a unique opportunity to address particular questions regarding the relationship between tumor suppressor gene silencing, histone modifications, and DNA methylation.

Results and discussion

Colorectal cancer cells in which the *DNMT1* and *DNMT3B* genes are disrupted (termed DKO for *d*ouble *k*nock*o*ut) were noted to grow very slowly compared to parental cells. With continued passage of these cells, the cells' growth rate increased (Figure 1A). To investigate the basis for the growth change, we first analyzed the expression of the *p16*^{INK4a} gene. In parental cells, one allele of *p16*^{INK4a} is wild-type (wt) and silenced while the other harbors a truncating frameshift mutation and is expressed (Myohanen et al., 1998). When DNA methylation is erased through disruption of DNMT1 and DNMT3B, silencing of the wt allele is eliminated (Rhee et al., 2002 and Figure 1B, passage 5 cells). By passage 22, when the growth rate has increased (Figure 1A), the wt allele is again completely silenced (Figure 1B). Evaluation of the DNA methylation status of the *p16*^{INK4a} gene by real time methylation-specific PCR (MSP) showed discordance between expression and DNA methylation at passage 22. Though the wt allele of *p16*^{INK4a} was silenced and methylated in parental HCT116 cells, no DNA methylation of the silenced wt allele was observed in the DKO cells at passage 22 (Figure

SIGNIFICANCE

The mechanisms underlying epigenetic silencing have been extensively investigated in various non-mammalian systems, but very little is known about this process in tumor suppressor genes in cancer cells. Our data show that the maintenance of epigenetic silencing and of associated histone modifications is dependent on DNA methylation. Because we used a genetic rather than pharmacologic approach to remove DNA methyl groups, the data leading to this conclusion were unambiguous. Moreover, we found that histone modifications associated with silencing of a tumor suppressor gene can occur independently of DNA methylation. These studies demonstrate similarities between the processes of epigenetic silencing of tumor suppressor genes and X inactivation and suggest that DNA methylation serves to "lock in" rather than initiate silencing.

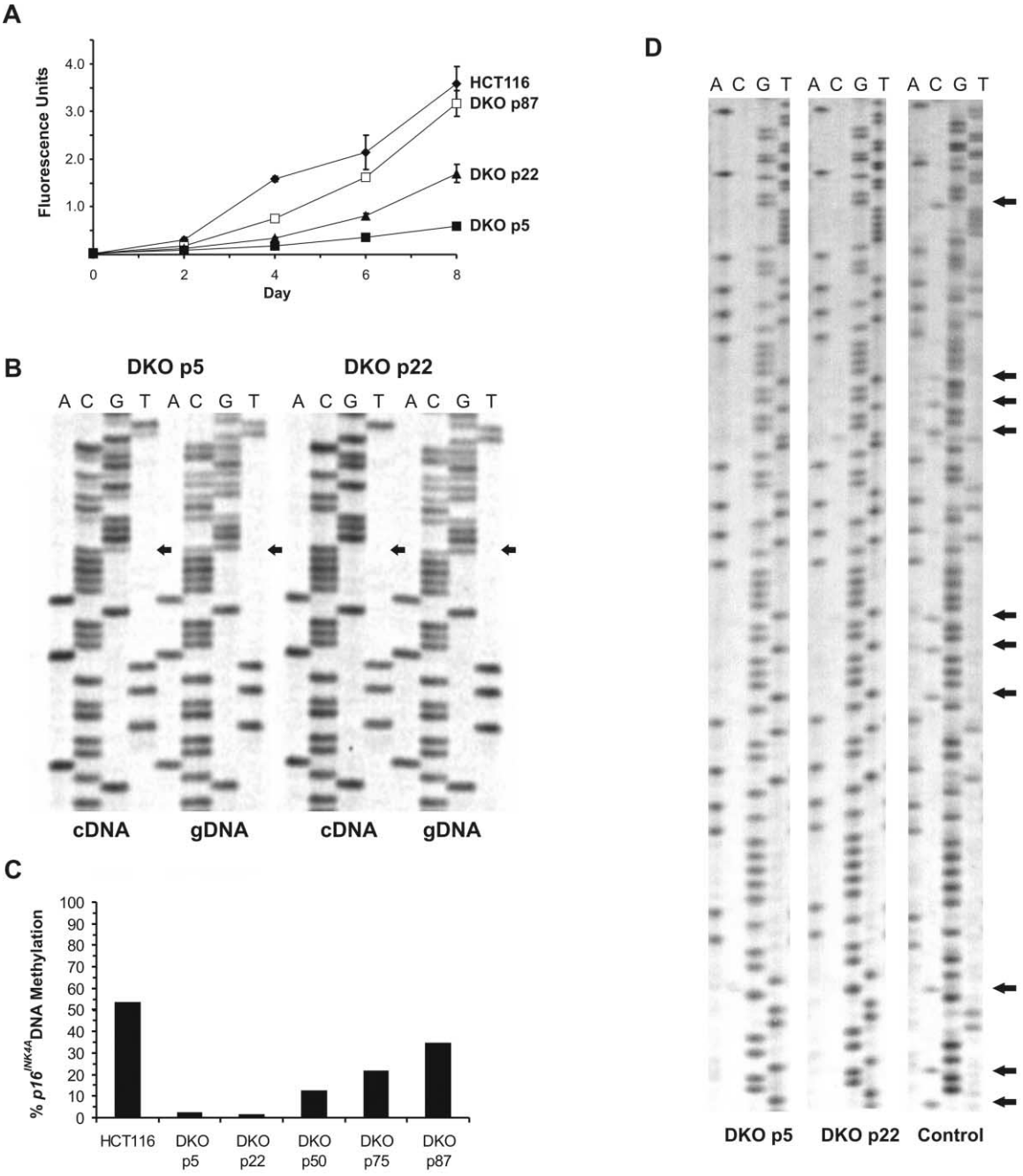

Figure 1. Changes in growth rate and *p16*^{INK4A} silencing in DKO cells after serial passage in culture

A: Growth curves of parental HCT116 and DKO cells at passages 5, 22, and 87. The points and bars represent the average and standard error, respectively, determined from triplicate measurements.

B: Sequence of genomic DNA and cDNA of exon 1 of the *p16*^{INK4A} gene from DKO cells at passages 5 and p22. Arrows point to the frameshift mutation in the mutant allele.

C: Quantitative assessment of the DNA methylation status of *p16*^{INK4A} by real time methylation-specific PCR of parental HCT116 and DKO cells at the indicated passage (p) number.

D: Genomic bisulfite sequencing of the *p16*^{INK4A} 5′ CpG island. A portion of the autoradiograph is shown. Arrows point to ten C residues that were unmethylated and converted to thymidines in passage 5 DKO cells, wherein *p16*^{INK4A} was active, but also in passage 22 DKO cells, wherein *p16*^{INK4A} was silenced. The control sequencing lanes were derived from RKO cells, wherein both alleles of the *p16*^{INK4A} gene are methylated and inactive.

1C). By passage 87, the wt allele had become methylated. Thus, silencing preceded DNA methylation. These changes in growth, DNA methylation, and gene silencing were observed in each of three independent DKO clones.

The MSP assay measures methylation at CpG sites shown previously to be invariably associated with silencing of the *p16*^{INK4a} gene (Herman et al., 1996). To ensure that no DNA methylation occurred elsewhere in this region of the gene, we preformed pooled genomic bisulfite sequencing. These studies revealed the absence of methylation at all 20 CpG sites analyzed at passage 22, at which time the *p16*^{INK4a} gene had already been silenced (Figure 1D). The 338 bp region analyzed extended from 108 nucleotides upstream of the translation start site to 230 nucleotides downstream and included the most CpG-rich region of the CpG island associated with methylation of the *p16*^{INK4a} gene (Herman et al., 1996).

To determine whether re-silencing of *p16*^{INK4a} was associated with modifications of histones, we performed chromatin immunoprecipitation (ChIP) with cells at various passages. By comparing the relative levels of wt and mutant *p16*^{INK4a} alleles in the immunoprecipitates, we could analyze chromatin modifications associated with silencing of the wt allele in a highly specific and quantitative manner. Immunoprecipitated DNA was used as a template for PCR, and the wt allele was discriminated from the mutant by virtue of a 1 bp insertion in the latter. One of the most important covalent modifications of chromatin proteins involves methylation at lysine 9 of histone H3 (H3-K9), observed in all eukaryotes studied to date (Bannister et al., 2001; Gendrel et al., 2002; Lachner et al., 2001; Nakayama et al., 2001; Noma et al., 2001; Volpe et al., 2002). In parental HCT116 cells, chromatin immunoprecipitation with an antibody recognizing this residue showed that the chromatin containing the wt allele of *p16*^{INK4a} was methylated at H3-K9, but that the chromatin containing the mutant allele was unmethylated (Figure 2). Erasure of the DNA methylation through disruption of the *DNMT1* and *DNMT3B* genes led to removal of the histone H3-K9 methylation from the wt allele (Figure 2, passage 5). By passage 22, however, the chromatin containing the wt allele had become re-methylated at H3-K9 while the mutant allele remained unmethylated.

Histone acetylation was also changed upon disruption of DNA methylation. In parental cells, the mutant *p16*^{INK4a} allele was acetylated to a greater extent than the silenced wt allele. After erasing DNA methylation, histone H4 acetylation of the wt allele reappeared, likely reflecting the absence of deacetylase activity (Figure 2, passage 5). The parental pattern of histone H4 acetylation was not fully restored until very late passages (Figure 2, passage 87). Thus gene silencing occurred in conjunction with histone H3-K9 methylation and well before histone H4 deacetylation of the chromatin or DNA methylation of the packaged DNA.

We next sought to test whether the silencing of the wt *p16*^{INK4a} allele was not only associated with enhanced growth of the subclones, but was essential for such growth. To perform this test in a rigorous manner, we disrupted the wt *p16*^{INK4a} allele through targeted homologous recombination prior to fully erasing methylation by disrupting the *DNMT1* and *DNMT3B* genes (Rhee et al., 2002). Exon 1 of *p16*^{INK4a} was targeted for deletion, leaving the *p14*^{ARF} gene, which shares exon 2 with *p16*^{INK4a}, unaltered. *p14*^{ARF} also contains one mutant and one wild-type allele, which were both expressed in the parental HCT116 cells as well as all derivatives employed in this study (data not shown). The *p16*^{INK4a} disruption strategy and documentation of targeting are shown in Figures 3A and 3B, respectively. Two *t*riple *k*nockout (TKO) clones with the wt *p16*^{INK4a} allele plus both *DNMT1* alleles and both *DNMT3B* alleles disrupted (called "TKO$^{mut/-}$" clones) were generated and compared to a clone generated analogously except that the mutant *p16*^{INK4a} allele was disrupted ("TKO$^{wt/-}$"). As expected, the TKO$^{wt/-}$ clones re-expressed the wt *p16*^{INK4a} allele following the loss of DNA methylation (data not shown). Genomic DNA methylation was erased in all clones, regardless of which *p16*^{INK4a} allele was targeted (Figure 3C). However, there was a distinct difference in growth rate of the TKO$^{wt/-}$ cells compared to the TKO$^{mut/-}$ cells (Figure

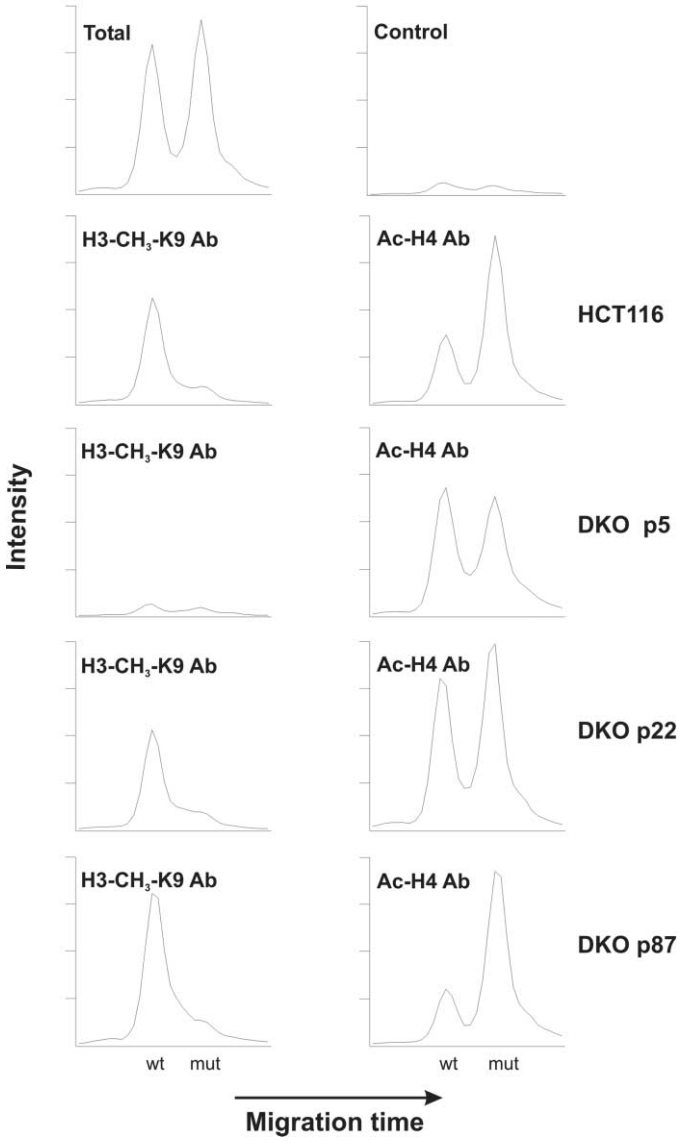

Figure 2. Chromatin immunoprecipitation of the *p16*^{INK4A} gene

HCT116 and DKO cells at passages 5, 22, and 87 were subjected to ChIP analysis. Fluorescently labeled PCR products from the immunoprecipitates were separated by capillary electrophoresis to discriminate the wild-type from the mutant allele. The antibodies used for ChIP were made against dimethyl-Histone H3-K9 (H3-CH_3-K9 Ab) and acetyl-Histone H4 (Ac-H4 Ab). The "Control" sample represents equivalent PCR products generated from immunoprecipitates in which no antibody was included.

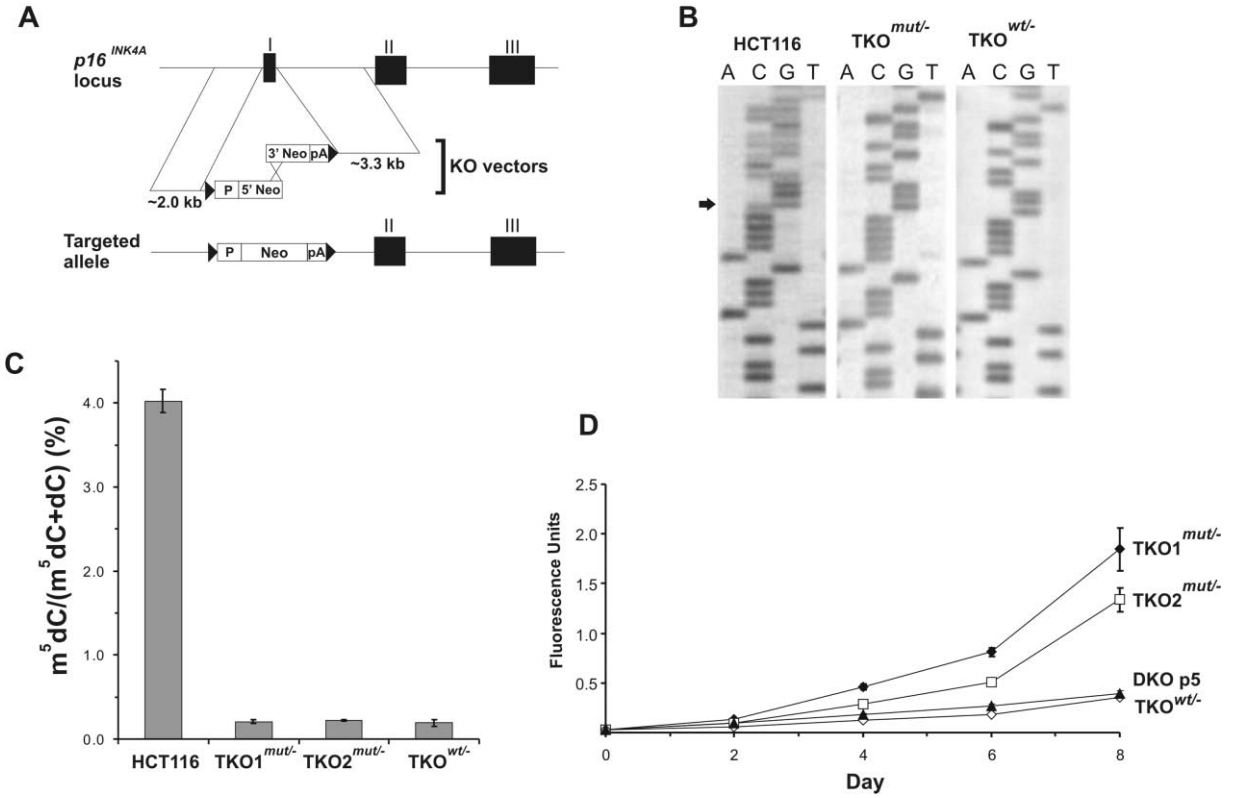

Figure 3. Targeted disruption of the *p16*^{INK4A} gene increases the growth rate of HCT116 cells in the absence of genomic DNA methylation

A: Schematic representation of the targeting constructs and the *p16*^{INK4A} locus before and after gene disruption. P, SV40 promoter; pA, polyadenylation site; black triangles correspond to loxP sites; and exons are indicated by Roman numerals.

B: Genomic sequence analysis of the *p16*^{INK4A} targeted region, demonstrating deletion of the wild-type and mutant alleles in TKO$^{mut/-}$ and TKO $^{wt/-}$ clones, respectively.

C: HPLC analysis of genomic DNA methylation in HCT116 cells and TKO cells.

D: Growth curves of DKO passage 5 cells and TKO passage 3 cells analyzed as described in Figure 1A.

3D). The TKO$^{wt/-}$ cells grew at a very slow rate, similar to that of early passages of DKO cells (Figure 3D). In contrast, The TKO$^{mut/-}$ clones grew much faster, approximately as fast as passage 22 of the DKO cells wherein the wt *p16*^{INK4a} allele was re-silenced (compare Figure 1A with Figure 3D).

These results have several implications. First, they prove that the growth rate of cancer cells can depend on silencing of a specific tumor suppressor gene due to epigenetic modifications. This conclusion was suggested previously, but the relative importance of *p16*^{INK4a} could not be determined or distinguished from the silencing of other growth-controlling genes. By disrupting a specific allele of *p16*^{INK4a}, we could show that *p16*^{INK4a} was responsible for approximately half of the growth rate decrease achieved by erasure of all DNA methylation. The other half was presumably due to changes in expression of other growth-controlling genes that are dependent on epigenetic silencing.

Second, and most importantly, our data show not only that DNA methylation is required to maintain silencing of the wt *p16*^{INK4a} gene, but also that erasure of the methylation signature of DNA leads to histone modifications. Moreover, there appeared strong selective pressure for re-silencing of the wt *p16*^{INK4a} allele in these cells. This re-silencing was independent of DNA methylation and was associated with histone H3-K9 methylation, but not with changes in histone H4 acetylation. Only after the H3-K9 methylation associated with gene silencing was replaced did other epigenetic changes come about. These results suggest that DNA methylation and H4 deacetylation serve to lock chromatin in a specific repressed state that was originally initiated by the methylation of histone H3-K9, as depicted in the model presented in Figure 4.

Certain caveats to this model should be pointed out. The fact that DNA methylation is required both to maintain gene silencing and to maintain the methylation signature of silenced chromatin should be applicable to many naturally occurring cancers, as the conclusions rest on removing DNA methyl groups through genetic means from tumor cells that have intact methyltransferase genes. Indeed it has been shown that silencing and DNA methylation of *p16*^{INK4a} are very common occurrences in cancer cells of diverse types (Jones and Baylin, 2002). Re-silencing of *p16*^{INK4a}, however, occurred in the near absence of methyltransferase activity, an experimentally contrived situation. Though this experimental approach was required to investigate the timing of the various events studied, it is certainly not physiological. Our experiments demonstrate that histone H3-K9 methylation *can* occur in association with gene silencing and long before DNA methylation. This formally demonstrates that the enzymatic mechanisms and recognition elements re-

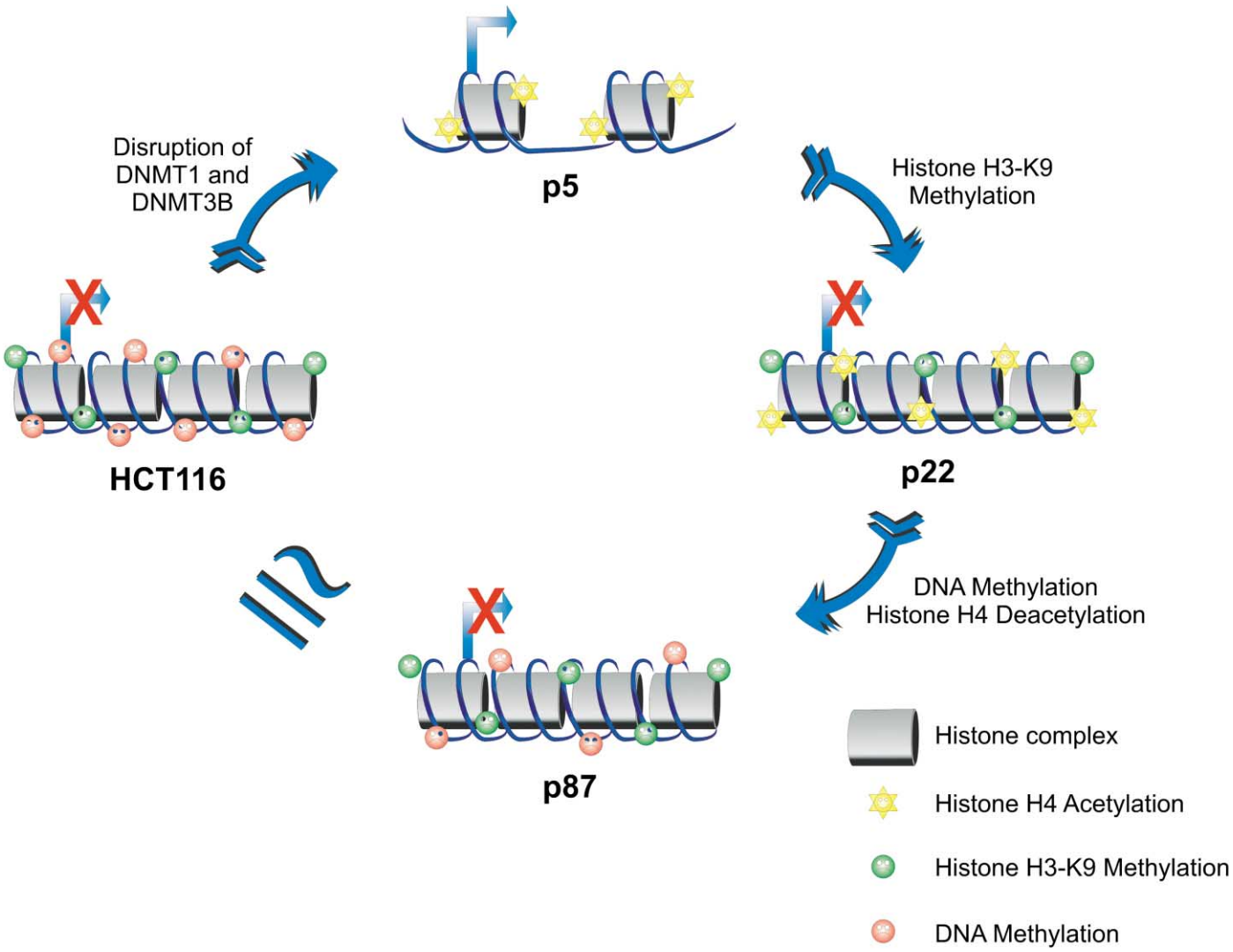

Figure 4. Epigenetic control of the wild-type *p16*^{INK4A} gene

In parental cells, the wt *p16*^{INK4a} allele is silenced, the DNA is methylated, histone H4 is deacetylated, and histone H3-K9 in surrounding chromatin is methylated. When DNA methylation is erased by disruption of the *DNMT1* and *DNMT3B* genes, the histone code is altered and the wt allele of *p16*^{INK4A} is no longer silenced. With passaging, methylation of histone H3-K9 occurs in conjunction with re-silencing of the wt *p16*^{INK4A} allele. Many passages later, histone H4 deacetylation and DNA methylation take place, rendering the chromatin similar to that of parental HCT116 cells. Thus maintenance of the histone code as well as gene silencing are dependent on DNA methylation, but the process of silencing can be initiated in conjunction with histone H3-K9 methylation in the absence of changes in DNA methylation or histone H4 acetylation.

quired for chromatin modifications are not dependent on prior DNA methylation. But the timing of these events may be different in genetically unmanipulated cells. Further research based on the results presented here may allow evaluation of this possibility in the future.

Our data support the primary importance of histone H3 methylation in gene silencing in general (Bird, 2001; Gendrel et al., 2002; Goll and Bestor, 2002; Jenuwein and Allis, 2001; Lachner and Jenuwein, 2002; Li, 2002; Nakayama et al., 2001; Noma et al., 2001; Volpe et al., 2002). Moreover, the epigenetic events associated with silencing of the *p16*^{INK4a} suppressor gene observed in our study have remarkable similarities to those associated with silencing of the inactive X chromosome (Heard et al., 2001; Keohane et al., 1996; Mermoud et al., 2002). As there is no evident Xist gene for autosomes as there is for X chromosomes, one wonders how the cell distinguishes the wt from the mutant *p16*^{INK4a} allele. One hypothesis to explain this result invokes a molecular mark on the wt allele that is not present on the mutant allele (or vice versa). This putative mark could be a DNA bound protein, a histone modification, or a DNA modification not detected in our assays. Arguing against this hypothesis is the fact that re-silencing took many generations; at least 30 cell-doublings occurred between the generation of each DKO clone and passage 5. One would have expected that a mark would have led to more rapid establishment of silencing, perhaps even within one generation. Our working hypothesis is therefore that the silencing is simply stochastic, and that it occurs randomly, much like mutations but at higher frequency.

In the case of the system analyzed here, the remarkable growth advantage resulting from the absence of functional *p16^{INK4a}* expression would allow cells with a silenced wt *p16^{INK4a}* allele to rapidly overtake the population. The epigenetic changes that accompany this silencing then would provide a heritable signal for its retention in the cell's progeny. It will be of interest to determine whether this hypothesis, as well as other features of the model depicted in Figure 4, applies to the many other genes that are epigenetically silenced during neoplastic development.

Experimental procedures

Cell culture, transfection, and screening for recombinants

HCT116 cells (American Type Culture Collection, Manassas, Virginia) were cultured in McCoy's 5A modified media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. *DNMT1* and *DNMT3B* targeting was performed as previously described (Rhee et al., 2002). To target the *p16^{INK4A}* locus, HCT116 cells were transfected with the linearized *p16^{INK4A}* targeting vectors described in Figure 3 using LipofectAmine (Invitrogen, Carlsbad, California) and selected in growth medium supplemented with 0.4 mg/ml geneticin (Invitrogen). General aspects of targeting with bipartite Neo vectors are described in Jallepalli et al. (2001). Homologous recombinants identified by PCR were confirmed by genomic sequencing using one primer from the Neo gene and another from the *p16^{INK4A}* locus outside the targeting arms. Each TKO clone had a total of five targeted alleles; the targeting order was *DNMT3B*, allele 1 → *DNMT3B*, allele 2 → *DNMT1*, allele 1 → *p16^{INK4A}* (wt or mutant allele) → *DNMT1*, allele 2. DKO and TKO clones were passaged by splitting 1:10 when the cells reached 90% confluency.

In vitro growth assays

Cells were seeded in triplicate in 12-well plates at a concentration of 3×10^4 cells/well. Cells were harvested at days 2, 4, 6, and 8 for analysis, lysed in 0.6% Igepal CA-630 (Sigma, St. Louis, Missouri) and 0.4% PicoGreen (Molecular Probes, Eugene, Oregon), and incubated at room temperature for 20 min. Fluorescence was measured using a Fluostar Galaxy (BMG, Durham, North Carolina) plate reader with excitation at 485 nm and emission at 530 nm.

Real time methylation-specific PCR

Approximately 1 μg of genomic DNA was treated with sodium bisulphite for analysis using MSP primers that recognize unmethylated or methylated *p16^{INK4a}* alleles as previously described (Herman et al., 1996). A 1:50,000 dilution of the intercalating dye SYBR Green I (Molecular Probes) was added to the reactions, and amplification products were quantified in real time using an iCycler (Bio-Rad, Hercules, California). The number of amplification cycles required to reach a statistical threshold using the primers specific for unmethylated alleles was subtracted from the corresponding number derived for the primers specific for methylated alleles. This cycle number difference (d) was converted to the percentage of *p16^{INK4a}* alleles that are methylated using the formula % methylation $= 2^d/(2^d + 1)$.

Genomic bisulfite sequencing

Approximately 1 μg of genomic DNA was treated with sodium bisulphite for manual sequencing analysis, using primers previously described (Herman et al., 1996). Cycle sequencing was performed with ^{33}P-labeled ddNTPs and Thermosequenase (Amersham, Piscataway, New Jersey) and incorporated 30 cycles of 94°C for 30 s, 58°C for 30 s, and 70°C for 1 min.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitations were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate, Lake Placid, New York) with modifications based on advice generously provided by B. Dynlacht. Approximately 1×10^7 cells were crosslinked using 1% formaldehyde in growth medium at 37°C for 10 min. Crosslinking was quenched by glycine at a final concentration of 0.125 M and incubated at room temperature for 5 min. Cells were rinsed with 1× PBS and trypsinized at 37°C for 5 min. Trypsinization was stopped by the addition of ice-cold 1× PBS containing 10% FBS and protease inhibitors. Cells were collected by scraping and centrifuged at 700 g at 4°C for 10 min. Pelleted cells were resuspended in 200 μl SDS Lysis Buffer (Upstate) and incubated on ice for 10 min. Chromatin was then sonicated to an average length of 1 kb using a Branson Sonifier 250 (Branson Ultrasonics, Danbury, Connecticut) with microtip at 40% duty cycle. Sonicated chromatin was the subjected to immunoprecipitation using a protocol provided by Upstate. Antibodies used for ChIP were anti-dimethyl-Histone H3 (Lys9) and anti-acetyl-Histone H4 (Upstate).

Allele-specific ChIP analysis

To discriminate between the wild-type and mutant *p16^{INK4A}* alleles, a PCR based assay was developed. The wild-type *p16^{INK4A}* allele contains four guanidine nucleotides in exon 1 while the mutant allele has an insertion of one additional guanidine residue at this sequence. PCR primers flanking this region were designed to yield an approximately 220 base pair product (forward primer 5′-TGGCTGGTCACCAGAGGGTG-3′ and reverse primer 5′-GACCGTAACTATTCGGTGCG-3′). The forward primer was labeled with fluoroscein at its 5′ end by Gene Link (Hawthorne, New York). Cycling conditions using Platinum Taq (Invitrogen) were: 94°C for 2 min followed by 30 cycles of 94°C for 10 s, 58°C for 15 s, and 68°C for 30 s. The PCR products were separated by capillary electrophoresis on a SpectruMedix 9610 instrument fitted with 192 capillaries (State College, Pennsylvania). Two peaks, representing the wt and mutant alleles, were observed in this analysis when it was performed on control HCT116 samples. The results obtained upon ChIP were confirmed by manual sequencing using ^{33}P-labeled ddNTPs and Thermosequenase (Amersham, Piscataway, New Jersey).

High performance liquid chromatography

Approximately 40 μg of RNA-free genomic DNA, prepared using the Blood and Cell Culture DNA Midi Kit (Qiagen, Valencia, California), was quantitatively digested with nuclease P1 (Roche Molecular Biochemicals, Indianapolis, Indiana) and calf intestinal alkaline phosphatase (Sigma) as described (Kuo et al., 1980). Samples were separated on a reversed-phase column (Supelcosil LC-18 DB, Sigma) at room temperature, monitoring absorbances at 275 nm and 285 nm (Feinberg et al., 1988). Peak assignments were confirmed using deoxyribonucleoside standards (Sigma). 5-Methylcytosine content was expressed as a percentage of the total cytosine pool, using peak areas after correction for extinction coefficients.

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