CHANGES IN DNA METHYLATION AND HISTONE MODIFICATION DURING EPIGENETIC TRANSITIONS

By

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Abstract

Aberrant epigenetic silencing is a prevalent mechanism of tumor suppressor gene inactivation that promotes cancer initiation and progression. Although repressive epigenetic modifications have been identified that are specific to tumor suppressor genes after aberrant silencing occurs, the factors that initiate these changes are not clearly understood. The aim of this thesis research was to examine epigenetic changes during the dynamic transition from active to inactive transcription states during epigenetic silencing; this strategy is in contrast to measuring epigenetic modifications after transcriptional silencing has stabilized. To achieve this goal I designed a system to directly test the hypothesis that transient reductions in gene activation are an initiating factor of aberrant epigenetic silencing. Aberrant silencing of an experimental transgene was induced by reversibly inhibiting transcriptional activation, and the frequency of silencing increased with longer durations of reduced transcriptional activation. Thus, these observations confirmed the hypothesis, and epigenetic modification associated with experimentally induced silencing were the same as those measured at silenced tumor suppressor genes in cancer cells. These data demonstrated that results from the experimentally induced silencing are relevant to epigenetic processes that contribute to tumorigenesis. In the experimental system, the initiation of silencing was dependent on the activity of class I/II histone deacetylases, but not DNA methylation. This demonstrated the value of this system of induced silencing as a way to examine specific molecular requirements for initiation and stabilization of aberrant epigenetic silencing.

Aberrant silencing of endogenous *Aprt* in mouse cells was also examined to identify characteristics of distinct silencing pathways and requirements for reactivation.

In D7 cells, *Aprt* silencing was correlated with a bivalent histone modification pattern, enrichment of methylation at H3K4 and H3K9, which did not require DNA methylation of the *Aprt* promoter. In contrast, Aprt silencing in D3 and D3S1 cells correlated with hypermethylation of the promoter region DNA, loss of methyl-H3K4, and increased methyl-H3K9. Comparison between D3 and D3S1 cells demonstrated that increased DNA methylation of the promoter stabilized transcriptional silencing and required demethylation of promoter DNA for reactivation. However, the silenced *Aprt* promoter in D3 cells that was only partially methylated, did not require a loss of DNA methylation for reactivation. Maintaining reactivated expression by selection did promote eventual removal of DNA methylation from *Aprt*, but re-silencing of *Aprt* still occurred at a high frequency. Therefore, some epigenetic modification other than DNA methylation is responsible for the instability of reactivated expression of silenced alleles.

In summary, these results identify transient reduction of gene activation as an early initiator of aberrant epigenetic silencing. This has allowed us to distinguish between early and late events during the silencing process and assign specific functions to individual repressive modifications. Additionally, these results demonstrate the existence of a stably inherited epigenetic state that does not require DNA methylation and prevents stable reactivation of previously silenced alleles. Finally, the experimental system designed for these experiments provides the means to examine steps in the silencing process upstream or independent of DNA methylation. This is a critical experimental tool for the study of both normal and aberrant epigenetic processes.

Chapter 1

INTRODUCTION

Epigenetics was a term initially used by Conrad Waddington during the mid 20th century to describe the heritability of a particular phenotype through cell division [1]. This classical definition has been updated to refer specifically to heritable changes in gene expression that occur without accompanying changes in the DNA sequence [2]. Epigenetic regulation of gene expression is mediated through chromatin that contains the genomic DNA wound together with histone proteins, condensed, and organized into higher-ordered structures. Higher-ordered structuring of genomic DNA is able to affect expression by controlling accessibility of genomic regions to proteins required for active expression or repression. In addition to the general association between DNA strands and histone octamers, the higher-order structure is affected by covalent chemical modifications made to DNA and the associated histone proteins. Differential physical conformations segregate genomic DNA into two distinct forms, heterochromatin and euchromatin, correlated with inactive and active transcriptional states, respectively. The measured differences that are seen between identical twins despite having identical genomes are sometimes cited as an example of epigenetic contributions to phenotype [3]. Although differences illustrated in this example are easily seen, they are also relatively subtle and do not reflect the magnitude or prevalence of epigenetic regulation in eukaryotic biology.

The vast array of individual cell types that compose higher eukaryotes is an example that better illustrates the magnitude of epigenetic regulation, albeit on a microscopic scale and could fittingly be termed “epigenetic diversity”. The fact that DNA sequences of the human and chimpanzee genomes are approxiamtely 98% identical [4], but yet that small amount of genetic diversity between humans and chimpanzees accounts for profound phenotypic differences, is surprising to some people. However, there are far more phenotypic similarities between humans and chimpanzees than there are when comparing fibroblasts to neurons, myoblasts, or pancreatic

β-cells, yet each of these unique cell types arises from one identical genomic sequence. Epigenetic mechanisms of gene regulation are required for generating these specialized cell types and ensuring heritability of the proper cell phenotype in daughter cells during proliferation. Therefore, "epigenetic diversity" would define the spectrum of unique cellular phenotypes that are generated from a single eukaryotic genome. This concept is presented here to emphasize that epigenetic regulation is not a rare mechanism with function restricted to a few specialized processes (*i.e.* X chromosome inactivation in females, genomic imprinting, and formation of cetromeric heterochromatin). Instead, epigenetic regulation is an absolute requirement for eukaryotic development and these mechanisms are critical for cell identity and function.

An ambitious effort has been recently initiated to experimentally define the human epigenome, *i.e.* measure epigenetic modifications that establish specific phenotype and function in each of the individual cell types within a human [5]. This information will provide a better understanding of how epigenetic mechanisms establish cell identity and function. The higher-order organization of genomic DNA serves multiple functions including dosage control, genome defense, structural elements, and establishment of cell type-specific expression patterns. Although epigenetic regulation affects gene expression without changing the sequence of the DNA, covalent modification of the DNA is a key mediator of epigenetic regulation.

Methylation of cytosine residues in a CpG dinucleotide context is a primary mediator of epigenetic regulation in eukaryotic genomes. The functional consequence of DNA methylation is dependent on genomic location and density. The genomes of humans and higher eukaryotes have a bimodal distribution of CpG sites (Fig. 1-1) [6]. The CpG dinucleotide is underrepresented in the genome as a consequence of the increased mutagenicity of methyl-cytosine. Therefore, many of the cytosine bases within the CpG context have been lost by spontaneous deamination and subsequent transitional mutation to thymines. The exception to this general rule is CpG islands that are frequently associated with gene promoters.

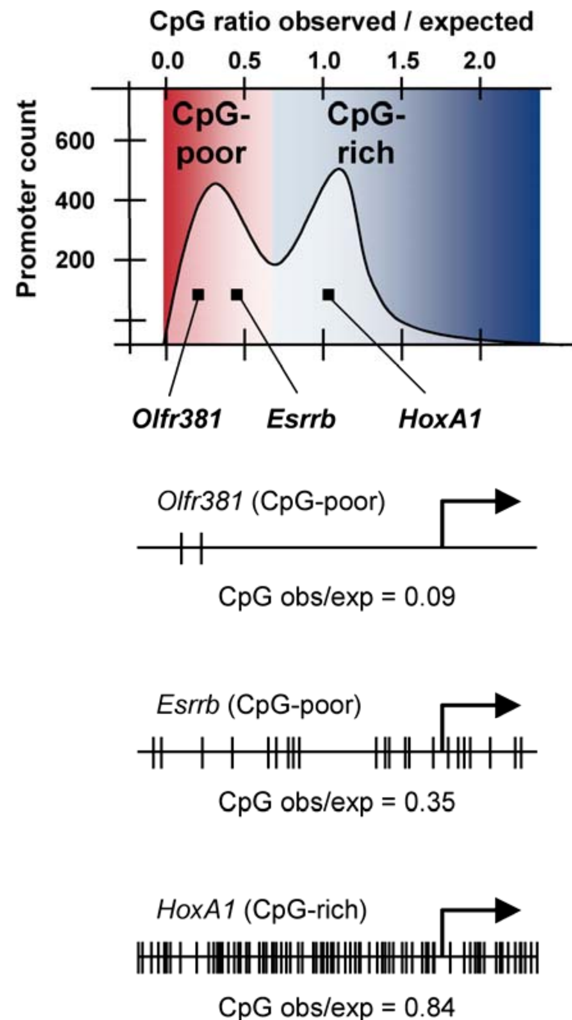

Figure 1-1. Bimodal CpG distribution in vertebrae genomes reflects two distinct classes of gene promoters, CpG-poor and CpG-rich. Approximation of CpG distribution in promoters of the *Mus musculus* genome. Representative examples of CpG distribution in gene promoters across a 1.3-kb window spanning the transcriptional start site are shown below the histogram. Two CpG-poor promoters are shown, *olfactory receptor 381* (*Olfr381*) and *estrogen-related receptor β* (*Esrrb*). One CpG-rich promoter is shown *Homeobox A1* (*HoxA1*). [Figure adapted from (F. Mohn et al. 2009)]

Gene / Function	Mutant phenotype
DNA methylation	
Dnmt1$^{-/-}$	Global loss of DNA methylation Embryonic lethality by day E8.5
Dnmt3a$^{-/-}$	Gut malformation and spermatogenesis defects Early postnatal lethality, ~1 month
Dnmt3b$^{-/-}$	Loss of DNA methylation at minor satellite repeats Embryonic lethality during E14.5-E18.5
Dnmt3a$^{-/-}$, 3b$^{-/-}$	Fail to initiate *de novo* DNA methylation Embryonic lethality at day E8.5
Lsh$^{-/-}$	Global loss of DNA methylation at day E13.5 Postnatal lethality
Mbd3$^{-/-}$	Early developmental arrest and lethality by day E6.5
Mecp2$^{-/-}$	Viable, but suffer from neurological defects
Histone modification	
G9a$^{-/-}$	Loss of H3K9 methylation in euchromatic regions Developmental arrest and lethality at day E8.5
Hdac1$^{-/-}$	Growth defects and embryonic lethality near day E9.5
Suv39h1$^{-/-}$, 2$^{-/-}$	Loss of H3K9 methylation in heterochromatic regions, genomic instability, and decreased embryonic viability
Ezh2$^{-/-}$	Growth defect with early embryonic lethality before implantation

Table 1-1. Phenotypes observed in mice deficient for key epigenetic regulators.

Embryonic DNA methylation patterns are established early during development beginning in the blastocyst through the cooperative functions of the maintenance DNA methyltransferase DNMT1 and *de novo* DNA methyltransferases DNMT3a and DNMT3b [7, 8]. Although DNMT1 is termed the "maintenance" methyltransferase, genetic knockout of DNMT1 causes embryonic lethality near day E9.5 with hypomethylation of genomic DNA and indicates DNMT1 is also required for establishment of proper DNA methylation patterns (Table 1-1) [9-11]. DNMT1 is considered the maintenance methyltransferase because it is ubiquitously expressed beyond development and is responsible for copying existing DNA methylation patterns onto newly synthesized daughter strands. This activity is in part due to a substrate preference for hemimethylated DNA templates [12, 13]. Additionally, protein interactions between DNMT1 and PCNA link replication and maintenance methylation by recruiting

DNMT1 to active transcription forks (LS Chuang 1997). The SNF2/helicase protein Lsh is an additional protein needed for maintenance of DNA methylation that reflects the requirement to increase DNA accessibility for efficient DNMT1 function [14, 15].

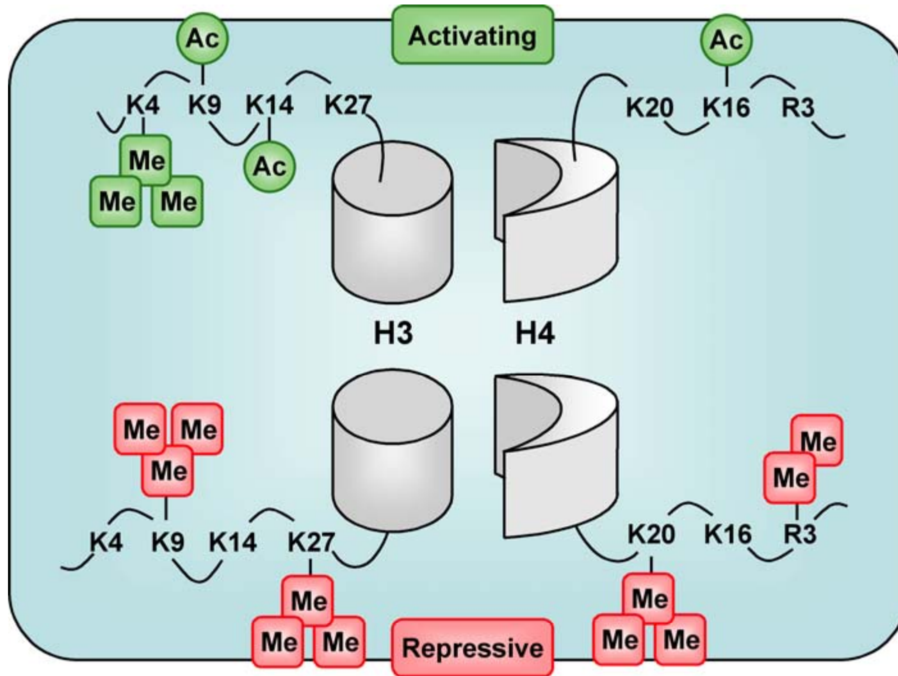

Figure 1-2. Several different covalent modifications are made to amino-terminal tails of the histone proteins.
A representative subset of potential histone modifications that affect transcriptional regulation are shown. Histone modifications of histone H3 and H4 generally associated with active chromatin are colored in green, and modifications generally associated with transcriptionally inactive chromatin are marked in red. Methylation is indicated by boxes marked Me, and acetylation is indicated by circles marked Ac. Up to three methyl groups can be attached to a single lysine residue (K), and two methyl groups can be attached to a single arginine residue (R).

DNA methylation is also tightly linked to repressive histone modifications. Probably the clearest and most direct relationship between repressive histone modifications and DNA methylation exists in the filamentous fungi *Neurospora crassa*. In these fungi, mutations in *dim-5* (*defective in methylation-5*) result in a complete loss of genomic DNA methylation, but *dim-5* encodes a homolog of the histone methyltransferase G9a that catalyzes methylation of H3K9 [16]. Thus, repressive histone methylation enables and directs methylation of the genomic DNA. A similar relationship exists in *Arabidopsis thaliana*, although genomic DNA methylation is only partially lost upon inactivation of the H3K9 methyltransferase *kryptonite* [17]. In Arabidopsis, methylation at H3K9 forms a binding substrate for the chromodomain of heterochromatin protein 1 (HP1), which interacts with the DNA methyltransferase to direct DNA methylation [17]. Mammalian DNA methyltransferases also interact with histone modifying enzymes (Table 1-2), including deacetylases (HDAC1 & HDAC2) and methyltransferases (G9a, SUV39H1, & EZH2), but it is somewhat unclear if DNA methylation promotes these repressive histone modifications or vice versa [18-22]. Surprisingly, efficient DNA methylation of reterotransposons, major satellite repeats, and CpG-rich promoters in human ES cells requires expression of G9a protein, but its methyltransferase activity and entire catalytic SET domain are unnecessary for normal DNA methylation [23]. The H3K9 methyltransferases SUV39H1 & SUV39H2 are required for DNA methylation at major satellite repeats in pericentric regions, but genetic inactivation does not affect DNA methylation in other genomic regions [24]. Methylation of histone H4R3 by the protein arginine methyltransferase PRMT5 creates a direct binding site for DNMT3A and establishes DNA methylation during silencing of the embryonically expressed γ-globin gene [25]. Although these proteins (HATs, HDACs, HMTs, and histone demethylases) have been termed histone modifying enzymes, modification of non-

histone proteins is a relatively common observation. The histone demethylase LSD1 influences DNA methylation through this mechanism by directly controlling lysine methylation of the DNMT1 enzyme, which affect its protein stability [26].

Enzyme	Residues Modified	Enzyme	Residues Modified
Acetyltransferase		***Lysine Methyltransferases***	
CBP/P300	H3 (K14, K18), H4K5	SUV39H1	H3K9
PCAF/GCN5	H3 (K9, K14, K18)	G9a	H3K9
TIP60	H3K14, H4 (K5, K16)	ESET/SETDB1	H3K9
Deacetylases		EuHMTase/GLP	H3K9
SirT2	H4K16	MLL1	H3K4
HDAC1	Low substrate specificity	SET1A, B	H3K4
Lysine Demethylases		SUV420H1	H4K20
LSD1	H3K4	EZH2	H3K27
JHDM1a	H3K36	RIZ1	H3K9
JHDM2a	H3K9	***Arginine Methyltransferases***	
JMJD2A	H3K9, H3K36	PRMT4	H4R3
GASC1	H3K9, H3K36	PRMT5	H3R8, H4R3

Table 1-2. Partial summary of histone modifications and modifying enzymes.

Establishment of DNA methylation patterns may also be influenced by methylation of histone residues that provides resistance against de novo DNA methylation. Unlike repressive methylation at H3K9, methylation at H3K4 is generally associated with transcriptional activation and catalyzed by MLL histone methyltransferases that are recruited to gene promoters via interaction with RNA polymerase II [27]. Global analysis of DNA methylation and methyl-H3K4 shows that non-overlapping distributions for these epigenetic modifications [28]. Additionally, during stem cell differentiation loss of H3K4 methylation from specific regions coincides with de novo DNA methylation [29, 30]. Specific histone variants are also preferentially associated with regions of the genome lacking DNA methylation. The histone variant H3.3 is enriched in activating histone modifications, including methylation at K4 and

localized in actively transcribed regions of the genome [31]. H2AZ is a histone variant that has mutually exclusive distribution from methylated DNA in Arabidopsis and enriched at transcriptional start sites in the human genome [32, 33]. DNA methylation represses transcription by specific recruitment of proteins that contain a conserved methyl-cytosine binding domain (MBD). The methyl-CpG binding protein MeCP2 is present in a complex with HDACs and the Sin3a co-repressor [34, 35]. MBD2 is a component of a multi-protein NuRD complex previously designated MeCP1 that represses transcription via ATP-dependent chromatin remodeling and HDAC activity [36, 37].

Cancer epigenetics

Cancer is a disease that is driven by selective forces. In other words, the acquisition of changes that increase a cell's capacity for proliferation and survival promotes tumor progression, and essentially, there are two ways for cells to increase capacity in these areas; increased expression of oncogenes or inactivation of tumor suppressor genes. Due to the opportunistic nature of cancer cells, any existing mechanism with capacity to either increase oncogene activity or reduce tumor suppressor function will be exploited at some frequency in cancer. Epigenetic transcriptional regulation is no exception, and epigenetic alterations are frequently observed in human tumor and cancer cell lines.

DNA hypomethylation in cancer

Reductions in genomic DNA methylation were some of the earliest epigenetic alterations observed in human cancers [38, 39]. DNA methylation levels were significantly reduced in a number of different tumor types, as well as pre-malignant and benign tumors, which suggested

DNA hypomethylation could be a common early event in tumor development [40, 41]. The observed consequences of DNA hypomethylation include reactivated expression of silenced genes, the oncogene *HRAS* [42], repetitive elements (L1 and Alu) [43], loss of imprinting [44], and miRNA upregulation [45, 46]. Re-expression of genes specific for testis or early development demonstrates that one consequence of cancer-related genomic hypomethylation is reactivation of silenced genes [47]. Additionally, *PAX2* reactivation associated with genomic DNA hypomethylation promotes proliferation of endometrial carcinoma cells and demonstrates this cancer-related mechanism can reactivate genes with significant oncogenic effects [48]. Loss of silencing at the imprinted gene *IGF2*, *insulin-like growth factor-2*, is associated with an increased risk of cancer [44, 49, 50]. Increased genomic instability is another potential consequence of genomic hypomethylation that promotes tumorigenesis. Loss of DNA methylation that occurs in DNMT-deficient cells or cancer cells coincides with increased aneuploidy and large-scale genomic mutations (deletions, translocations, etc.) [51, 52].

Aberrant epigenetic silencing in cancer

Although it initially seems counterintuitive, the same human tumors that display hypomethylation may also have tumor suppressor genes inactivated by DNA hypermethylation. The key to resolving this contradiction is the genomic context where the changes in methylation occur. The alterations in DNA methylation that correlate with cancer could be summarized succinctly and fairly accurately that normal patterns are reversed. That is to say the regions of the genome that are normally methylated lose their DNA methylation and become structurally unstable or reactivated, and the genomic regions that are normally unmethylated (CpG islands & promoter regions) are prone to methylation and silencing.

The first tumor suppressor gene in which promoter DNA hypermethylation was observed to correlate with cancer was the retinoblastoma gene *Rb* [53-55], which incidentally was also the first tumor suppressor gene to be identified [56]. Once promoter DNA hypermethylation was identified as a mechanism for gene inactivation, several other significant tumor suppressor genes were also found to be silenced in human cancers, including *p16*^{INK4a}, *hMLH1*, *VHL*, *BRCA1*, and *E-cadherin* [57-62]. Just among this small number of tumor suppressor genes a diverse group of cellular processes are represented; DNA repair, cell cycle control, and cell adhesion. Thus demonstrating that genes prone to aberrant silencing are not limited to one particular cellular function. In addition to the numerous genetic targets of aberrant silencing associated with DNA hypermethylation, it is also a mechanism that occurs across the whole spectrum of human cancers, from leukemias to solid tumors [63]. The specific mechanisms that initiate aberrant silencing and DNA hypermethylation are not clear at this point, but observations in cancer suggest aberrant silencing can result from either normal pathways of epigenetic regulation acquiring an aberrant gene target or dysregulation of the epigenetic enzymes that results in a global increase of genes targeted by aberrant silencing [64, 65].

Reactivation of aberrantly silenced tumor suppressor genes

The potential reversibility of epigenetic silencing and the prevelance of aberrant silencing events in cancer make development of epigenetic cancer therapies an area of great interest. DNA methyltransferase inhibitors are used as effective treatments of certain leukemias, although it is not clear if the efficacy is due to gene reactivation [66]. HDAC inhibitors have also shown positive effects in clinical trials [67]. Reactivation of silenced tumor suppressor genes is commonly observed after treating tumor cells with inhibitors of DNA methylation, but the gene

expression re-silences unless inhibition of DNA methylation is maintained [68]. The levels of reactivated mRNA expression can be increased further if inhibition of DNA methylation and HDACs are combined, but re-silencing still occurs. This memory of transcriptional silencing that causes re-silencing may be due to other repressive modifications. Inhibiting DNA methylation induces *hMLH1* promoter demethylation, reexpression, and reversal of histone modifications (H3 acetylation, methyl-H3K4, and dimethyl-H3K9), but these effects cannot be induced by HDAC inhibition alone [69]. However, some repressive histone modifications (tri-methyl H3K9 and tri-methyl H3K27), were detected to remain or increase after loss of DNA methylation and reexpression of multiple genes including *hMLH1* in colon cancer cells [70]. Despite reversal of histone modifications and apparent reactivation, the $p16^{INK4a}$ promoter eventually re-silences with histone modifications (H3K9 methylation, then H4 deacetylation) preceding DNA methylation of the promoter [71]. Inhibition of class I/II HDACs is reported not to have measurable effects on reactivation of silenced alleles with promoter DNA methylation unless it is combined with DNA methylation inhibitors. Inhibition of the class III HDAC SIRT1 has been shown to reactivate expression of silenced promoters with DNA hypermethylation [72]. Reactivation of silenced alleles by inhibition of the class III HDAC SIRT1 does not require loss of DNA methylation from the promoter. After reactivating silenced genes by inhibiting DNA methylation, continuous treatment with HDAC inhibitors fails to block re-silencing of the *P16* gene [73]. The histone H3 variant H3.3 is specifically localized to actively expressed regions of the genome, enriched covalent modifications associated with activation, and may influence memory of the transcriptional state [74]. Studies that measured ability of a promoter to remain transcriptionally competent in the absence of active expression showed histone H3.3 and methylation at lysine 4 were required to retain transcriptional competence [75]. Physical

localization within the nucleus may also influence epigenetic regulation in yeast [76, 77].

Initiation of aberrant silencing

Much of the research in cancer epigenetics is descriptive in nature because the general aim is to identify the significant targets of aberrant silencing. Those results cataloging the numerous examples of specific genes silenced in different tumors have been critical in establishing aberrant silencing as an important mechanism in tumorigenesis, but they only provide limited details about how silencing occurred. Identification of steps that occur during the dynamic process of aberrant silencing would provide a better understanding of what initiates the process and how it may be prevented. Experimental models have shown silencing can be induced by DNA methylation. When DNA methylation is directly established by site directed integration of *in vitro* methylated constructs, transcription is silenced in conjunction with chromatin changes characterized by histone deacetylation and loss of DNase I-hypersensitivity [78]. Human mammary epithelial cells with increasing passage silence the tumor suppressor gene *RASSF1A*. DNA methylation and stable silencing is preceded by loss of transcription, which coincided with decreased Sp1 binding, histone deacetylation, and H3K9 methylation [79]. Based on the observation that TGF-β signaling pathway components (*TGF-β2*, *TGF-βR1*, and *TGF-βR2*) are epigenetically inactivated in breast cancer, epigenetic modifications were measured early during the selection phase of human mammary epithelial cells in culture and gene suppression was associated with methylation of H3K9 and H3K27, but not DNA methylation [80].

Decreased expression as an initiating factor

Loss of *estrogen receptor-α* in breast cancer cells disrupts activation of the downstream target gene *progesterone receptor* and leads to stable epigenetic silencing [81]. Also, inactivation of the *GATA6* transcription factor in ovarian cancer is associated with reduced expression and eventual silencing of the downstream target gene *disabled-2* [82]. While both of these examples involve loss of activating factors, increased gene repression may also initiate aberrant silencing. Genetic targets of polycomb repressor complexes are maintained at low levels of gene expression and appear to be highly prone to aberrant silencing and DNA hypermethylation in tumors [83-85]. Histone modifications established by polycomb repressor complexes may predispose genes to silencing associated with DNA hypermethylation [86]. The bivalent histone modification pattern frequently observed in stem cells is present at genes that are frequently silenced later in cancer with the additional modification of H3K9 methylation and DNA methylation [83]. Additionally, cases of B-cell chronic lymphocytic leukemia have been associated with an inherited mutation in the *death-associated protein kinase 1* (*DAPK1*) promoter. This mutation stabilizes binding of a transcriptional repressor and leads to epigenetic silencing [87]. Moreover, a sequence polymorphism in the promoter of the *recombination-induced LIM protein* (*RIL*) tumor suppressor gene destabilizes binding of Sp1/Sp3 transcription factors and increases susceptibility to silencing although there is no effect on initial gene expression levels [88]. Examination of mouse *Aprt* transgenes identified a similar Sp1 consensus site that is not required for expression but functions to protect against epigenetic silencing [89].

Environmental Factors

Evidence suggests that certain environmental factors may initiate aberrant silencing.

Helicobacter pylori infection of gastric mucosae is associated with increased risk of gastric cancer and infection has been shown to reduce expression of a subset of genes that are subsequently silenced in conjunction with DNA methylation in gastric tumors [90]. Nickel (Ni) exposure increases relative cancer risks although evaluation in culture based assays suggest Ni is not a mutagen [91]. The carcinogenic activity of Ni may be due to induction of epigenetic silencing via a pathway dependent on HDAC activity [92].

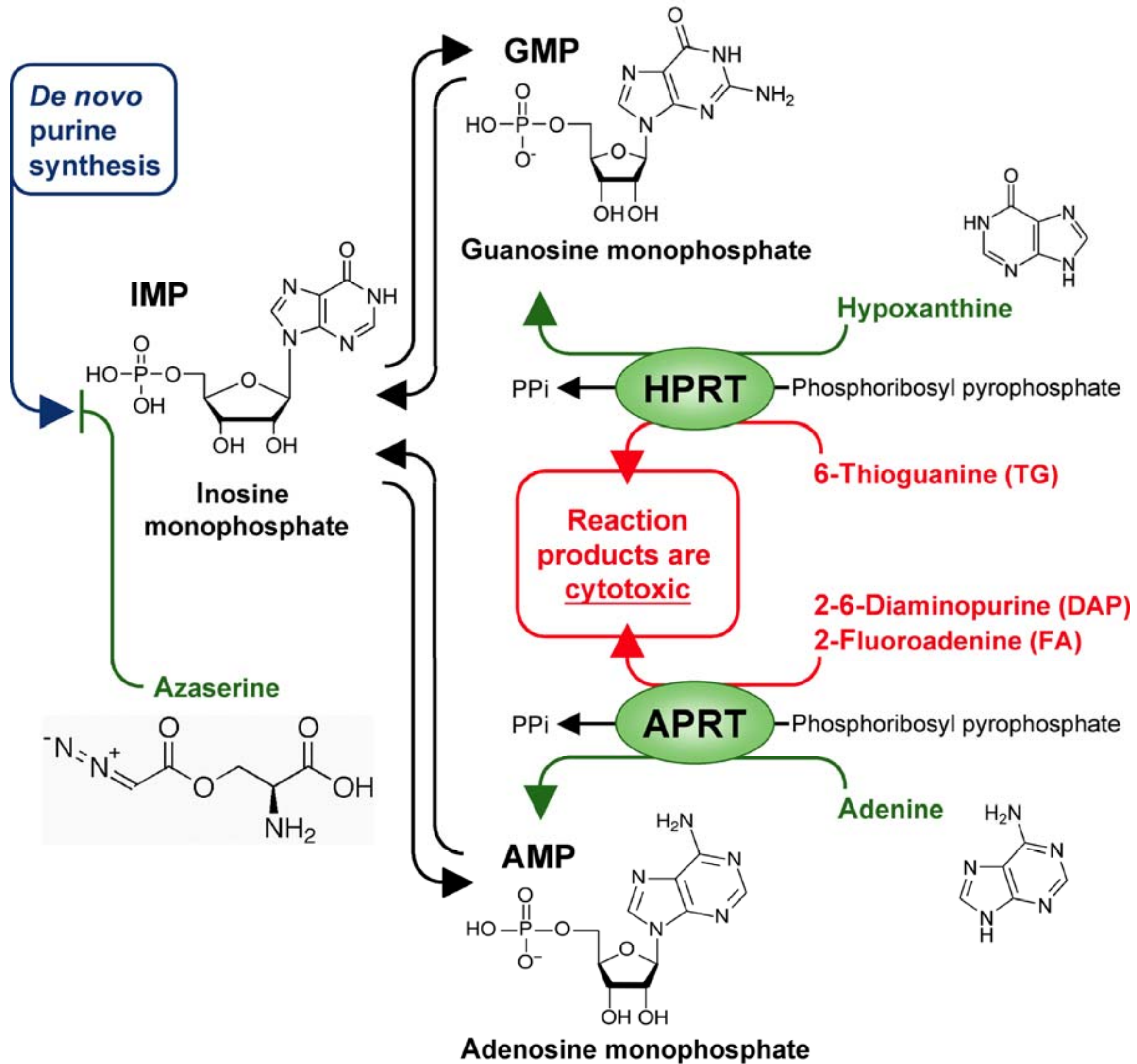

Figure 1-3. Outline for positive or negative selection in the purine salvage pathway. The general biochemical basis for using selection in the purine salvage pathway is detailed in the diagram. Purine nucleosides (IMP, AMP, & GMP) are required for cell survival and must be generated by either the *de novo* pathway or the salvage pathway. APRT and HPRT are both enzymes in the purine salvage pathway. Reagents needed to select for APRT or HPRT activity are colored in green, Nucleoside analogues, TG, DAP, and FA, are converted into toxic compounds by purine salvage enzymes, and thus, select against APRT or HPRT activity.

Chapter 2

ABERRANT EPIGENETIC SILENCING IS TRIGGERED BY A TRANSIENT REDUCTION IN GENE EXPRESSION

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Preface

This work was published in PLoS One on March 12th by Jon Oyer, Adrian Chu, Sukhmani Brar, and Mitchell Turker.

My contributions to the manuscript include conception of the project, experimental design, cloning design and construction of *P_{TRE}-HPRT* vector, cell culture, bisulfite sequencing experiments, ChIP experiments, data analysis, and preparation of all figures included in the manuscript, and writing the manuscript.

Adrian Chu contributed to this work by assisting with cloning procedures used to generate *P_{TRE}-HPRT*, establishment of stably transfected cell lines by electroporation, and cell culture assays measuring the induction of silencing.

Sukhmani Brar contributed to this work by assisting with qRT-PCR assays measuring mRNA induction in silenced cell lines, and cell culture assays measuring induction of silencing after inhibitor treatments.

Mitch Turker contributed to conception and design of this project, data analysis, writing the manuscript, as well as the space, supplies, and funding used to perform the experiments.

Summary

Aberrant epigenetic silencing plays a major role in cancer formation by inactivating tumor suppressor genes. While the endpoints of aberrant silencing are known, i.e., promoter region DNA methylation and altered histone modifications, the triggers of silencing are not known. We used the tet-off system to test the hypothesis that a transient reduction in gene expression will sensitize a promoter to undergo epigenetic silencing. The tet responsive promoter (P_{TRE}) was used to drive expression of the selectable human *HPRT* cDNA in independent transfectants of an *Hprt* deficient mouse cell line. In this system, high basal *HPRT* expression is greatly reduced when doxycycline (Dox) is added to the culture medium. Exposure of the P_{TRE}-*HPRT* transfectants to Dox induced *HPRT* deficient clones in a time dependent manner. A molecular analysis demonstrated promoter region DNA methylation, loss of histone modifications associated with expression (i.e., H3 lysine 9 and 14 acetylation and lysine 4 methylation), and acquisition of the repressive histone modification H3 lysine 9 methylation. These changes, which are consistent with aberrant epigenetic silencing, were not present in the Dox-treated cultures, with the exception of reduced H3 lysine 14 acetylation. Silenced alleles readily reactivated spontaneously or after treatment of cells with inhibitors of histone deacetylation and/or DNA methylation, but re-silencing of reactivated alleles did not require a new round of Dox exposure. Inhibition of histone deacetylation inhibited both the induction of silencing and re-silencing, whereas inhibition of DNA methylation had no such effect. This study demonstrates that a transient reduction in gene expression triggers a pathway for aberrant silencing in mammalian cells and identifies histone deacetylation as a critical early step in this process. DNA methylation, in contrast, is a secondary step in the silencing pathway under study.

Introduction

Aberrant epigenetic silencing is a common and significant mechanism in cancer development and progression [93]. Like mutational events, aberrant silencing frequently inactivates tumor suppressor genes in both sporadic tumors and human cancer cell lines [63]. Unlike mutations, however, silencing is a stepwise process [94, 95] with potential for reversal [96]. These observations have led to research to identify the molecular changes that accompany silencing. Such changes include promoter region DNA methylation, histone deacetylation, histone methylation at specific residues (*e.g.* H3K9, H3K27), and densely packed nucleosomes that create a closed chromatin structure [97]. However, a caveat is that these changes are most often documented at stably silenced alleles that were under continuous selective pressure within the tumor microenvironment for maintenance of the silenced state. Therefore, reported epigenetic modifications represent an ultimate endpoint and do not reveal how silencing initiates, nor do they reveal the order of epigenetic modifications that occur during the transition from active expression to stable silencing. Such information is required to create strategies to prevent the initiation or progression of aberrant epigenetic silencing.

Many models designed to examine initiation of silencing track normal epigenetic changes during development at imprinted genes [98] or during X chromosome inactivation [99], but developmentally programmed silencing may progress differently than aberrant silencing occurring in cancer. Promoter DNA methylation is the most common modification associated with epigenetic silencing, and has previously been thought to play a causal role [100], but evidence is accumulating to suggest DNA methylation as a late step in the silencing process. For example, DNA methylation occurs after histone modifications for silenced, stably integrated transgenes [101]. A similar progression of epigenetic modifications occurs for silencing of the

endogenous tumor suppressor gene *RASSF1A* [79]. Previous studies in our laboratory showed that silencing of an integrated *Aprt* transgene allows the spread of DNA methylation into a promoter region, which stabilizes the silenced transcriptional state [95]. Although DNA methylation has been the most common modification associated with cancer-related silencing, examples of epigenetic silencing occurring independent of DNA methylation show it is not an absolute requirement [102-104]. Collectively these data suggest that DNA methylation primarily functions to maintain and stabilize the silenced state and that other epigenetic processes are required to initiate silencing.

If DNA methylation is neither a required nor an initiating step for aberrant silencing, how is this process triggered? Recent studies suggest reduced expression as one possibility. For example, in ovarian cancer loss of the *GATA6* transcription factor results in reduced expression and subsequent epigenetic silencing of the downstream target *Disabled-2* [82]. Also, inhibition of ERα (estrogen receptor-α) signaling in breast cancer cell lines reduces expression and induces silencing of the downstream target gene, *PR* (progesterone receptor) [81]. These are two instances that involve loss of transcriptional activators, but evidence also exists that reducing expression by inappropriate recruitment of transcriptional repressors can lead to silencing. An inherited mutation in the *DAPK1* promoter apparently causes B-cell chronic lymphocytic leukemia by increased localization of a transcriptional repressor that reduces expression and correlates with silencing [87]. In addition to altered signaling pathways, some environmental changes accompanying tumor progression also reduce gene expression, which could initiate silencing. For example, hypoxia. a common feature of tumor microenvironments, represses expression of tumor suppressor genes (e.g., *E-CAD*, *BRCA1*, *hMLH1*, and *RUNX*) [105-108] frequently silenced in cancer [63, 109, 110].

In the current study we have developed a system to directly test the hypothesis that a transient reduction in gene expression can sensitize a promoter to undergo epigenetic silencing. The results demonstrate that this principle is correct. Additionally, we find that induction of silencing is dependent on histone deacetylase activity, but does not require DNA methylation.

Results

A system to study transient reductions in gene expression.

To test the hypothesis that a transient reduction in gene expression can initiate epigenetic silencing, we used the tet-off system [111] to control transcription levels of human *HPRT* cDNA in the mouse Dif-6 cell line, which lacks expression of endogenous *Hprt* [112]. In this system the tet-Transcriptional Activator (tTA) localizes to the tet-responsive promoter (P_{TRE}) and promotes *HPRT* expression (Fig. 2-1 A). Adding the tetracycline analog doxycycline (Dox) to the growth medium reduces *HPRT* expression by directly binding tTA and inhibiting its localization to the promoter (Fig. 2-1 B). Three stable transfectants, HP11, HP13, and HP14, expressing high levels of *HPRT* were established. After 48 hours growth in Dox medium, *HPRT* expression was reduced by more than 90% relative to untreated controls, with the HP11 cell line exhibiting the strongest Dox response (Fig. 2-1 C). Although *HPRT* expression is significantly reduced, cell cultures growing in Dox media remain sensitive to selection against *HPRT* (Fig. 2-1 D) and can grow under conditions that require *HPRT* expression (data not shown). A concentration of 1 µg/ml Dox showed a maximum effect on expression without causing toxicity (data not shown) and was used in all subsequent Dox treatments.

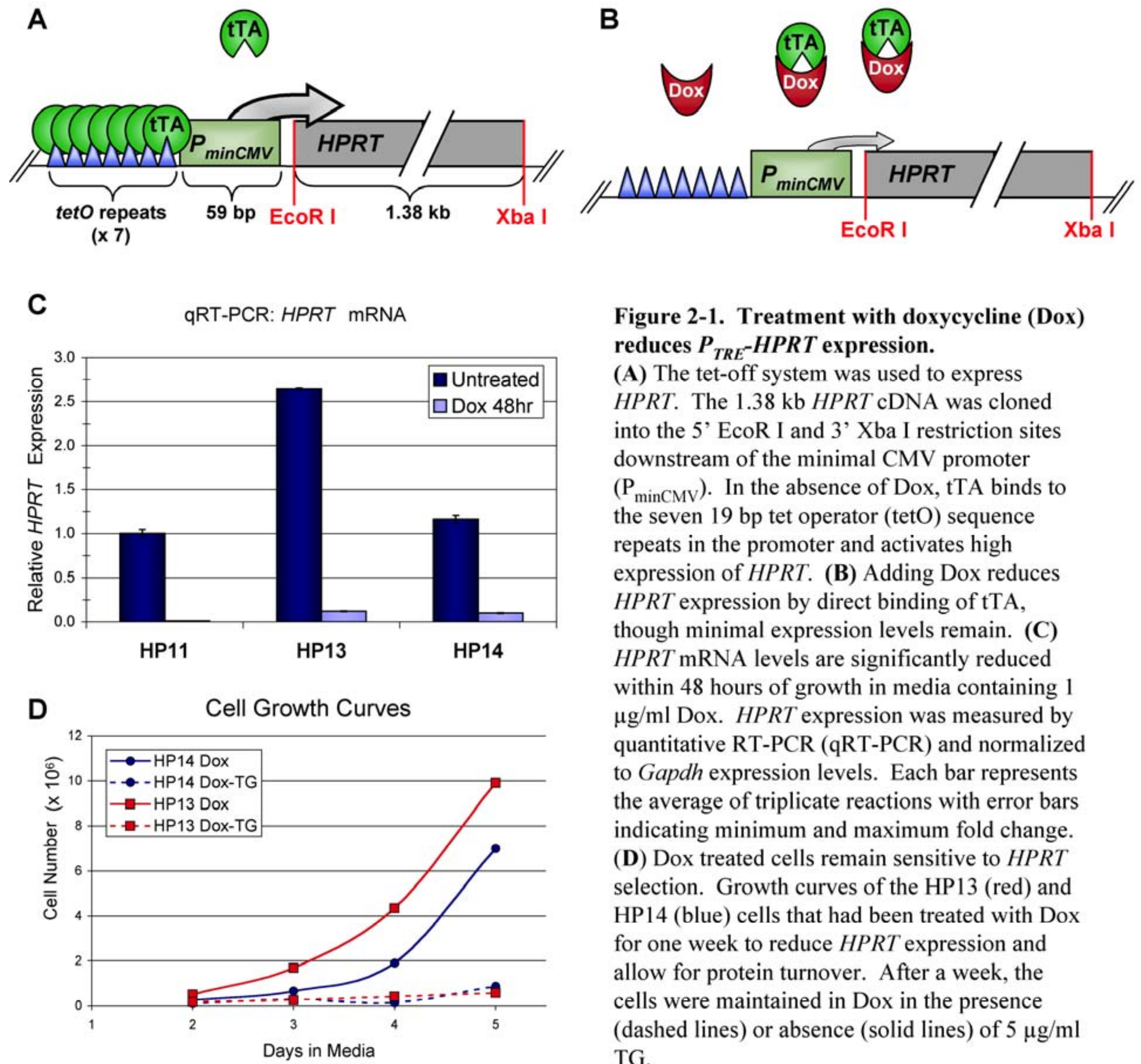


Figure 2-1. Treatment with doxycycline (Dox) reduces P_{TRE}-*HPRT* expression. **(A)** The tet-off system was used to express *HPRT*. The 1.38 kb *HPRT* cDNA was cloned into the 5' EcoR I and 3' Xba I restriction sites downstream of the minimal CMV promoter (P_{minCMV}). In the absence of Dox, tTA binds to the seven 19 bp tet operator (tetO) sequence repeats in the promoter and activates high expression of *HPRT*. **(B)** Adding Dox reduces *HPRT* expression by direct binding of tTA, though minimal expression levels remain. **(C)** *HPRT* mRNA levels are significantly reduced within 48 hours of growth in media containing 1 µg/ml Dox. *HPRT* expression was measured by quantitative RT-PCR (qRT-PCR) and normalized to *Gapdh* expression levels. Each bar represents the average of triplicate reactions with error bars indicating minimum and maximum fold change. **(D)** Dox treated cells remain sensitive to *HPRT* selection. Growth curves of the HP13 (red) and HP14 (blue) cells that had been treated with Dox for one week to reduce *HPRT* expression and allow for protein turnover. After a week, the cells were maintained in Dox in the presence (dashed lines) or absence (solid lines) of 5 µg/ml TG.

A transient reduction in gene expression induces phenotypic gene inactivation.

Following removal of Dox from the culture medium the tTA protein can again bind to P_{TRE} and restore *HPRT* expression. However, our hypothesis predicts that during the period of reduced expression a fraction of alleles will become epigenetically silenced and thus will be unable to restore *HPRT* expression upon removal of Dox. To test this hypothesis, cells were grown in Dox media for a week to reduce expression and allow adequate time for HPRT protein turnover before removing Dox and selecting for *HPRT* deficient cells with the purine analog 6-

thioguanine (TG). The fraction of surviving TG-resistant cells reflects the gene inactivation frequency for P_{TRE}-*HPRT*. Dox exposure was found to induce TG-resistant cells for all three cell lines, at frequencies ranging from 1.4×10^{-3} to 9.4×10^{-2}, which were several orders of magnitude higher than untreated cultures (Fig. 2-2 A). Moreover, P_{TRE}-*HPRT* inactivation frequency increased with longer durations of initial Dox exposure (Fig. 2-2 B). These results demonstrate that transient reductions in gene expression correlate with greatly increased frequencies of P_{TRE}-*HPRT* inactivation.

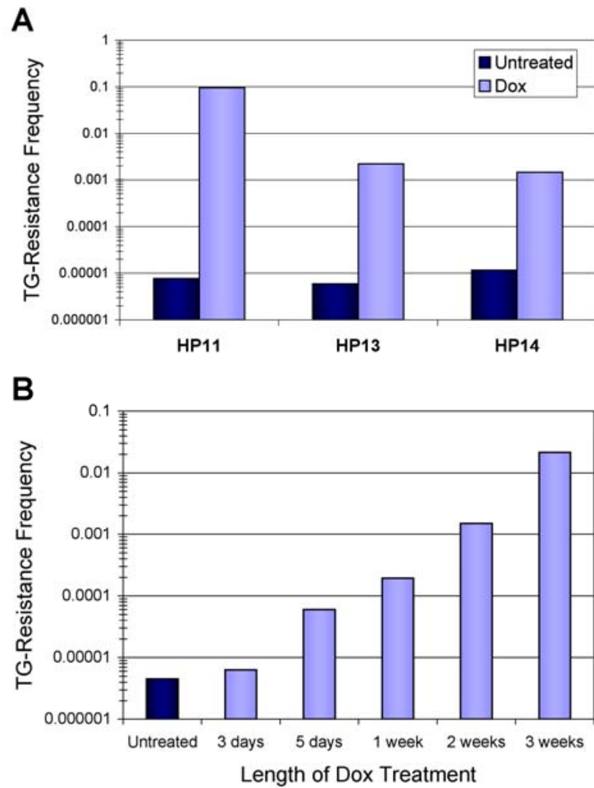


Figure 2-2. Dox exposure induces P_{TRE}-*HPRT* inactivation.
(A) Reducing expression of P_{TRE}-*HPRT* by growing cells for 1 week in medium containing 1 μg/ml Dox increased the frequency of gene inactivation, as measured by TG-resistance. TG-resistance was measured by washing out Dox, selecting cells with 5 μg/ml TG, and counting surviving colonies after 2 weeks of continuous selection. During Dox treatment or the equivalent period without treatment, cells were maintained in medium containing puromycin and G418 to maintain the P_{TRE}-*HPRT* and tTA constructs respectively, but without azaserine/hypoxanthine (AzHx) selection. Only cells that express *HPRT* can grow in AzHx selection. **(B)** TG-resistance frequencies increased as a function of time HP13 cells were exposed to Dox before starting TG selection. HP13 cells were continuously cultured in 1μg/ml Dox for 3 weeks, and TG-resistance was measured at different points during the Dox treatment (3, 5, 7, 14, and 21 days). A parallel control culture was maintained in medium containing puromycin and G418 without Dox, and TG-resistance was measured after 21 days (Untreated).

Epigenetic modifications consistent with silencing characterize inactivated P_{TRE}-*HPRT* alleles.

Individual TG-resistant clones were characterized to identify molecular changes correlating with P_{TRE}-*HPRT* inactivation. TG-resistant clones were isolated from the HP13 and HP14 parental lines following one week of Dox treatment. *HPRT* mRNA levels in all TG-resistant clones were substantially lower than those observed in the Dox treated parental cells (Fig. 2-3). DNA methylation at the P_{TRE}-*HPRT* promoters of HP14-derived TG-resistant cells was measured via bisulfite sequencing (Fig. 2-4). As expected, all CpG sites within the P_{CMVmin}, *tetO* repeats, and nearby regions were unmethylated in actively expressing HP14 cells. Moreover, these sites remained unmethylated in the same cells grown in the presence of Dox for one week. In contrast, all TG-resistant clones analyzed exhibited DNA methylation in the promoter region, though the density of DNA methylation varied (Fig. 2-4). TG1 and TG2 both exhibited low levels of DNA methylation and contained some alleles without any methylated CpG sites in the minimal CMV promoter (P_{minCMV}). In contrast, TG5 and TG6 exhibited substantially more DNA methylation within the promoter, including the core P_{minCMV} region. The other two TG-resistant lines, TG3 and TG4, contained intermediate to high levels of DNA methylation within the promoter relative to the other cell lines (Fig. 2-4).

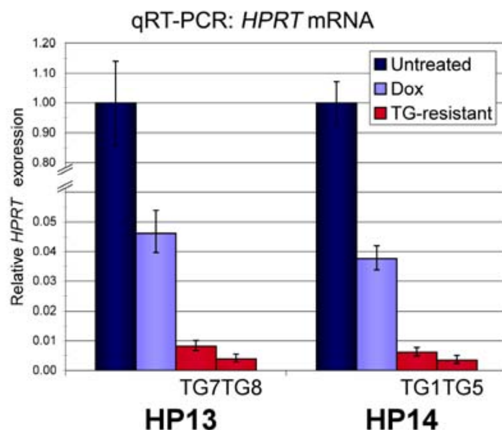


Figure 2-3. P_{TRE}-*HPRT* inactivation correlates with reduced mRNA levels.
Dox-induced TG-resistant clones were isolated and expanded. *HPRT* mRNA levels in TG-resistant clones from the HP13 (TG7 and TG8) and HP14 (TG1 and TG5) parental cells are lower than both active expression levels (Untreated) and reduced expression levels after exposure to Dox for one week (Dox). *HPRT* mRNA was measured by qRT-PCR and normalized to *Gapdh* expression levels. Each bar represents the average of triplicate reactions with error bars indicating minimum and maximum fold change.

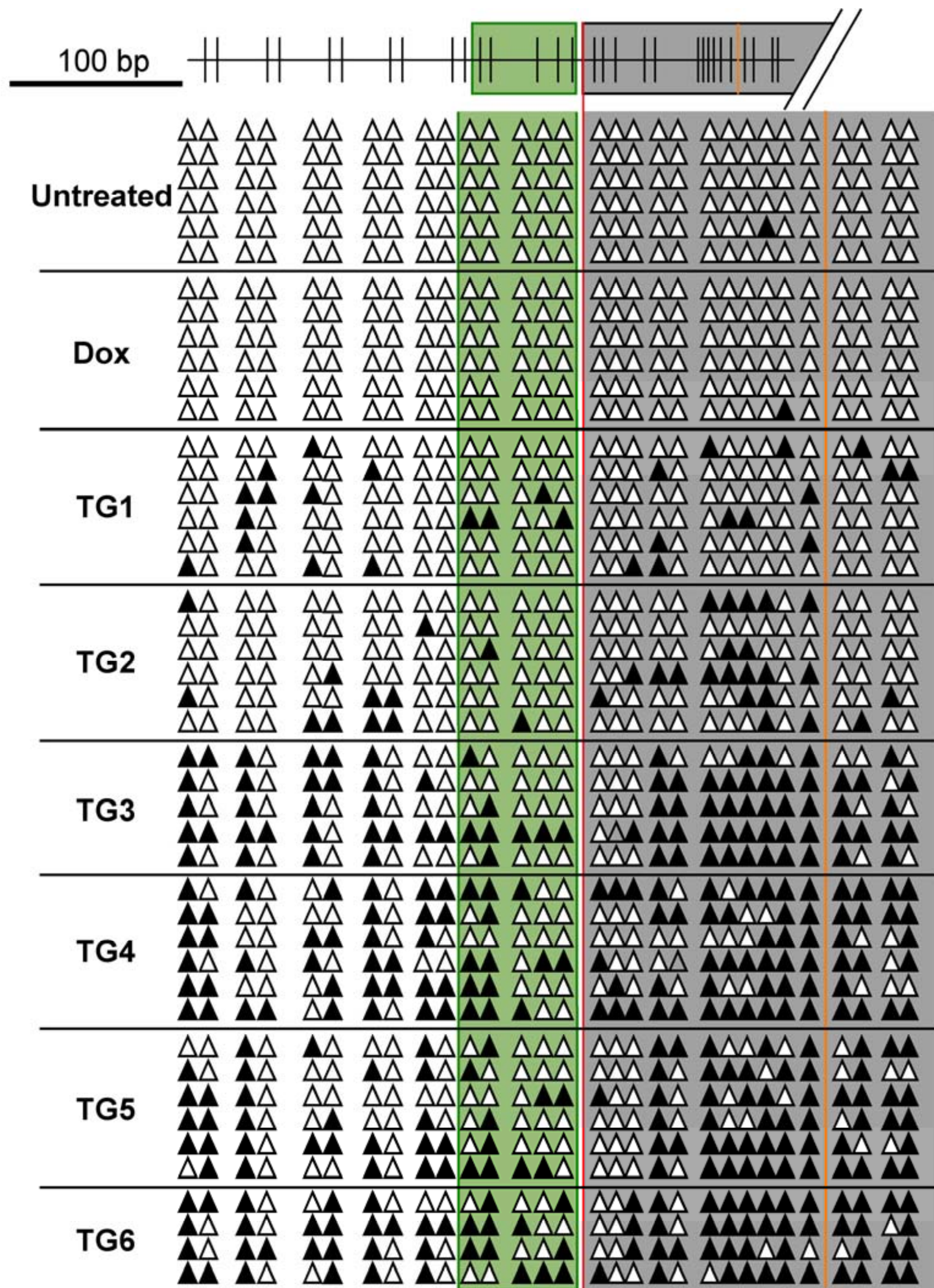


Figure 2-4. ***P_{TRE}-HPRT*** **inactivation correlates with increased promoter DNA methylation.** Expanded allelic methylation patterns for parental HP14 cells expressing high levels of *HPRT* (Untreated), reduced levels of *HPRT* after a 1 week Dox treatment (Dox), and HP14-derived TG-resistant clones (TG1-TG6). Bisulfite sequencing identified methylated (closed triangles) and unmethylated (open triangles) CpG sites within individual alleles. Schematic of the promoter shows approximate positioning of CpG sites (vertical bars) within the minimal CMV promoter (green shaded box) and the 5' region (~112 bp) of the *HPRT* cDNA sequence (grey shaded box). The start of the *HPRT* cDNA sequence, EcoR I restriction site (red vertical bar), has been designated base position +1. The *HPRT* start codon is marked by the orange vertical bar.

The absence of dense promoter DNA methylation in some clones suggested additional mechanisms were contributing to P_{TRE}-*HPRT* inactivation. Chromatin immunoprecipitation (ChIP) analysis was used to measure specific histone modifications associated with either active transcription (methyl-K4, acetyl-K9, and acetyl-K14 of histone H3) or silenced transcription (dimethyl-K9 of histone H3) at the P_{TRE}-*HPRT* promoter (Fig. 2-5). As expected, cells expressing high levels of *HPRT* have a histone modification pattern at the promoter consistent with active transcription. Specifically, the actively transcribed P_{TRE}–*HPRT* promoter was associated with high levels of H3 acetylation (Fig. 2-5 A) and methylation at lysine 4 (methyl-K4 H3) (Fig. 2-5 B) relative to modification levels measured at the active *Gapdh* promoter (*P-Gapdh*). The repressive modification dimethyl-K9 H3 was low in the *HPRT* expressing cells (Fig. 2-5 C), measured relative to the silenced *Mage-a* locus (*P-Mage*) [113, 114]. Reducing *HPRT* expression by treating cells with Dox did not reduce levels of methyl-K4 H3 (Fig. 2-5 B) or significantly change the levels of dimethyl-K9 H3 (Fig. 2-5 C) at the P_{TRE}-*HPRT* promoter. However, H3 acetylation decreased significantly after reducing *HPRT* expression by Dox treatment (Fig. 2-5 A). The antibody used for the acetyl-H3 ChIP recognizes both acetyl-K9 H3 and acetyl-K14 H3 [115]. To probe this decrease further, an additional ChIP was conducted with antibody directed specifically against acetyl-K9 H3. In this case, no decrease was observed after the one-week exposure to Dox (Fig. 2-5 D). Therefore, reducing expression by Dox treatment caused loss of acetyl-K14 H3 without decreasing other modifications associated with transcriptional activity, i.e., methyl-K4 H3 or acetyl-K9 H3. Dox treatment had no effect on histone modifications measured at the control promoters (*P-Gapdh* and *P-Mage*) used for normalization.

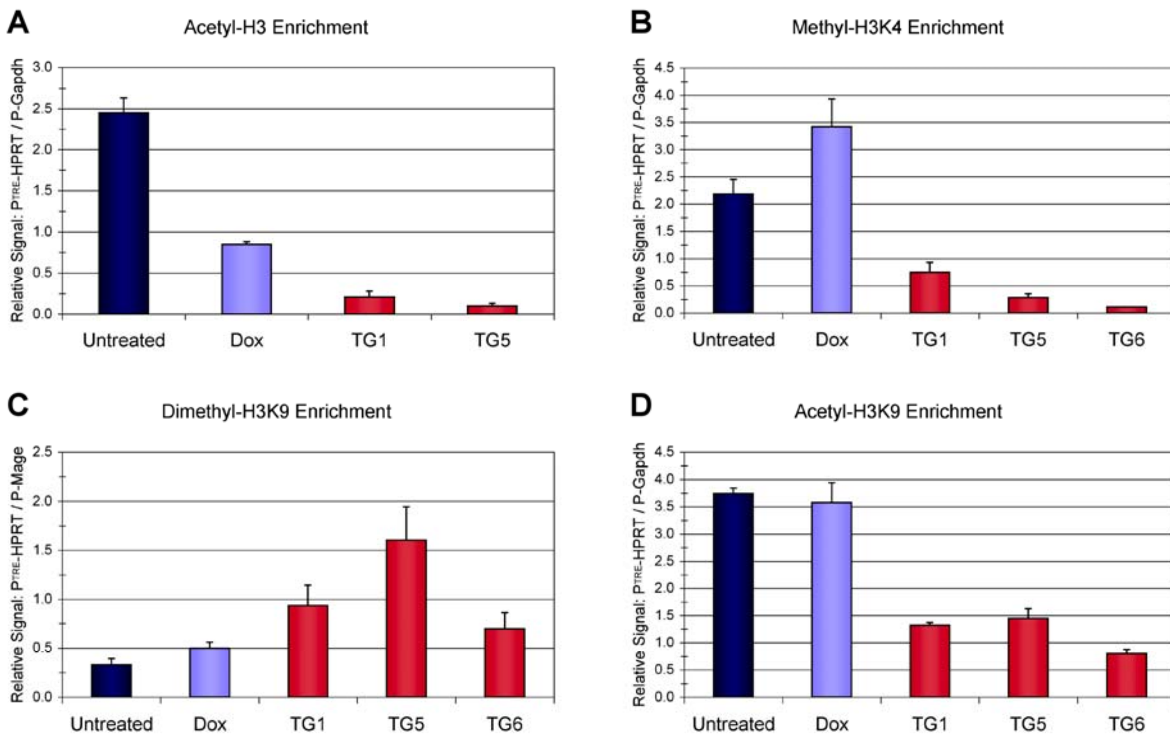


Figure 2-5. P_{TRE}-*HPRT* inactivation correlates with repressive histone modifications.
ChIP analysis measuring histone H3 modifications at the P_{TRE}-*HPRT* promoter in HP14 cells expressing high levels of *HPRT* (Untreated), reduced levels of *HPRT* after 1 week Dox treatment (Dox), and HP14-derived TG-resistant cell lines (TG1, TG5, and TG6). **(A)** ChIP analysis measuring acetylated histone H3 using a polyclonal antibody raised against a peptide corresponding to acetyl-K9 and acetyl-K14. **(B)** ChIP analysis measuring methylation at lysine 4 of histone H3 (methyl-H3K4). The antibody used for immunoprecipitation recognizes all three forms of methylation at K4, mono-, di-, and tri-methyl. **(C)** ChIP analysis measuring the repressive modification of dimethylation at lysine 9 of histone H3 (di-methyl-H3K9). **(D)** ChIP analysis measuring acetylation at lysine 9 of histone H3 (acetyl-H3K9). Immunoprecipitated DNA levels were quantified by qRT-PCR, and levels at P_{TRE}-*HPRT* are displayed relative to either the *Gapdh* or *Mage* promoter. Error bars indicate the SD from triplicate reactions.

In contrast to the observations for histone modifications in the presence of Dox, ChIP analysis for the Dox-independent, TG resistant clones revealed markedly reduced levels of methyl-K4 H3 and acetyl-K9 H3. Increased dimethyl-K9 H3 was also observed at the P_{TRE}-*HPRT* promoter in the TG-resistant cells, ranging from a 2-fold increase in TG6 to a nearly 5-fold increase in TG5 (Fig. 2-5 C). These results demonstrate that the transition from reduced expression to gene inactivation is associated with a shift from activating to repressive histone modifications consistent with epigenetic silencing.

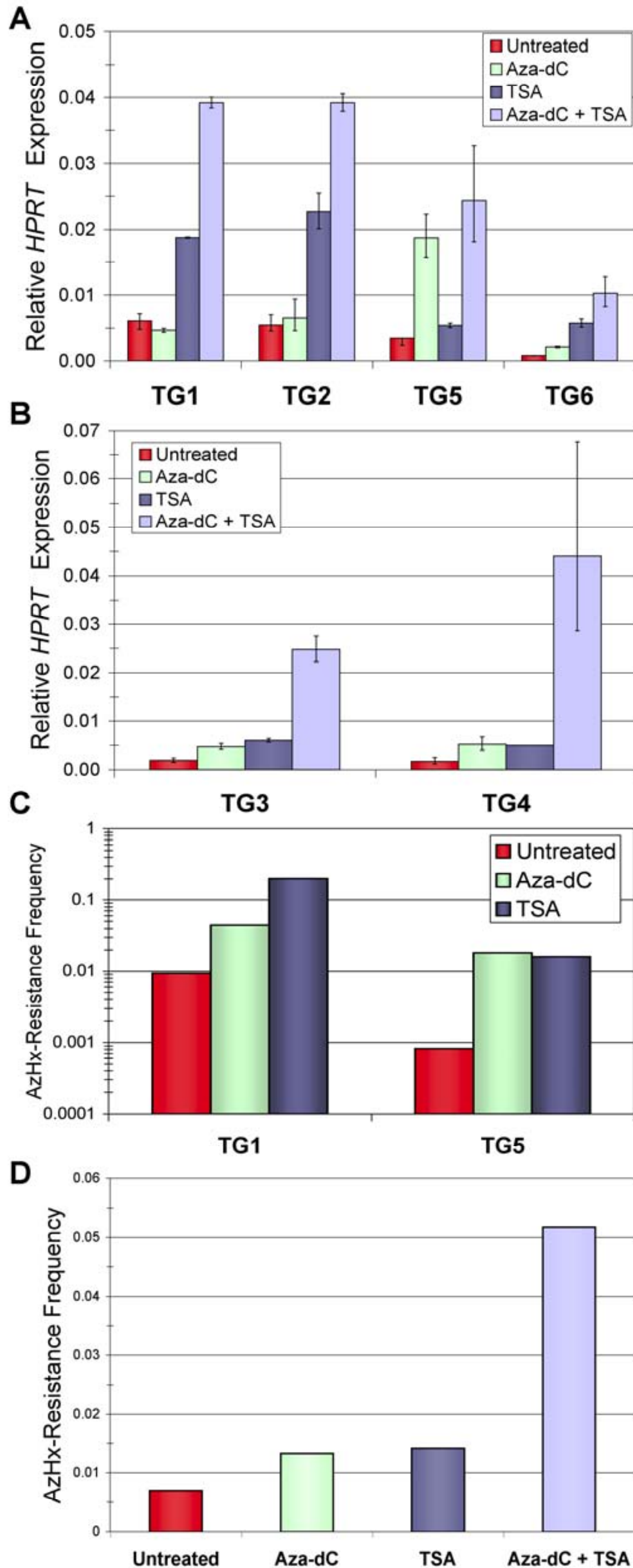


Figure 2-6. Silenced P_{TRE}-*HPRT* alleles are reactivated by inhibiting histone deacetylation or DNA methylation.
(A & B) Inhibition of DNA methylation and histone deacetylation increased *HPRT* mRNA levels. HP14-derived TG-resistant cell lines (TG1-TG6) were treated with 300 nM 5-aza-dC (Aza-dC), inhibiting histone deacetylation with 100 nM trichostatin A (TSA), or a combination of the 300 nM 5-aza-dC and 100 nM TSA treatments (Aza-dC + TSA). Cells were treated with inhibitors overnight (~16 hours), and RNA was harvested 24 hours later. The units shown along the Y-axis are relative to those measured in the untreated parental HP14 cells (see Figure 3). *HPRT* expression was measured by qRT-PCR and normalized to *Gapdh* expression levels. Each bar represents the average of duplicate reactions with error bars indicating minimum and maximum fold change. **(C)** TG-resistant cell lines were capable of reactivating P_{TRE}-*HPRT* expression. Cells were plated with azaserine/hypoxanthine (AzHx) selection, which requires HPRT enzyme activity for cell survival, to isolate and measure the number of cells that reactivated *HPRT* expression. Before plating and selection, cells were treated overnight with 300nM 5-aza-dC (Aza-dC), 100nM TSA (TSA), or vehicle control (untreated) and allowed to recover for 24 hours. Frequencies represent the fraction of cell colonies surviving after two weeks of continuous AzHx selection. **(D)** TG-resistant cell lines were capable of stably reactivating P_{TRE}-*HPRT* expression as shown by reversion assay. Cells were plated with azaserine/hypoxanthine (AzHx) selection, which requires HPRT enzyme activity for cell survival, to isolate and measure the number of cells that stably reactivated *HPRT* expression. Before plating and selection, cells were treated overnight with 300 nM 5-aza-dC (Aza-dC), 100 nM TSA (TSA), both 300 nM 5-aza-dC and 100 nM TSA (Aza-dC + TSA), or vehicle control (Untreated) and allowed to recover for 24 hours. Frequencies represent the fraction of cell colonies surviving after two weeks of continuous AzHx selection.

Silenced P_{TRE}-*HPRT* alleles are reactivated by inhibiting histone deacetylases or DNA methylation.

One of the hallmarks of epigenetic silencing is reversibility. To confirm definitively that the induced P_{TRE}–*HPRT* inactivation was due to silencing, we measured the effects of inhibiting histone deacetylation and/or DNA methylation on gene reactivation in the TG-resistant cells. First, changes in *HPRT* mRNA levels were measured for the TG-resistant cells after inhibiting histone deacetylation with trichostatin A (TSA) treatment or inhibiting DNA methylation with 5-aza-2'-deoxycytidine (5-aza-dC) (Fig. 2-6 A & B). The different TG-resistant clones had varied responses to histone deacetylase (HDAC) inhibition ranging from an approximately 3-fold increase in *HPRT* mRNA (TG1 and TG2) to no response (TG5). Inhibiting DNA methylation gave a nearly reciprocal result with the TG5 cell line showing the largest 5-aza-dC induction of *HPRT* mRNA, an approximately 5-fold increase, and little response in the TG1 and TG2 clones, which exhibited the strongest response after HDAC inhibition. Combining inhibition of histone deacetylases and DNA methylation by treating the cells with 5-aza-dC and TSA simultaneously resulted in synergistic induction of *HPRT* expression for every TG-resistant cell lines except for TG5, which exhibited at best an additive effect (Fig. 2-6 A & D).

Next we determined if the silenced alleles could phenotypically reactivate by selecting for reactivant cells in media requiring *HPRT* expression for survival (azaserine / hypoxanthine or AzHx). Two TG-resistant cell lines, TG1and TG5, spontaneously gave rise to AzHx-resistant colonies at frequencies of $9.3x10^{-3}$ and $8.2x10^{-4}$, respectively (Fig. 2-6 C). TSA and 5-aza-dC treatments were used to determine if inhibiting histone deacetylases or DNA methylation, respectively, would induce phenotypic reactivants similar to their effects on induced reactivation at the RNA level (Fig. 2-6 A). Phenotypic reactivants were induced, though the results did not

mimic precisely those obtained by measuring *HPRT* mRNA levels. For example, TSA treatment increased the frequency of phenotypic reactivation of the TG5 cell line despite the apparent lack of induction when measuring *HPRT* mRNA one day after TSA treatment. While these discrepancies reveal differences between the two assays, the combined results clearly demonstrate that TG-resistance was due to epigenetic mechanisms.

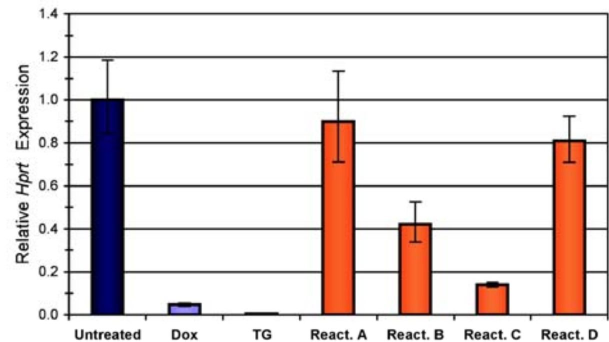


Figure 2-7. Reactivant cell lines have increased *HPRT* expression levels.
HPRT mRNA levels were measured in reactivant cell lines (Reactivants A, B, C, and D) and displayed relative to the expression level in the initial HP13 parental cell line (Untreated). Also shown for comparison are *HPRT* expression levels in the TG-resistant cell line before reactivation (TG), and the parental line after treatment with Dox for one week (Dox). *HPRT* expression was measured by qRT-PCR and normalized to *Gapdh* expression levels. Each bar represents the average of duplicate reactions with error bars indicating minimum and maximum fold change.

Reactivated alleles exhibit memory of transcriptional silencing.

Several laboratories have reported that 5-aza-dC reactivated promoters exhibit rapid re-silencing [70, 73]; however these experiments could not use selection to maintain expression. Our system allowed continuous selection to ensure maintenance of the reactivated promoter state by growing the cells in AzHx medium, which requires HPRT enzyme activity for cell survival. We therefore asked whether promoter reactivation stabilized under selective conditions or alternatively whether the reactivated promoters retained a memory of silencing, as defined by high frequency re-silencing. Although selection ensures *HPRT* expression, the absolute expression levels were variable ranging from 14% to 90% of *HPRT* expression in the parental cells (Fig. 2-7). Two clones, reactivants 1 and 2, were isolated from TG-resistant HP13 cells that had spontaneously reactivated *HPRT* expression and grew well in AzHx medium. Spontaneous

and Dox-induced silencing frequencies were determined for both clones (Fig. 2-8 A). These reactivant cell lines spontaneously re-silenced at high frequencies, $1.8x10^{-2}$ and $6.8x10^{-3}$, relative to the initial HP13 silencing frequency of $4.5x10^{-6}$, with Dox treatment only inducing an approximately 3-fold increase in silencing frequencies.

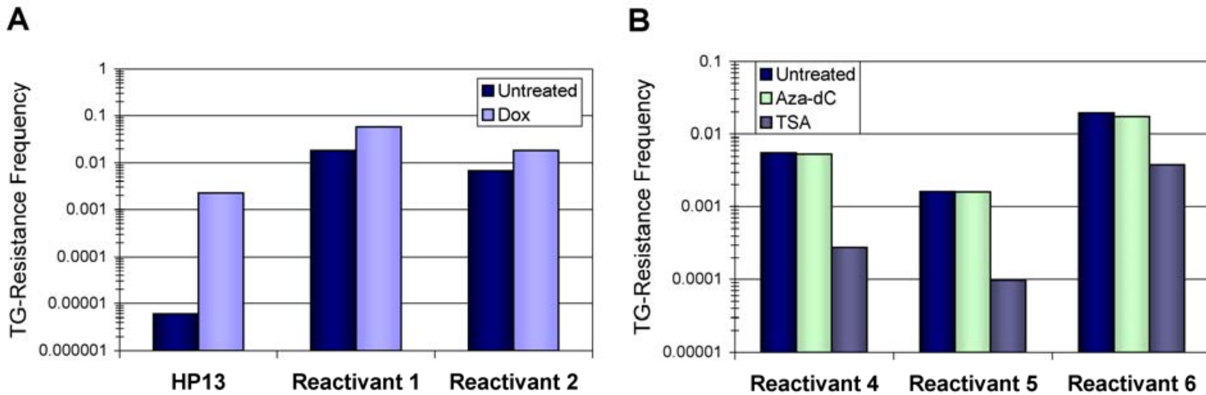


Figure 2-8. Reactivation of silenced P_{TRE}-*HPRT* alleles is unstable.
(A) P_{TRE}-*HPRT* inactivation frequencies for HP13-derived clones with epigenetically silenced and then reactivated *HPRT* expression (Reactivant 1 and 2) were measured after one week without Dox exposure (Untreated) or after one week Dox treatment (Dox). The silencing frequency for the parental HP13 cell line (HP13) is shown for comparison. **(B)** P_{TRE}-*HPRT* inactivation frequencies for HP14-derived clones with epigenetically silenced and then reactivated *HPRT* expression (Reactivants 4-6) were measured after overnight treatment with 300nM 5-aza-dC (Aza-dC), 100nM TSA (TSA), or vehicle control (Untreated) before selecting against *HPRT* activity with TG. Frequencies represent the fraction of cell colonies surviving after two weeks of TG selection.

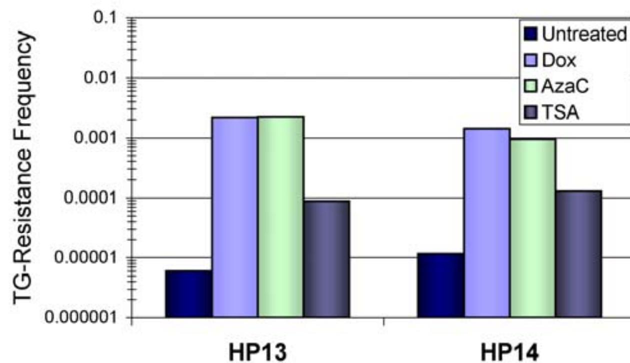
Three reactivant clones from the HP14 TG1 cell line (reactivants 4-6) were examined and also showed that Dox treatment was not required for high frequency P_{TRE}-*HPRT* re-silencing (Fig. 2-8 B). The spontaneous silencing frequency for parental HP14 cells was less than $6x10^{-6}$ (Fig. 2-2 A), but all reactivant cell lines had spontaneous silencing frequencies (10^{-3} to 10^{-2}) equal to or higher than that induced by the week-long Dox treatment (~10^{-3}). Knowing that the silenced state was reversible by inhibiting DNA methylation or histone deacetylation, we examined whether either of these events were required for re-silencing. After inhibiting DNA methylation with 5-aza-dC, the re-silencing frequencies were essentially unchanged relative to

the high spontaneous frequencies. In contrast, HDAC inhibition by treatment with TSA reduced the re-silencing frequencies from 5- to 20-fold. In total, these results showed that the reactivated cells no longer required a period of Dox-mediated transcriptional reduction to silence expression and suggested that re-silencing was dependent on histone deacetylation, but not DNA methylation.

Initiation of silencing is dependent on histone deacetylase activity but not DNA methylation.

After demonstrating that HDAC inhibition reduced re-silencing of reactivated alleles, we tested whether inhibiting HDACs or DNA methylation would affect initial silencing induced by Dox treatment. Induced silencing frequencies were measured again for the HP13 and HP14 parental *HPRT* expressing cell lines, with the modification of adding TSA or 5-aza-dC for the last 16 hours the cells were in Dox media. Inhibiting DNA methylation did not affect the Dox-induced silencing frequency, but HDAC inhibition drastically reduced the silencing frequency (Fig. 2-9). These results show that HDAC activity is an early requirement for silencing induced by decreased transcription in our model, but DNA methylation is not required.

Figure 2-9. Histone deacetylase inhibition prevents Dox-induced P_{TRE}-*HPRT* silencing. P_{TRE}-*HPRT* inactivation frequencies for HP13 and HP14 cells were measured after treatment exposure to 1 mg/ml Dox for one week (Dox), exposure to Dox for one week plus 300nM 5-aza-dC for the last 16 hours (Aza-dC), exposure to Dox for one week plus 100nM TSA for the last 16 hours (TSA), or no treatments (Untreated). Frequencies represent the fraction of cell colonies surviving after two weeks of continuous TG selection.



Discussion

Aberrant epigenetic silencing is a significant mechanism of tumor suppressor gene inactivation, but how this process initiates in mammalian cells is poorly understood. We used the tet-off system to test the hypothesis that a transient and reversible reduction in gene expression could sensitize a promoter to undergo silencing. This hypothesis was based on observations showing reduced gene expression correlates with subsequent tumor suppressor gene silencing (see Introduction) and results from our laboratory showing transcriptional silencing allowed DNA methylation to spread into a promoter region [95]. Moreover, some tumor suppressor genes that are frequently silenced in cancer are also repressed by specific environmental exposures. For example, the tumor microenvironment causes hypoxia, which represses the *E-Cadherin* [107], *BRCA1* [106], and *MLH1* [105] tumor suppressor genes. All are epigenetically silenced in one or more cancer types [63], which suggests a relation between transcriptional repression and silencing in cancer. Here we report results from an experimental system that allowed us to demonstrate that a reduction in gene expression can trigger epigenetic silencing.

Dox treatment reduces expression in the tet-off system by preventing association of the tTA activator protein with the promoter, but reduced expression is not equivalent to epigenetic silencing. For example, the Dox-treated cultures remained sensitive to TG while silenced clones are TG-resistant (Fig. 2-1 D). However, a small fraction of cells exposed to Dox exhibited *HPRT* levels that are reduced further, which provided TG-resistance, and the fraction of cells increases with longer durations of Dox exposure. The induced TG-resistance was also relatively stable because it did not require continued exposure to Dox. These observations demonstrate that the reduced expression in the presence of Dox sensitized some alleles to undergo epigenetic

silencing, but was insufficient to confer TG-resistance by itself. All evidence obtained in these experiments supported the conclusion that Dox-induced TG-resistance was due to epigenetic silencing as opposed to mutational events. The best evidence was the ability of TG-resistant cells to reactivate expression and restore functional HPRT activity, which was evident by growth of the cells in AzHx media. Besides the nine different TG-resistant clones described in this paper, we examined an additional fifteen TG-resistant clones induced by Dox treatment. At least one characteristic of epigenetic silencing (i.e., TSA or 5-aza-dC induction of *HPRT* mRNA or reactivant cell clones) was measured in each of these TG-resistant clones. In total, all twenty-four of the examined Dox-induced clones were shown to have silenced P_{TRE}-*HPRT* alleles. Additionally, the silencing frequencies induced by Dox treatment were orders of magnitude higher than that expected for *HPRT* inactivating mutations ($< 10^{-6}$), and a previous study that characterized base-pair substitutions in the Dif-6 cells showed they do not have a mutator phenotype [116]. Dox treatment has also been used extensively in cell culture without having displayed mutagenic properties.

High-level promoter expression in the tet-off system occurs via localization of the tTA protein and activity of its VP16 activation domain; this domain promotes expression through recruitment of TBP, TFIIB, and the SAGA complex [117]. Then reduced expression during Dox treatment likely results from losing recruitment of these factors. The resultant disruption in recruitment of the SAGA complex and its associated histone acetyltransferases may therefore cause the concurrent decrease in acetyl-K14 H3. In contrast, acetyl-K9 H3 did not decrease when gene expression was reduced by Dox treatment, which demonstrates that the acetylation state of K9 and K14 of H3 may be regulated independently. Previous studies have also observed acetylation at K9 H3 can remain high despite decreased gene expression levels [118]. Levels of

the repressive histone modification dimethyl-K9 H3 remained relatively low during reduced expression in the presence of Dox, which is not surprising considering the continuing presence of acetyl-K9 H3 should prevent the addition of methyl groups at K9 H3. Hypoxic conditions have been reported to increase dimethyl-K9 H3 upon repression of the mouse *Mlh1* [119] and human *RUNX3* promoters [108]. Increased dimethyl-K9 H3 has also been reported to result from nickel exposure [120], which can induce silencing of a *gpt* transgene in hamster cells [121]. While reduced expression alone did not induce methylation of K9 H3 in our system, increased levels of dimethyl-K9 H3 were observed after alleles transitioned to the silenced state identified by TG-resistance.

Results provided by our experiments help establish specific distinctions between the states of transcriptional repression and epigenetic silencing. In our system epigenetic silencing was defined as *HPRT* expression that was reduced to levels that allowed growth in TG selection. Therefore, the most evident difference was that clones with silenced alleles were TG-resistant while cells growing in Dox remained sensitive to TG selection (Fig. 2-1 D). The molecular basis of this phenotypic difference was demonstrated by showing TG-resistant cells had lower levels of *HPRT* mRNA than cells treated with Dox (Fig. 2-3) and molecular changes associated with epigenetic silencing (Figs. 2-4 & 2-5). While the reduced expression after Dox treatment correlated with a loss of acetyl-K14 H3 at the P_{TRE}-*HPRT* promoter, TG-resistance and epigenetic silencing correlated with additional molecular changes including DNA methylation, reduced methyl-K4 H3, loss of acetyl-K9 H3, and increased dimethyl-K9 H3 at the P_{TRE}-*HPRT* promoter. Although increased DNA methylation was one of the molecular changes observed at silenced promoters in our system, DNA methylation was not required for the initiation of silencing because 5-aza-dC treatment had no effect on the frequency of silenced clones induced

by Dox treatment. Evidence that the 5-aza-dC treatment used here was sufficient to inhibit DNA methylation was provided with experiments showing 5-aza-dC treatment induced reactivation of silenced P_{TRE}-*HPRT* promoters that were hypermethylated (Fig. 2-6). Additionally, bisulfite sequencing analysis showed *HPRT* silencing in the TG1 and TG2 cell lines did not require high levels of DNA methylation (Fig. 2-4). In contrast to inhibition of DNA methylation, inhibiting HDAC activity prevented most, but not all, of the Dox-dependent increase in *HPRT* silencing. This observation suggests the presence of two populations of silenced alleles at the end of the Dox treatment. One population would be silenced alleles that are readily reactivated by TSA treatment, and the second population would be alleles that are more stably silenced and fail to restore *HPRT* expression after TSA treatment. Presumably, the second population would have acquired additional repressive epigenetic modifications that cooperate with histone deacetylation to stabilize the silenced state.

A speculative model (Fig. 2-10) to explain the results obtained herein is that promoters with high transcriptional activity are resistant to silencing and are characterized by epigenetic modifications commonly associated with active expression (Fig. 2-10 A). After transcriptional activity decreases at the promoter, acetyl-K14 H3 levels are reduced, and the promoter is more susceptible to epigenetic silencing (Fig. 2-10 B). Although decreased acetyl-K14 H3 alone is not sufficient to induce epigenetic silencing, loss of this modification could decrease protection of the promoter from epigenetic silencing. Similarly, histone H3 acetylation has been shown to establish a protective boundary against spreading of DNA methylation [122]. The transition from reduced expression to epigenetic silencing initiates with histone deacetylation based on the observations that acetyl-K9 H3 levels were low at silenced P_{TRE}-*HPRT* promoters and inhibiting class I and II HDACs reduced the frequency of epigenetic silencing (Fig. 2-10 C).

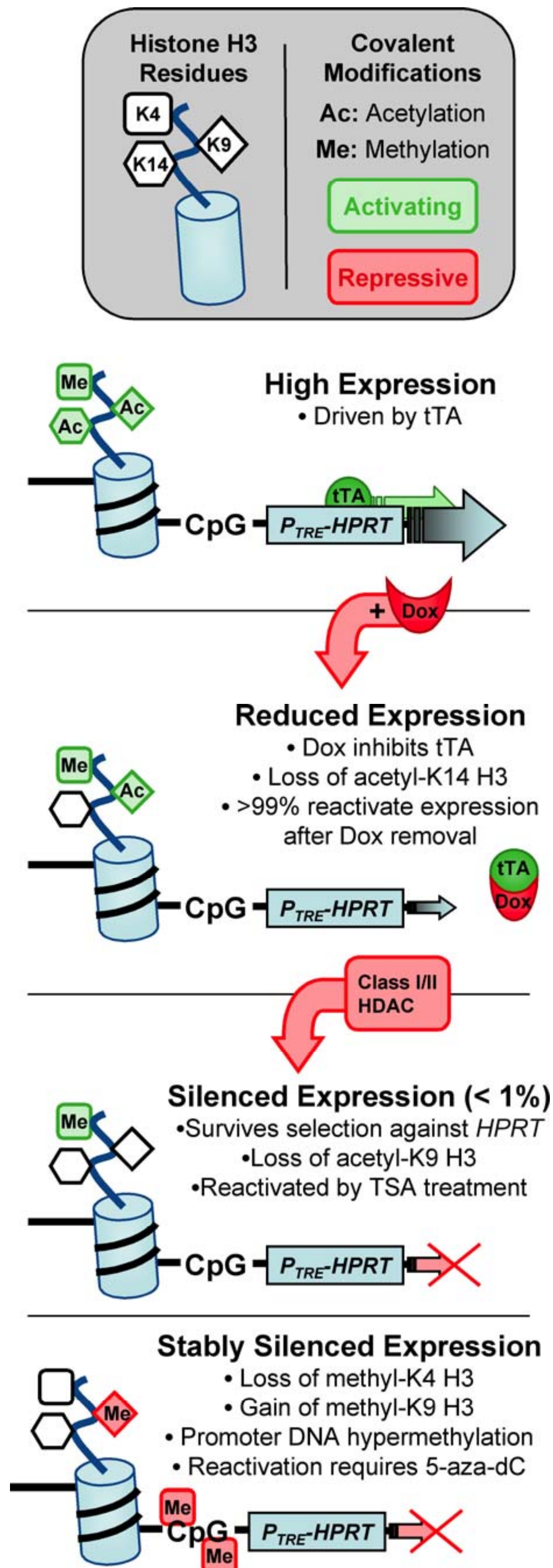


Figure 2-10. A model for induced silencing via reduced gene expression.
(A) The VP16 activation domain promotes high levels of expression. DNA in the promoter region DNA is unmethylated and histone H3 is enriched for activating modifications (methyl-K4, acetyl-K9, and acetyl-K14). **(B)** Adding Dox reduces expression levels and acetylation at K14 of histone H3. **(C)** Reduced expression sensitizes alleles to undergo silencing; silenced alleles become unable to restore expression after Dox removal. The transition to silencing correlates with a further reduction in detectable mRNA and hypoacetylation at K9 H3 and is inhibited by TSA treatment (Class I/II HDAC inhibitor). **(D)** Additional epigenetic changes (loss of methylation at K4-H3, methylation at K9-H3, and DNA methylation) occur with continued TG selection against *HPRT* expression, as the silenced state stabilizes.

Initially, the silenced alleles are unstable and can be reactivated by TSA treatment, but as additional epigenetic modifications occur the silenced state stabilizes and is resistant to TSA treatment alone (Fig. 2-10 D). We propose DNA methylation as a late step in epigenetic silencing because 5-aza-dC treatment did not affect the initiation of silencing. Although loss of methyl-K4 H3 is also shown as a secondary step, our results are not inconsistent with this loss being an early step in epigenetic silencing similar to loss of acetyl-K9 H3. While future experiments are required to test this model directly, aspects of it are consistent with prior observations. One is that silencing is a multistep

process in which DNA methylation occurs downstream of silencing initiation [95]. This conclusion is supported by multiple observations of DNA methylation occurring after histone modification [71, 79, 95, 101] and examples of DNA methylation-independent silencing [102-104].

A current focus in cancer treatment is reactivating silenced tumor suppressor genes in malignant cells through the use of pharmacological agents [96]. Although inhibiting DNA methylation and histone deacetylation usually reactivates expression of silenced alleles [69, 123], such renewed expression is often unstable and quickly re-silences at a high frequency, possibly as a consequence of retention of some repressive histone modifications [70, 73]. Although temporary reactivation of tumor suppressor genes may be sufficient to induce anti-tumor effects, re-silencing would ensure that these effects are short-lived. Thus, it would be helpful to know if high frequency re-silencing reflects a lack of prolonged expression, or alternatively if silenced and reactivated alleles have a persistent memory of the silenced state. To distinguish these possibilities, we isolated subclones from cells with silenced P_{TRE}-*HPRT* that spontaneously reactivated expression and used selection for *HPRT* to maintain the reactivated state for at least one month (~50 cell divisions). Despite the prolonged time of reactivated expression, the absolute level of expression is not always restored to the original level (Fig. 2-7), and the reactivated P_{TRE}-*HPRT* alleles still re-silence at a high frequency. Additionally, re-silencing did not require the Dox-mediated reduction in expression that was required for the initial silencing event. Thus, the memory of silencing was clearly persistent and likely reflects retention of epigenetic modifications. The inhibition of re-silencing with TSA suggests similarities with the initiation of silencing, which was also inhibited with TSA treatment.

We propose that the P_{TRE}-*HPRT* system presented in this study represents a valid model

for initiation and progression of aberrant silencing in cancer because silenced P_{TRE}-*HPRT* alleles display the hallmarks of tumor suppressor gene silencing (promoter region DNA methylation, histone hypoacetylation, loss of methyl-K4 H3, and gain of methyl-K9 H3). In other words, we believe that the principle of reduced expression as a trigger for silencing will apply to *bona fide* mammalian promoters. Although our system utilized a non-mammalian promoter, endogenous levels of enzymes that control histone modifications and DNA methylation were responsible for the transition from repression to silencing. This is a unique and significant difference between our experimental system and previous systems that induced silencing by direct recruitment of repressive protein domains [124] or direct establishment of DNA methylation [78, 125] at promoters. Hence, our system has the potential to detect multiple independent pathways of epigenetic silencing, which could be cell type specific. For example, histone modifications and DNA methylation are both observed at silenced promoters in colon cancer cells, whereas some of the same promoters only exhibit histone modifications when silenced in prostate cancer cells [103].

In summary, we used the tet-off system to provide a clear demonstration that reduced transcriptional potential can sensitize a promoter to undergo epigenetic silencing. Consistent with prior work, the results demonstrate that silencing is a multistep process in which promoter region DNA methylation is secondary to altered histone modification. We propose that these results are applicable to tumor suppressor promoters that are repressible by internal or external environmental exposures and that the model we created will be useful for identifying molecular determinants of aberrant silencing in mammalian cells.

Materials & Methods

Tet-Off Constructs

The Tet-Off system has been described previously [111]. The pTet-Off plasmid (Clontech) expresses the neomycin (*Neo*) resistance gene (Neo^r) and tTA, a fusion protein composed of the amino-terminus of the tetracycline repressor and the activation domain of the VP16 protein. The 1.38 kb *HPRT* full-length cDNA sequence (Accesion # NM_000194.1) was isolated by EcoRI and XbaI digestion of the TrueClone *HPRT* cDNA expression vector (Origene, catalog #TC120047). pTRE-tight-HPRT was created by directionally cloning the *HPRT* fragment into the EcoRI and XbaI restriction sites within the pTRE-Tight (Clontech) multiple cloning site.

Cell Culture

Dif-6 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 5% fetal bovine serum (Hyclone) and 5% Serum Plus (SABC Biosciences). 5×10^6 Dif-6 cells were transfected by electroporation [25] with 4 µg of pTet-Off plasmid (Clontech) that expressed the tTA activating protein and selected for linked Neo^r with 500 µg/ml of G418. A transfectant showing high tTA expression was selected for a second transfection with 10 µg of P_{TRE}-*HPRT* and 2 µg of a separate plasmid containing a bacterial puromycin (*pur*) resistance gene. Stable transfectants expressing functional *HPRT* were selected with media containing 10 µg/ml azaserine (Sigma) and 10 µg/ml hypoxanthine (Sigma) (AzHx medium). Selection for the *pur* gene was with 1.5 µg/ml puromycin (Invitrogen). Individual clones were expanded and screened for physical linkage between *pur* and P_{TRE}-*HPRT* by identifying clones with low frequency P_{TRE}-*HPRT* inactivation (via TG selection) while retaining resistance to

puromycin. Comparing the P_{TRE} promoter signal to the *Gapdh* promoter signal from genomic DNA samples by quantitative-PCR measured P_{TRE}-*HPRT* copy number in the cell lines. HP11 and HP14 contained single copies and HP13 contained two copies of P_{TRE}-*HPRT*. These parental cells were routinely cultured in AzHx, G418, and puromycin to retain expression of all constructs. Doxycycline hyclate (Dox) (Sigma) was added to DMEM at a concentration of 1 µg/ml for silencing experiments. Dox medium also contained G418 and puromycin to retain the tTA and P_{TRE}-*HPRT* constructs, respectively. These drugs were also used during TG selection to retain both constructs in clones with silenced alleles. Cell exposed to Dox were not exposed to AzHx, unless indicated.

RNA Preparation and Analysis

Total RNA was isolated from cell cultures with the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Total RNA samples were converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen) with removal of genomic DNA contamination. 100 ng cDNA was used as input in subsequent quantitative-PCR analysis for either *HPRT* (TaqMan assay Hs99999909_m1, Applied Biosystems) or *Gapdh* (Mouse TaqMan Endogenous Control, Applied Biosystems) with iQ Supermix (Bio-Rad) and a Bio-Rad iCycler. *HPRT* Results were normalized in relation to *Gapdh* mRNA levels and displayed relative to an arbitrary value.

Silencing and Reactivation Cell Cloning Assays

To measure P_{TRE}-*HPRT* inactivation or reactivation, cells were plated into 100 mm plates at densities ranging from $1x10^4$ to $1x10^5$ cells per plate. The next day the medium was removed, cells were rinsed with DMEM, and TG or AzHx selective medium was used to select against or

for *HPRT* expression, respectively. Cells were cultured for approximately two weeks in the appropriate selective media before staining live colonies with crystal violet solution. To estimate cloning efficiencies, additional cells were plated under identical conditions as selective plates but at lower densities, 250 to 1000 cells per plate and without selection for or against *HPRT* expression. Silencing or reactivation frequencies were calculated by dividing the number of clones growing under selection by the effective number of cells plated (as determined with the cloning efficiency plates).

Drug Treatments

Cells were treated with media containing 100 nM TSA (Wako) overnight (~16 hours) to inhibit histone deacetylation. Cells were treated with media containing 300 nM 5-aza-dC (Sigma) overnight (~16 hours) to inhibit DNA methylation. For *HPRT* mRNA analysis, cells were allow to recover 24 hours in DMEM after drug treatment (TSA or 5-aza-dC) before harvesting for RNA purification.

DNA Methylation Bisulfite Sequencing Assay

Genomic DNA was isolated from cell cultures using DNAzol (Molecular Research Center) according to the manufacturer's instructions. For each treatment, 4 µg of genomic DNA was digested with Bsr I, and modified in a solution of 6.24 M urea, 4 M sodium bisulfite, and 10 mM hydroquinone as described previously [95]. PCR amplification of modified DNA, cloning of PCR products, and sequence analysis were also described elsewhere [126], with the following exceptions. The primers used in the initial PCR reaction were the TRE-NaBis-S sense primer 5'-GTA TTT ATT AGG GTT ATT GTT TTA TGA G-3' and the HPRT NaBis-A antisense primer

5'-CAA AAT AAA TCA AAA TCA TAA CCT AAT TC-3'. 1 µl of the PCR product was used as input in the subsequent semi-nested PCR reaction using the TRE-NaBis-NS primer 5'-GTA TTT AGA AAA ATA AAT AAA TAG GGG TTT-3' and HPRT-NaBis-A for amplification. PCR products were cloned using Strataclone PCR cloning kit (Stratagene). Sequencing analysis showed all cytosine bases not present in the CpG dinucleotide context were converted to thymine indicating complete bisulfite modification of the genomic template occurred.

Chromatin Immunoprecipitation

ChIP assays were carried out using EZ ChIP chromatin immunoprecipitation kit (Millipore) with the following specific details or modifications. Proteins were cross-linked to DNA in 5×10^7 cells by adding formaldehyde to a final concentration of 1% and incubating for 10 minutes at room temperature. The cross-linking reaction was stopped by addition of glycine to a final concentration of 125 mM and incubating for 5 minutes at room temperature. Cells were rinsed with cold PBS containing complete protease inhibitor cocktail (Roche) and resuspended in SDS lysis buffer. Lysates were sonicated using a Branson 450 microtip sonicator to shear DNA into 100-1000 bp fragments. Protein-DNA complexes were immunoprecipitated using antibodies to acetyl-K9/K14 H3 (06-599, Millipore), acetyl-K9 H3 (07-352, Millipore), mono/di/trimethyl-K4 H3 (05-791, Millipore), and dimethyl-K9 H3 (ab1220, Abcam). 5 µl of each specific antibody was added to lysates from $\sim 1 \times 10^6$ cells and incubated overnight at 4º C. Immunocomplexes were isolated by incubating for 3 hours at 4º C with a 3:1 mixture of Protein A and Protein G conjugated magnetic Dynabeads (Invitrogen) that had been blocked with salmon sperm DNA and BSA. Beads were washed once with each of the following: low salt buffer, high salt buffer, LiCl buffer, and 1X TE. Immunocomplexes were eluted by incubating

beads at 65° C for 15 minutes in 200 µl elution buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS), and the cross-links were reversed by incubating at 65ºC overnight. After incubation with 0.2 µg/ml RNase A at 37ºC for 2 hours and 0.2 µg/ml Proteinase K at 55° C for 2 hours, DNA was purified using QiaQuick PCR purification kit (Qiagen). Quantitative PCR using an Icycler and iQ SYBR Green Supermix (Bio-Rad) was used to analyze the immunoprecipitated DNA. The P_{TRE}-*HPRT* promoter was amplified using the 5'-AAC GTA TGT CGA GGT AGG CGT GTA-3' sense primer and the 5'-ATC TCC TTC ATC ACA TCT CGA G-3' antisense primer. The active *Gapdh* promoter was amplified using the 5'-TTG AGC TAG GAC TGG ATA AGC AGG-3' sense primer and the 5'-AAG AAG ATG CGG CCG TCT CTG GAA-3' antisense primer. The silenced *Mage-a* promoter was amplified using the 5'-GTT CTA GTG TCC ATA TTG GTG-3' sense primer and the 5'-AAC TGG CAC AGC ATG GAG AC-3' antisense primer. The specific signal from each immunoprecipitation relative to signal from input was calculated for the three promoters, P_{TRE}-*HPRT*, *Gapdh*, and *Mage*. For activating modifications, levels at P_{TRE}-*HPRT* are displayed relative to the *Gapdh* promoter; for the repressive modification, dimethyl-K9 H3, results are displayed relative to the *Mage* promoter.

Chapter 3

DNA METHYLATION MODULATES BUT DOES NOT CONTROL EPIGENETIC SILENCING AND REACTIVATION OF MOUSE *APRT*

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Manuscript is currently in preparation.

Preface

This work is currently in preparation for submission.

My contributions to the manuscript include conception of the project, experimental design, cell culture, qRT-PCR, bisulfite sequencing, ChIP experiments, data analysis, preparation of all figures included in the manuscript, and writing the manuscript.

Phil Yates contributed to this work by designing and optimizing the bisulfite sequencing assay and primers used in these experiments, bisulfite sequencing analysis of the P19H22 cells.

Sarah Godsey contributed to this work by performing bisulfite sequencing and data analysis for the P19H22 and D3 cell lines.

Mitch Turker contributed to conception and design of this project, data analysis, writing the manuscript, as well as the space, supplies, and funding used to perform the experiments.

Summary

Two distinct epigenetic pathways that silence endogenous *Aprt* in mouse embryonal carcinoma cells were examined to model epigenetic processes that inactivate tumor suppressor genes in cancer. Epigenetic silencing associated with a bivalent chromatin pattern of enriched methyl-H3K4 and dimethyl-H3K9 occurred independent of DNA methylation at the *Aprt* promoter. By comparison, silenced *Aprt* promoters that contain high levels of DNA methylation were enriched in dimethyl-H3K9 and had reduced acetyl-H3K9 and methyl-H3K4. Higher levels of DNA methylation within the *Aprt* promoter correlated with stabilized silencing that was more resistant to reactivation. However, reactivation of silenced *Aprt* did not always require loss of DNA methylation in the promoter. DNA methylation could be removed from the *Aprt* promoter by maintaining reactivated expression through selection, but reactivation remained unstable even after complete loss of DNA methylation from the *Aprt* promoter. This demonstrated that DNA methylation is not required for memory of epigenetic silencing. Additionally, no specific histone modification was identified that could explain the high frequency of re-silencing in reactivants, but absolute levels of methyl-H3K4, acetyl-H3K9, and methyl-H3K9 were unstable at the reactivated promoters. Collectively, these results show DNA methylation modulates but does not control epigenetic silencing and reactivation of mouse *Aprt*.

Introduction

DNA methylation is the epigenetic modification most commonly associated with transcriptional silencing. This modification is present at most non-transcribed regions of the eukaryotic genome including alleles silenced during imprinting, the inactive X chromosome, repetitive elements, and heterochromatin at chromosome centromeres and telomeres [127]. In addition to these examples of transcriptional silencing that contribute to normal genomic regulation, DNA methylation is consistently observed at aberrantly silenced tumor suppressor genes in a diverse array of human tumors and cancer cell lines [63, 65]. The strong correlation between aberrant tumor suppressor silencing and DNA methylation is functionally significant because expression in cancer cell lines can be reactivated by inhibiting DNA methylation [128-130]. However, examples of aberrant silencing that occur independent of DNA methylation show that this modification is not an absolute requirement [102-104, 131]. Moreover, some tumor suppressor promoters silenced independent of DNA methylation in prostate cancer cells are silenced in conjunction with DNA hypermethylation in colon cancer cells, which suggests the mechanisms responsible for epigenetic silencing are influenced by cell-type specific differences [103]. Finally, distinct silencing mechanisms may function within a single cell type because analysis of ovarian tumors shows aberrant transcriptional silencing of *BRCA2* expression either independent of DNA methylation or with promoter DNA hypermethylation [132].

Alleles that are epigenetically silenced in the absence of DNA methylation are associated with specific repressive histone modifications including histone hypoacetylation, loss of methylation at H3K4, and increased methylation at H3K9 and H3K27 [103, 133]. However, none of these epigenetic changes are unique to silencing that occurs independent of DNA methylation, and most repressive histone modifications are also observed in conjunction with

DNA methylation at silenced tumor suppressor promoters [133, 134]. While DNA methylation and histone modifications have distinct functional activities in silencing transcription, establishment of these different repressive epigenetic modifications is often related [135]. DNA methylation recruits histone deacetylases (HDAC) via the methyl-binding protein MeCP2 [34, 35]. Reversal of histone deacetylation by treatment with HDAC inhibitors acts in synergy with DNA methylation inhibitors for reactivation of silenced alleles and shows that repressive histone modifications function cooperatively with DNA methylation [123]. Additionally, histone deacetylation has been identified as an early initiating event during epigenetic silencing of transgenes [101, 136] and endogenous tumor suppressor genes [79]. Enrichment of methyl-H3K27 at tumor suppressor promoters may increase susceptibility to epigenetic silencing [83, 85, 86] and is also capable of silencing expression independent of DNA methylation [103]. Discerning the specific functional consequences of individual repressive modifications is required for an effective understanding of normal and aberrant epigenetic regulation.

DNA methylation inhibitors are being tested as a potential cancer therapy because reactivation of silenced tumor suppressors is commonly observed in cancer cell lines after 5-aza-dC treatment [96]. However, induced reactivation is unstable, and reactivated expression will re-silence without continued DNMT inhibition [68, 137]. Certain repressive histone modifications that remain after demethylation and reactivation of silenced promoters may contribute to the high frequency of re-silencing [70], but continuous HDAC inhibition following reactivation is unable to prevent demethylated alleles from re-silencing [73]. It is unclear if the high frequency of re-silencing is due to incomplete reactivation of most alleles within a cell population or if the memory of silencing persists even at alleles where expression levels have been restored. One approach to answer this question is to select for gene function to isolate the population of cells

that have reactivated expression. The *Aprt* allele meets this criterion and has been used frequently in studies examining mechanisms of epigenetic silencing [89, 95, 100, 138-145]. In this study, we use the *Aprt* model to characterize two distinct, DNA methylation dependent and independent,epigenetic pathways that silence endogenous *Aprt* expression as well as examining the relationship between DNA methylation and reactivation of silenced *Aprt* promoters.

Results

DNA methylation is established upstream of the active *Aprt* promoter

The mouse P19H22 embryonal carcinoma cell line contains a single expressed *Aprt* allele; the other allele was removed by a spontaneous deletion [146]. Previous work using methylation sensitive restriction enzymes and Southern blot analysis of the P19H22 cells revealed a gradient of heavy DNA methylation in an upstream region of the *Aprt* promoter that transitions to little or no DNA methylation near the *Aprt* promoter [126]. The heavily methylated region includes two B1 repetitive elements and is located 1 kb upstream from the *Aprt* transcriptional start site.

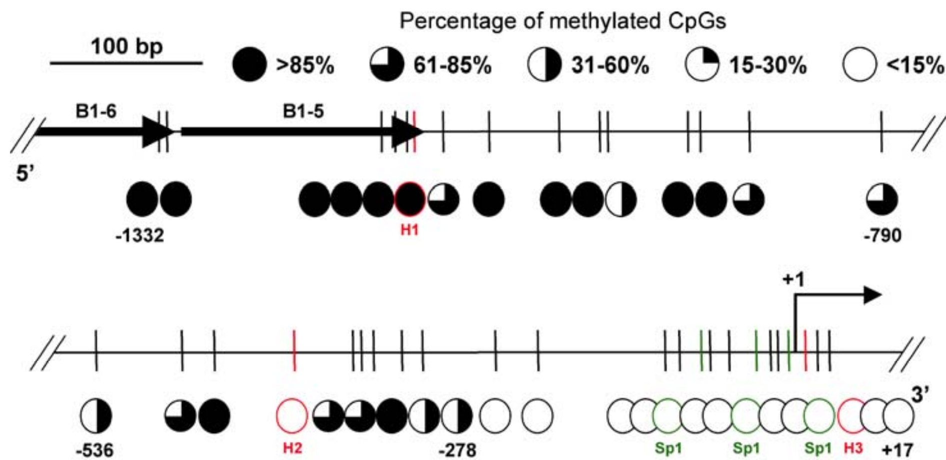


Figure 3-1. DNA is methylated upstream of the active *Aprt* promoter. The DNA methylation pattern of the *Aprt* promoter region in P19H22 cells was measured by bisulfite sequencing analysis. Vertical dashes mark the relative locations of CpG sites and the shaded circles indicate the percentage of total alleles (n=14) that were methylated at the corresponding CpG. Base pair positions are labeled relative to the *Aprt* transcriptional start site (arrow marked +1). Other DNA elements marked are the CpG sites within an Sp1 binding site (green dashes and green circles), CpG sites within a HpaII restriction site (red dashes and circles labeled H1, H2, and H3), and B1 elements (horizontal arrows labeled B1-5 and B1-6).

Figure 3-2. Allelic DNA methylation patterns in the *Aprt* promoter region of P19H22 cells.
Methylation patterns are shown for P19H22 cells expressing *Aprt*. Vertical dashes mark the relative locations of CpG sites and the triangles indicate the methylation status of the corresponding CpG (shaded triangles are methylated and empty triangles are unmethylated). Base pair positions are labeled relative to the *Aprt* transcriptional start site. Other DNA elements marked are the CpG sites within an Sp1 binding site (green dashes) and CpG sites within a HpaII restriction site (red dashes labeled H2 and H3).

To obtain a more detailed picture of the methylation pattern for this region, bisulfite sequencing was used to measure methylation status at 38 CpG sites across a 1450 bp region that includes the *Aprt* promoter and a portion of the upstream repetitive B1 elements (Figs. 3-1 & 3-2). CpG sites within the B1 elements were methylated at levels exceeding 85% of the alleles examined, and a high level of methylation (> 60% methylation at all but one CpG site) was maintained until a CpG site at position -790 bp relative to the transcriptional start site.

The region from position -536 to position -278 contains nine CpG sites that exhibited

variable levels of DNA methylation with an average frequency of methylated CpGs of 62%. This region includes one CpG site that is never methylated and another methylated in all alleles. The CpG site with no methylation detected by bisulfite sequencing analysis is a HpaII site (H2) that was shown by Southern blot analysis to be methylated at a level of approximately 15% [138]. The reason for the discrepancy between the two assays for DNA methylation is unclear. Only sporadic DNA methylation (< 1% of all CpG sites examined) was detected beyond the CpG site at position –278. This result suggests that aside from rare, isolated methylated CpG sites, active *Aprt* expression is associated with a boundary that prevents DNA methylation from spreading beyond the CpG site at –278 and into the Sp1 binding sites that compose the *Aprt* promoter.

***Aprt* silencing can occur either in association with promoter DNA methylation or independent of DNA methylation**

Two cell lines from P19H22 with silenced *Aprt* alleles, D3 and D7 were isolated in a prior study, and a Southern blot analysis revealed some methylation at a HpaII site (H3) near the *Aprt* promoter region of the D3 cells, but not in the D7 cells [138]. In this study, bisulfite sequencing provided a more comprehensive analysis of CpG methylation to identify specific changes in the DNA methylation pattern that occur after silencing of the *Aprt* promoter. The region analyzed contains 22 CpG sites from base position -470 through +17, relative to the *Aprt* transcriptional start site, and includes CpG sites within each of the three Sp1 binding sites that compose the *Aprt* promoter. Despite the silenced *Aprt* expression in D7 cells, all Sp1 sites within the *Aprt* promoter were unmethylated, and no significant difference in the DNA methylation pattern was observed as compared with the actively expressed *Aprt* allele in the

parental P19H22 cells (Figs. 3-3 & 3-4). In contrast, increased levels of DNA methylation were observed in the promoter region of the D3 clone, including significant methylation at the H2 site, which is unmethylated in the P19H22 cells, and complete methylation of the CpG site within the first Sp1 binding site (Figs. 3-3 & 3-5). CpG methylation beyond the first Sp1 binding site was variable with some alleles in the D3 cells exhibiting no DNA methylation at the downstream Sp1 binding sites.

Figure 3-3. *Aprt* silencing is associated with multiple distinct methylation patterns.
The DNA methylation patterns of the *Aprt* promoter region in silenced cell lines were measured by bisulfite sequencing analysis. Vertical dashes mark the relative locations of CpG sites and the shaded circles indicate the percentage of total alleles (n=14) that were methylated at the corresponding CpG. Base pair positions are labeled relative to the *Aprt* transcriptional start site (arrow marked +1). Other DNA elements marked are CpG sites within an Sp1 binding site (green dashes) and CpG sites within a HpaII restriction site (red dashes labeled H2 and H3).

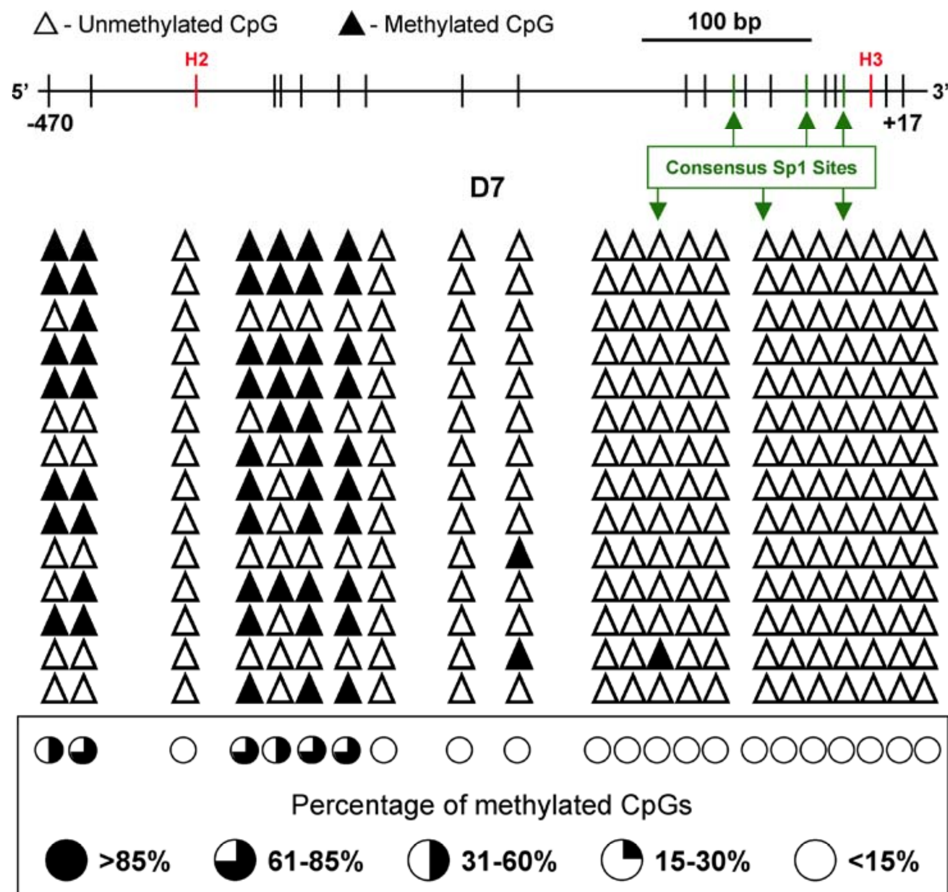


Figure 3-4. Allelic DNA methylation patterns in the silenced *Aprt* promoter region of D7 cells.

Methylation patterns are shown for D7 cells at the silenced *Aprt* allele. Vertical dashes mark the relative locations of CpG sites and the triangles indicate the methylation status of the corresponding CpG (shaded triangles are methylated and empty triangles are unmethylated). Base pair positions are labeled relative to the *Aprt* transcriptional start site. Other DNA elements marked are the CpG sites within an Sp1 binding site (green dashes) and CpG sites within a HpaII restriction site (red dashes labeled H2 and H3).

△ - Unmethylated CpG ▲ - Methylated CpG

100 bp

H2 H3

5' 3'

-470 +17

Consensus Sp1 Sites

A

D3

Percentage of methylated CpGs

>85% 61-85% 31-60% 15-30% <15%

B

D3S1

Percentage of methylated CpGs

Figure 3-5. Allelic DNA methylation patterns in the silenced *Aprt* promoter region of D3 and D3S1 cells.

Methylation patterns are shown for D3 (A) and D3S1 (B) cells at the silenced *Aprt* allele. Vertical dashes mark the relative locations of CpG sites and the triangles indicate the methylation status of the corresponding CpG (shaded triangles are methylated and empty triangles are unmethylated). Base pair positions are labeled relative to the *Aprt* transcriptional start site. Other DNA elements marked are the CpG sites within an Sp1 binding site (green dashes) and CpG sites within a HpaII restriction site (red dashes labeled H2 and H3).

Both cell lines, D3 and D7, yielded spontaneous reactivants at very high frequencies, greater than 10% (Table 3-1). Reactivant cells were selected in medium with azaserine and adenine (AzA), which requires *Aprt* expression for cell survival. The lack of DNA methylation in the *Aprt* promoter of D7 cells and incomplete DNA methylation on many alleles in the D3 cells could account for the very high spontaneous *Aprt* reactivation. To determine if clones with increased DNA methylation could be isolated, subclones of both the D3 and D7 cells were analyzed by bisulfite sequencing. One such subclone was isolated from D3 and designated D3S1. The *Aprt* promoter region in the D3S1 cells was almost completely methylated for all CpG sites examined, including within the three consensus Sp1 binding sites (Figs. 3-3 & 3-5). In addition to the increased DNA methylation of the *Aprt* promoter, the spontaneous reversion frequency for the D3S1 cells was decreased, approximately 20-fold lower than for D3 cells (Table 3-1). Subclones with increased DNA methylation could not be isolated from D7 even if selected with 2-fluroadenine (FA) (Fig. 3-6), which provides more stringent selection against *Aprt* expression than DAP [147]. These subclones yielded spontaneous reactivants at levels similar to that of D7 (data not shown) and were not examined further.

Cell Line	**Viable Cells Plated**	**AzA^r Clones**	***Aprt* Reactivation Frequency**
D7	5.0×10^3	522	1.04×10^{-1}
D3	5.2×10^3	960	1.86×10^{-1}
D3S1	2.4×10^5	1120	4.69×10^{-3}

Table 3-1. **Reactivation of silenced *Aprt* alleles**.

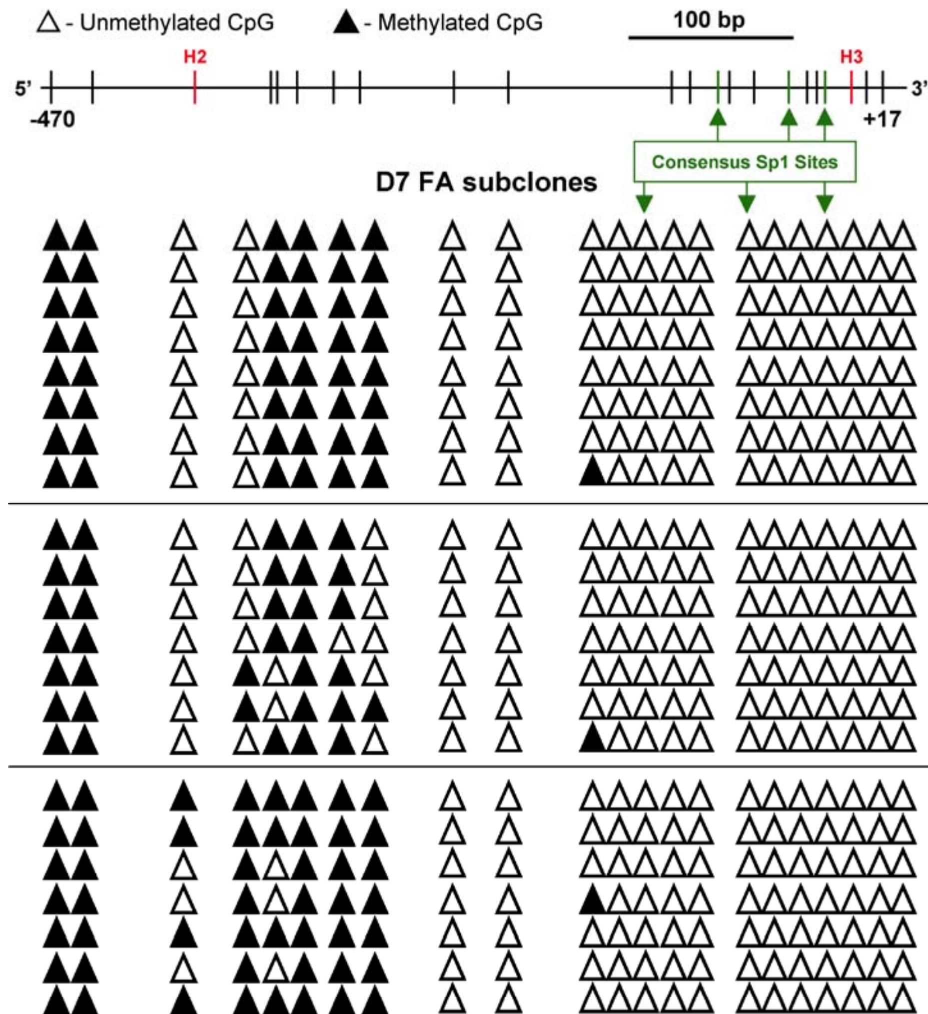


Figure 3-6. Allelic DNA methylation patterns of the silenced *Aprt* promoter in D7 subclones selected in FA media.
Methylation patterns are shown for three different FA resistant subclones from the silenced cell line D7. Vertical dashes mark the relative locations of CpG sites and the triangles indicate the methylation status the corresponding CpG (shaded triangles are methylated and empty triangles are unmethylated). Base pair positions are labeled relative to the *Aprt* transcriptional start site. Other DNA elements marked are the CpG sites within an Sp1 consensus site (green dashes) and CpG sites within a HpaII restriction site (red dashes labeled H2 and H3).

Relative to expression in P19H22, *Aprt* mRNA levels were reduced to 18%, 5%, and 1% in the silenced D7, D3, and D3S1 cell lines, respectively (Fig. 3-7). Inhibiting DNA methylation by 5-aza-dC treatment reactivated expression in D3 and D3S1, the two cell lines with hypermethylated *Aprt* promoters but did not alter expression in the D7

clone, which lacked DNA hypermethylation in the *Aprt* promoter (Fig 3-3). Inhibition of histone deacetylases by TSA treatment also had no affect on *Aprt* mRNA levels in the D7 cell line. By comparison, a small induction of *Aprt* mRNA levels was detected in the D3S1 and D3 cell lines after TSA treatment, an 8- and 1.5-fold increase, respectively. Although this measured effect was small, HDAC inhibition has some biological effect on the silenced *Aprt* promoter in the D3 and D3S1 cell lines as demonstrated in reactivation assays. TSA treatment increased the frequency of reactivant colonies selected in AzA medium as well as the number of cells in reactivant colonies, which reflects better cell growth under selection for *Aprt* expression (Fig. 3-8 A & B). In a final attempt to induce reactivation of the silenced *Aprt* promoter in the D7 cell line, these cells were treated with both 300 nM TSA and increasing concentrations of 5-aza-dC (from 0-100 nM), but no increase in *Aprt* mRNA expression was measured (Fig. 3-8 C). In contrast, a robust induction was observed for the D3S1 cells after simultaneous 5-aza-dC and TSA treatment combining inhibition of DNA methylation and HDACs (Fig. 3-8 D).

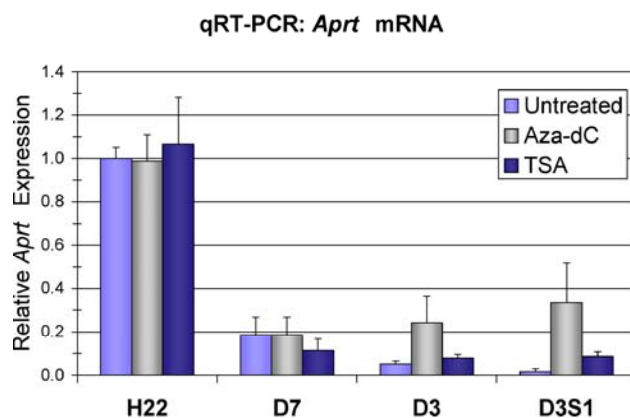


Figure 3-7. Inhibiting DNA methylation or histone deacetylases increases reactivation of silenced *Aprt* promoters with DNA hypermethylation. *Aprt* mRNA measured by qRT-PCR in parental cells with active Aprt expression (H22) and the Dap-resistant cell lines with silenced *Aprt* expression (D7, D3, & D3S1). *Aprt* expression was also measured after inhibiting DNA methylation by treatment with 3 μM Aza-dC or inhibiting HDACs by treatment with 300 nM TSA. Displayed results are the average of triplicate reactions with error bars indicating the standard deviation.

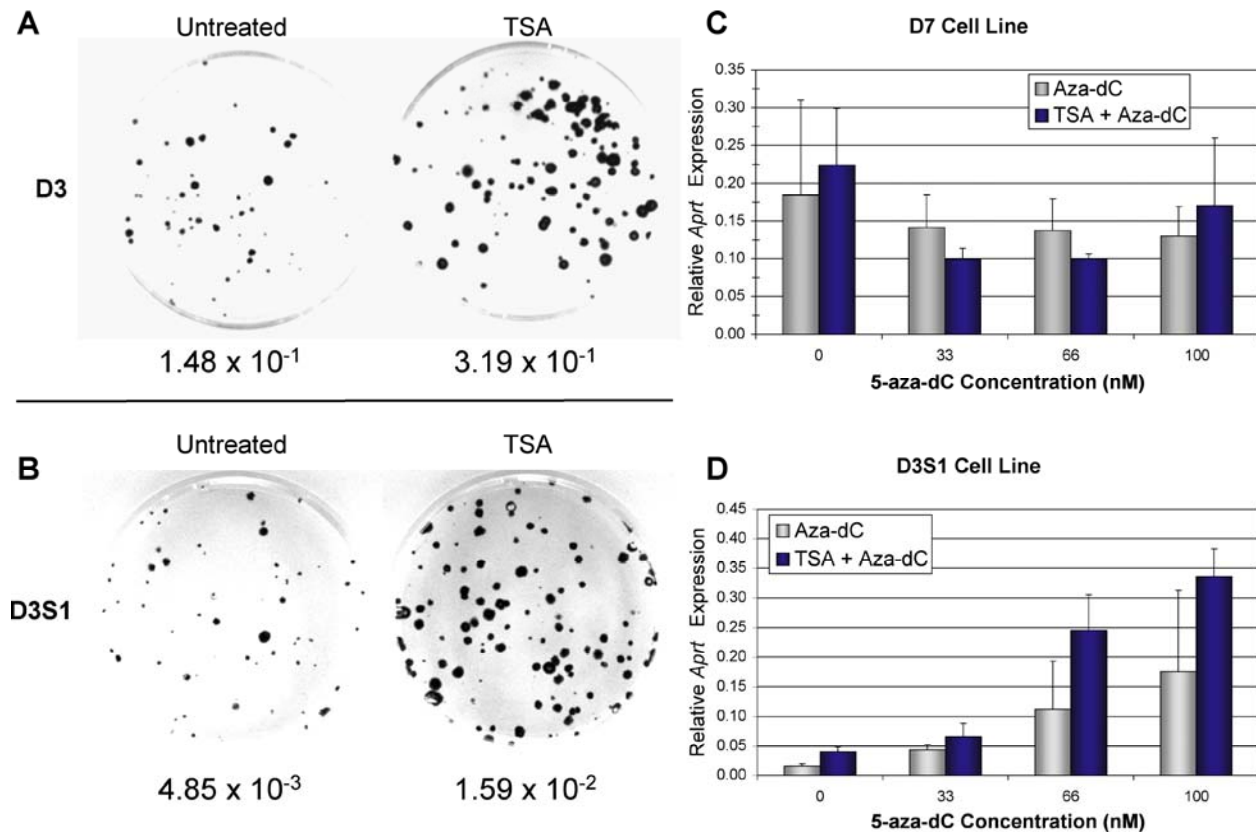


Figure 3-8. Inhibiting histone deacetylases increases reactivation of silenced *Aprt* promoters with DNA hypermethylation.
Azaserine / Adenine (AzA) reversion assays measured the number of D3 (A) or D3S1 (B) cells that reactivate *Aprt* expression and form viable clones either spontaneously (Untreated) or following HDAC inhibition with 300 nM TSA treatment (TSA). The number of cells originally plated was 1×10^3 D3 cells and 1×10^5 D3S1 cells. The calculated reactivation frequency is listed below the corresponding plate. (C) & (D) *Aprt* expression in D7 (C) and D3S1 (D) cells after combining inhibition of DNA methylation and HDACs by simultaneous treatment with Aza-dC and TSA. The 300 nM TSA concentration was kept constant, and multiple Aza-dC concentrations were used ranging from 0 to 100 nM. mRNA levels were measured by qRT-PCR and displayed results are the average of triplicate reactions with error bars indicating the standard deviation.

Differentially methylated *Aprt* promoters are associated with distinct histone modification patterns

The bisulfite sequencing analysis identified three distinct DNA methylation patterns among the silenced cell lines, no promoter region DNA methylation (D7), variable promoter region DNA methylation (D3), and nearly complete promoter region DNA methylation (D3S1). To determine if these methylation patterns correlated with different histone modifications, a

chromatin immunoprecipitation (ChIP) analysis was performed using antibodies against the activating modifications acetylation at H3K9 and methylation at H3K4 and the repressive modifications methylation at H3K9 and H3K27. Histone modifications measured at the *Aprt* promoter in the P19H22 cells were consistent with active expression, i.e., high levels of acetyl-K9 H3 (Fig. 3-9 A) and methyl-K4 H3 (Fig. 3-9 B) and low levels of dimethyl-K9 (Fig. 3-9 C).

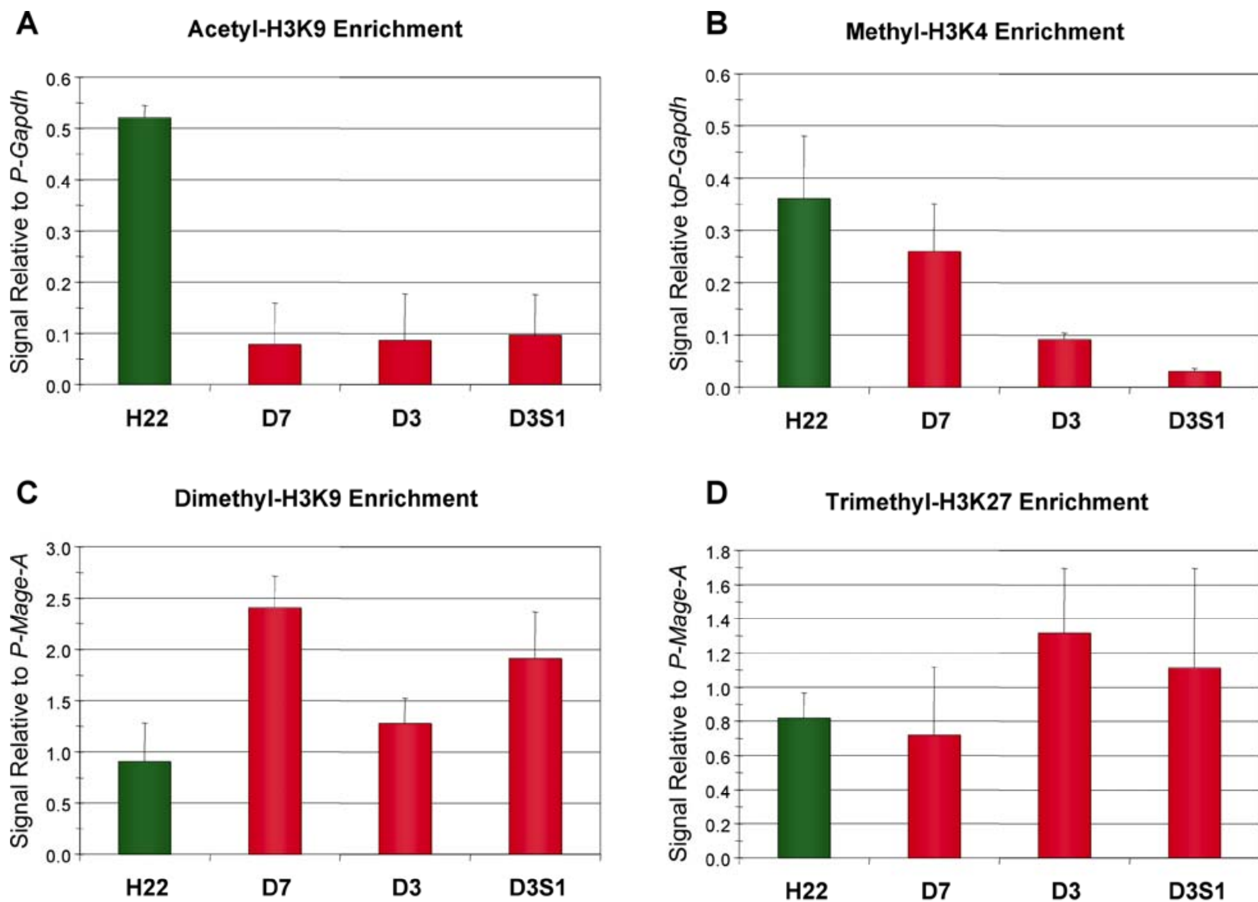


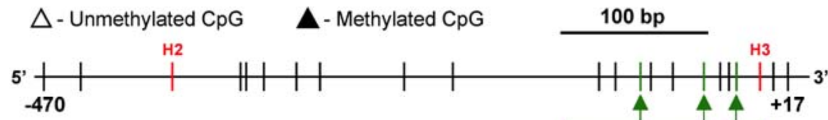
Figure 3-9. *Aprt* silencing correlates with altered histone modification patterns.
ChIP analysis measured histone H3 modifications at the *Aprt* promoter in H22 cells expressing *Aprt* (H22) and cell lines with silenced *Aprt* (D7, D3, and S1). (A) ChIP analysis measuring acetylation at lysine 9 of histone H3 (Acetyl-H3K9). (B) ChIP analysis measuring methylation at lysine 4 of histone H3 (Methyl-H3K4). The antibody used for immunoprecipitation recognizes all three forms of methylation at K4, mono-, di-, and tri-methyl. (C) ChIP analysis measuring the repressive modification of dimethylation at lysine 9 of histone H3 (Dimethyl-H3K9). (D) ChIP analysis measuring the repressive modification of trimethylation at lysine 27 of histone H3 (trimethyl-H3K27). Immunoprecipitated DNA levels were measured by qRT-PCR and normalized to enrichment at either the *Gapdh* promoter or the *Mage-a* promoter.

In D7 cells, which had no DNA hypermethylation in the *Aprt* promoter, levels of acetyl-K9 H3 were decreased, and a corresponding increase in dimethyl-K9 was observed (Fig 3-9 A & C). However, levels of methyl-K4 H3 were comparable to those measured at the active *Aprt* promoter in P19H22 (Fig. 3-9 B). Thus, the *Aprt* promoter in D7 cells exhibited a bivalent modification pattern with enrichment of both repressive and activating modifications. In contrast, the D3 and D3S1 clones had decreased levels of both modifications associated with active expression, i.e., acetylation at K9 and methylation at K4 of histone H3 (Figs. 3-9 A & B). The stably silenced D3S1 cells had higher levels of the repressive histone modification dimethyl-K9 H3 at the *Aprt* promoter than the D3 cell line, though both were higher than that observed in the P19H22 parental cells (Fig. 3-9 C).

Levels of trimethyl-K27 H3 were also measured at the *Aprt* promoter, but no significant differences were observed between P19H22 and the silenced cell lines (Fig. 3-9 D), which indicates that this histone modification was not altered as a result of promoter silencing.

Reactivation of silenced *Aprt* promoters does not require loss of DNA methylation

The results comparing spontaneous reactivation of the D3 and D3S1 cell lines suggest that higher levels of promoter DNA methylation increase the stability of the epigenetic silencing because spontaneous *Aprt* reactivation is reduced 20-fold for D3S1 cells. To study the relationship between DNA methylation and promoter reactivation, DNA methylation at the *Aprt* promoter was measured in D3 and D3S1 spontaneous reactivant clones after maintaining active expression for two weeks and one month. D3S1 reactivants exhibited complete loss of DNA methylation in the *Aprt* promoter when measured as early as two weeks after cells were selected in AzA medium (Fig. 3-10 A).



A D3S1 Reactivants: 2 weeks in AzA media

B D3 Reactivants: 2 weeks in AzA media

C D3 Reactivants: 1 month in AzA media

Figure 3-10. Allelic DNA methylation patterns after maintaining reactivated *Aprt* expression.
(A) DNA methylation patterns at the *Aprt* promoter are shown from D3S1 subclones that have been continuously growing in AzA media for two weeks. (B & C) DNA methylation patterns at the *Aprt* promoter are shown from D3 subclones that have been continuously growing in AzA media for two weeks (B) or one month (C). Vertical dashes mark the relative locations of CpG sites and the triangles indicate the methylation status the corresponding CpG (shaded triangles are methylated and empty triangles are unmethylated). Base pair positions are labeled relative to the *Aprt* transcriptional start site. Other DNA elements marked are the CpG sites within an Sp1 binding site (green dashes) and CpG sites within a HpaII restriction site (red dashes labeled H2 and H3).

In contrast, D3 reactivants exhibited high levels of promoter region DNA methylation within the *Aprt* promoter at the two-week time point (Fig. 3-10 B) and significant promoter region methylation persisted in some alleles measured at the one-month time point (Fig. 3-10 C). DNA methylation patterns for D3S1 reactivants after expression was maintained for one month were similar to those at two weeks, i.e., DNA methylation was absent from the *Aprt* promoter (data not shown).

Maintaining *Aprt* expression does not stabilize reactivation

The above results demonstrated that loss of DNA methylation from the *Aprt* promoter after reactivation clearly differed between the D3 and D3S1 cell lines because DNA methylation persisted at the *Aprt* promoter in D3 cells weeks after the DNA methylation pattern had returned to normal in D3S1 cells. To determine if this difference in DNA methylation affected the stability of reactivated *Aprt* expression, the re-silencing frequencies were measured for D3 and D3S1 reactivant clones after reactivated expression had been maintained continuously for one month. Consistent with the expectation that expression would be de-stabilized in promoters with higher levels of DNA methylation, *Aprt* re-silencing frequencies of reactivant clones isolated from D3 cells were approximately 10-fold higher than those of D3S1 reactivants. However, in both cases the re-silencing frequencies were found to be remarkably high, ~ 10% for the D3 reactivants (Fig. 3-11 A) and 1% for the D3S1 reactivants (Fig. 3-11 B). These frequencies were several orders of magnitude higher than that for the P19H22 parental cells (approximately from 10^{-5} to 10^{-6}) [146, 148]. This suggests that the reactivated *Aprt* alleles were metastable because a population of reactivants could be stably maintained with selection, but expression itself was not stabilized in a significant fraction of cells. This high frequency *Aprt* re-silencing observed in

reactivant clones is not a consequence of low expression levels in reactivant clones because after one month of growth in AzA medium *Aprt* expression in several reactivant clones was equal to or greater than that measured in the P19H22 cells (Fig. 3-11 C & D). For example, the D3A21 reactivant cell line exhibited an *Aprt* re-silencing frequency of 22% despite expressing higher levels of *Aprt* mRNA than P19H22.

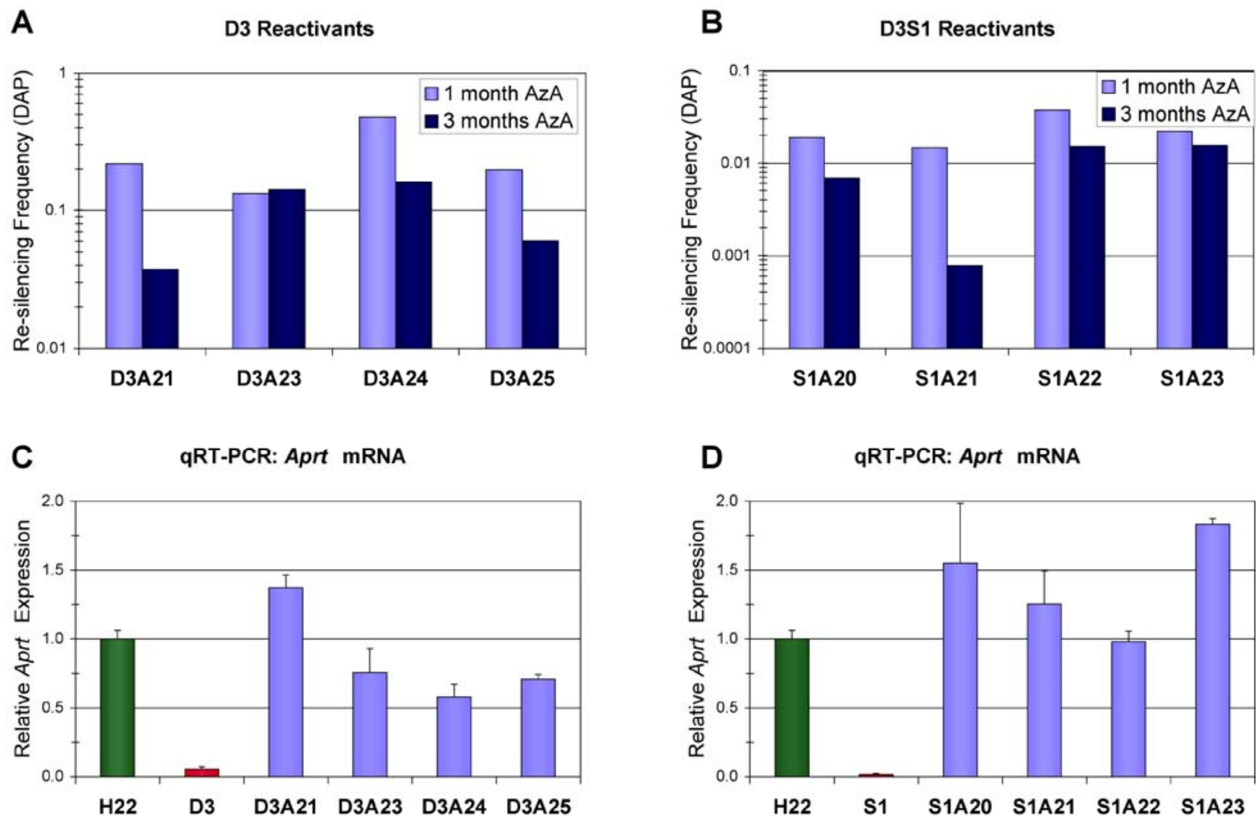


Figure 3-11. *Aprt* reactivants re-silence expression at high frequencies despite maintaining expression for three months.
Aprt re-silencing frequencies of D3-derived reactivants (A) and S1-derived reactivants (B) measured by cloning assays in DAP media. Re-silencing frequency was measured after reactivant clones had been growing continuously in AzA for one month and three months. *Aprt* expression was measured in reactivant clones from D3 (C) and from S1 (D) after growing continuously in AzA for one month. mRNA levels were measured by qRT-PCR and displayed results are the average of triplicate reactions with error bars indicating the standard deviation.

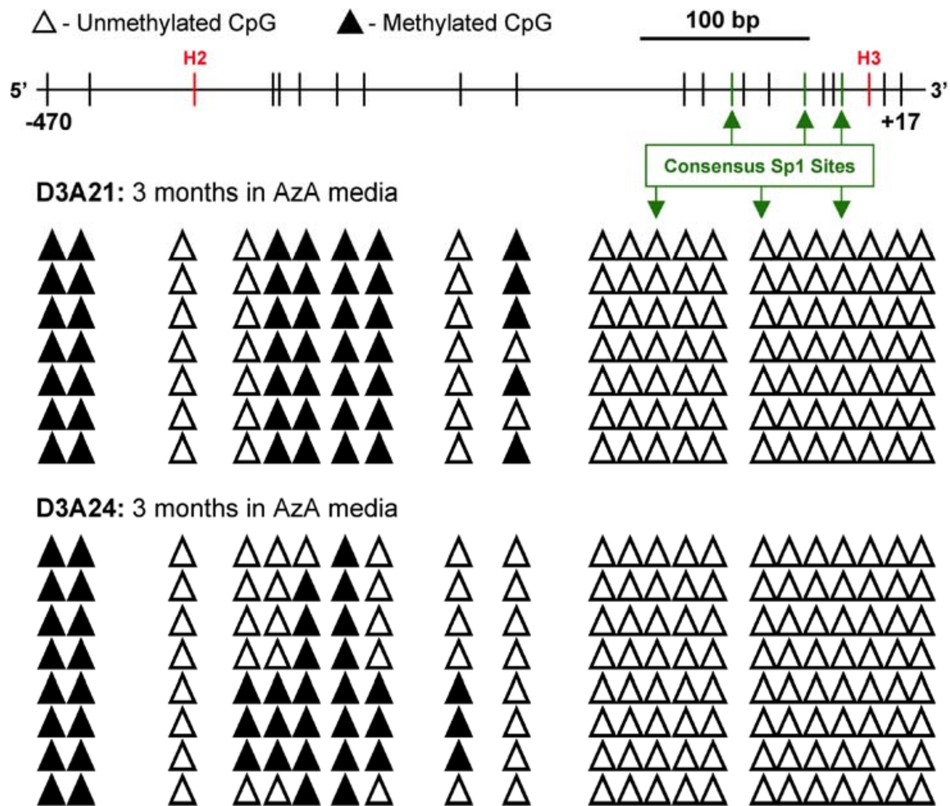


Figure 3-12. The reactivated *Aprt* promoter is eventually demethylated in D3 cells. DNA methylation patterns at the *Aprt* promoter are shown from D3 reactivant subclones that have been continuously growing in AzA media for three months. Vertical dashes mark the relative locations of CpG sites and the triangles indicate the methylation status the corresponding CpG (shaded triangles are methylated and empty triangles are unmethylated). Base pair positions are labeled relative to the *Aprt* transcriptional start site. Other DNA elements marked are the CpG sites within an Sp1 binding site (green dashes) and CpG sites within a HpaII restriction site (red dashes labeled H2 and H3).

To determine if *Aprt* expression would eventually stabilize under selective conditions, reactivant clones were grown for an additional two months (i.e. for a total of three months) in AzA medium. However, even after three months of maintained *Aprt* expression, re-silencing still occurred at high frequencies for both D3-and D3S1-derived reactivants and in most cases changed little from those observed at one month (Fig. 3-11 A & B). Although the *Aprt* re-silencing frequency remained high in D3 reactivants after three months of growth in AzA medium, DNA methylation was not detected in the *Aprt* promoter at this point (Fig. 3-12).

Therefore, promoter region DNA methylation did not appear to account for the very high frequency of re-silencing observed in the D3 reactivant clones. Additional data consistent with this assumption were the observations that re-silencing in D3 reactivants did not require DNA methylation in the *Aprt* promoter region (Fig. 3-13).

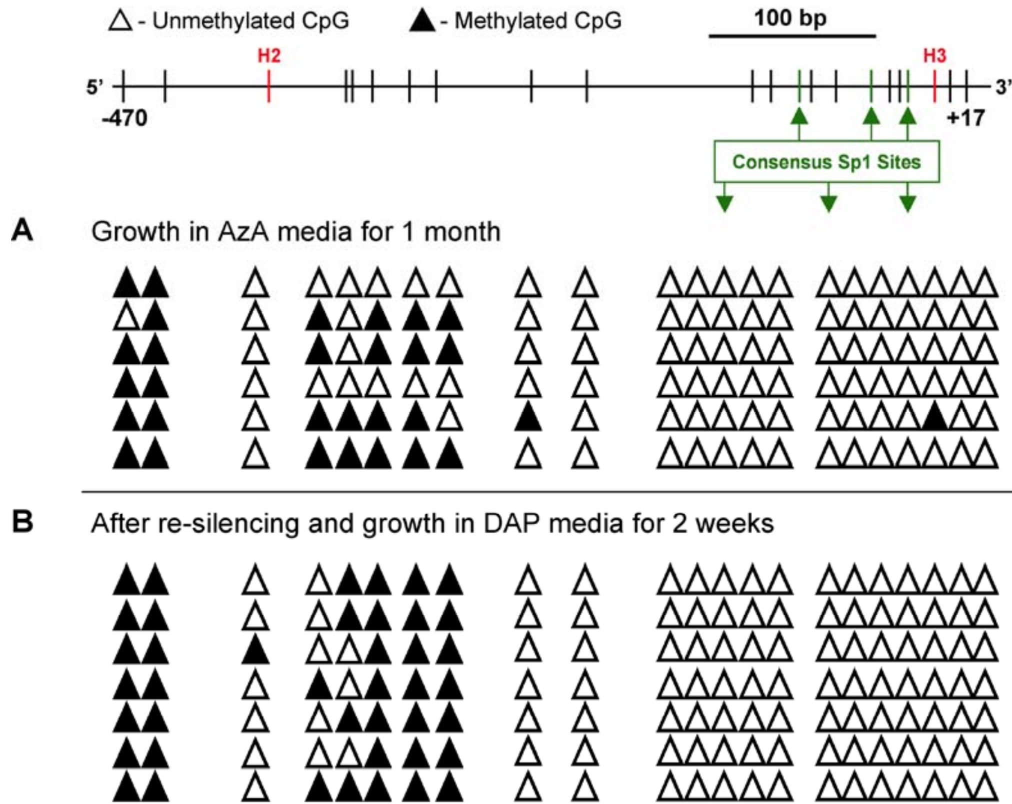


Figure 3-13. Re-silencing of *Aprt* does not require DNA methylation in D3 cells.
(A) DNA methylation patterns at the *Aprt* promoter are shown for a D3 reactivant subclone after continuous growth in AzA media for one month. Aprt expression re-silenced at a frequency of 4.7×10^{-2}.
(B) DNA methylation pattern of alleles that have re-silenced Aprt expression after two weeks continuous growth in DAP media. Vertical dashes mark the relative locations of CpG sites and the triangles indicate the methylation status the corresponding CpG (shaded triangles are methylated and empty triangles are unmethylated). Base pair positions are labeled relative to the *Aprt* transcriptional start site. Other DNA elements marked are the CpG sites within an Sp1 binding site (green dashes) and CpG sites within a HpaII restriction site (red dashes labeled H2 and H3).

Histone modifications are unstable at reactivated *Aprt* promoters

To determine if a failure to restore activating histone modifications could explain the high frequency re-silencing, the levels of acetyl-H3K9, methyl-H3K4, and dimethyl-H3K9 were measured at the *Aprt* promoter in reactivant cell lines and compared to those in P19H22 cells (Fig. 3-14). The results of this analysis failed to identify any specific histone modification, or lack thereof, that consistently correlated with high frequency re-silencing. The general histone modification pattern observed at reactivated *Aprt* promoters more closely resembled that of the active promoter in the P19H22 cells than the histone modifications at the silenced promoters in D3 or D3S1 cells. However, in some instances histone modifications changed from the one to three month time points in ways that were contrary to expectations. For example the levels of dimethyl-K9 H3 increased from the one month to three month time points for three D3S1 reactivant clones (S1A20 and S1A23). These dimethyl-K9 H3 levels were lower than those for P19H22 at the one month time point, but increased beyond that observed in P19H22 at the three month time point. The presence of activating histone modifications acetyl-K9 H3 and methyl-K4 H3 at the *Aprt* promoter in the reactivant cells were often in excess of those observed in the P19H22 parental cells, and again sometimes changed from one to three months. In summary, while these results failed to identify a histone modification to explain high frequency re-silencing in the reactivants, they did reveal remarkable and unstable clonal variations in the reactivants.

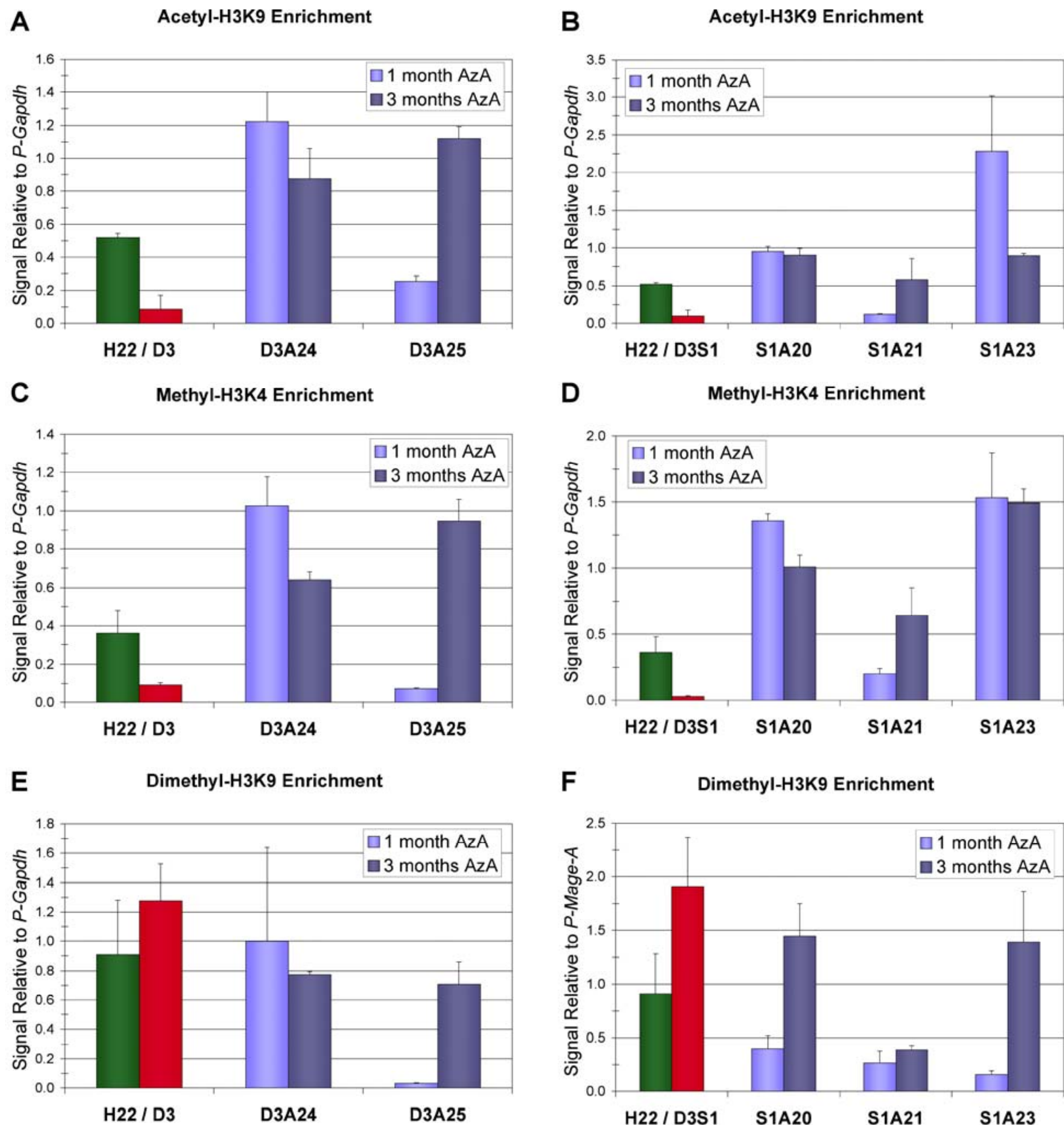


Figure 3-14. Histone modification patterns fail to stabilize at reactivated *Aprt* promoters.
ChIP analysis measured histone H3 modifications at the *Aprt* promoter in D3 and D3S1 reactivant cell lines. (A) ChIP analysis measuring acetylation at lysine 9 of histone H3 (Acetyl-H3K9). (B) ChIP analysis measuring methylation at lysine 4 of histone H3 (Methyl-H3K4). The antibody used for immunoprecipitation recognizes all three forms of methylation at K4, mono-, di-, and tri-methyl. (C) ChIP analysis measuring the repressive modification of dimethylation at lysine 9 of histone H3 (Dimethyl-H3K9). Immunoprecipitated DNA levels were measured by qRT-PCR and normalized to enrichment at either the *Gapdh* promoter or the *Mage-a* promoter.

Discussion

The results of this study revealed two distinct pathways of transcriptional epigenetic silencing at a single endogenous locus, mouse *Aprt*, in embryonal carcinoma cells. In D3 and D3S1 cells, *Aprt* silencing is correlated with increased levels of DNA methylation within the promoter. In contrast, DNA methylation is not present within the silenced *Aprt* promoter of D7 cells. Similar results demonstrating that the epigenetic silencing pathway is not determined solely by DNA sequence in the promoter were shown in a study that identified DNA methylation-associated silencing of tumor suppressor promoters in colon cancer cells and DNA methylation independent silencing of the same promoters in prostate cancer cells [103]. However, here we are showing both pathways acting at the same promoter in a single cell type.

The DNA methylation pattern for the active *Aprt* allele is due to the combination of a cis-acting sequence that includes B1 repetitive elements located 1 kb upstream of the promoter from which DNA methylation spreads [126, 149], and a redundant (with regards to transcription) Sp1 binding site that prevents DNA methylation from spreading into the promoter [89, 139]. This protection creates a DNA methylation boundary upstream of the *Aprt* promoter between the CpG sites at positions –279 and -222 (Fig. 3-1). Despite *Aprt* silencing in D7 cells, this boundary of DNA methylation was maintained because the silenced *Aprt* promoter exhibited a DNA methylation pattern essentially identical to that for the active *Aprt* allele in the parental P19H22 cells (Fig. 3-3). Even D7 subclones isolated under more stringent selection against *Aprt* expression still exhibited the same boundary for DNA methylation (Fig. 3-6). Epigenetic silencing independent of DNA methylation has been previously described [102-104, 133], but it is less well characterized than silencing associated with DNA methylation. The lack of *Aprt* reactivation after D7 cells were treated with 5-aza-dC provided a functional demonstration that

DNA methylation does not contribute to *Aprt* silencing in D7 cells (Fig. 3-8).

Despite the absence of DNA hypermethylation, the silenced *Aprt* promoter in D7 cells was highly enriched in the repressive histone modification dimethyl-H3K9 and showed a corresponding loss in the activating modification acetyl-H3K9. However, levels of methyl-H3K4, which usually decrease with epigenetic silencing [86, 136, 150], remained high (Fig. 3-9). These results demonstrate that loss of methyl-H3K4 is not required for *Aprt* silencing in D7 cells and that this silenced promoter exhibits a bivalent modification pattern (i.e., the co-existence of repressive and activating histone modifications). Promoters that are maintained in a bivalent modification state in embryonal stem cells also exhibit low levels of expression despite relative enrichment of methyl-H3K4 [83, 151]. Thus, acquisition of a bivalent histone modification pattern similar to that commonly observed in embryonal stem cells is sufficient to silence *Aprt* in D7 cells. The simultaneous presence of high methyl-H3K4 levels and resistance to DNA methylation at the silenced *Aprt* promoter in the D7 cells suggests a causal link between these observations. Genomic insulator elements and the histone variant H2A.Z have both been shown to function as barriers to DNA methylation and protect against silencing, but methyl-H3K4 was not examined in these studies [32, 122]. However, mapping of global methyl-H3K4 levels have shown a distribution that overlaps with enrichment of the histone variant H2A.Z at insulator elements and near transcriptional start sites [33]. Our results, combined with those studies, suggest that methyl-H3K4 contributes to blocking the spread of DNA methylation.

In contrast to the D7 cells, *Aprt* silencing in D3 and D3S1 cells correlated with increased DNA methylation, and inhibiting DNA methylation significantly increased *Aprt* reactivation (Figs. 3-5 & 3-7). In both the D3 and S1 cells, the hypermethylated and silenced *Aprt* promoter exhibited reduced levels of methyl-H3K4 and acetyl-H3K9 combined with enrichment of the

repressive histone modification dimethyl-H3K9 (Fig. 3-9). By comparison to the D3 cell line, the higher levels of dimethyl-H3K9 at the *Aprt* promoter in D3S1 cells correlated with increased DNA methylation, decreased *Aprt* mRNA expression, and an approximately 20-fold reduction in the spontaneous reactivation frequency.

While D3S1 cells had nearly complete DNA methylation of the *Aprt* promoter, D3 cells exhibited low to intermediate frequencies of methylated CpG sites in the 3' region (-70 to +17) of the *Aprt* promoter. Interestingly, the Sp1 binding site that was found to contain a fully methylated CpG site in the silenced *Aprt* promoter of D3 cells was the same one found to be redundant for expression from an *Aprt* transgene, but required to resist epigenetic silencing [89]. A similar result has been observed at the reversion-induced LIM protein (*RIL*) locus that is frequently silenced in cancer. Within the *RIL* promoter, a polymorphism was identified that does not initially affect expression levels, but does prevent binding of the Sp1 and Sp3 transcription factors and correlates with eventual DNA hypermethylation and silencing of the *RIL* promoter [88]. Surprisingly, the intermediate methylation pattern at the *Aprt* promoter in the D3 cells has been stable over extended periods of continuous growth in culture. In other words, a progressive increase in DNA methylation is not inherent for the *Aprt* promoter in the D3 cells. However, stably silenced subclones with extensive DNA methylation spread through the entire *Aprt* promoter (such as in D3S1 cells), can be isolated from the D3 cell line.

The comparison of epigenetic characteristics for the silenced *Aprt* promoter in the D3 and D3S1 cells strongly suggests that DNA methylation at a promoter plays a significant role in the relative stability of the silenced state. Examination of spontaneous reactivation allowed us to probe this relationship further. Spontaneous reactivation was examined, as opposed to induced reactivation by direct inhibition of DNA methylation via 5-aza-dC treatment, to determine

whether *Aprt* reactivation would coincide with a gradual retreat of DNA methylation from the promoter, or alternatively if *Aprt* reactivation required a complete loss of promoter region DNA methylation. Surprisingly, both scenarios were observed. Although the stably silenced Aprt promoter in D3S1 cells contained higher levels of DNA methylation, its reactivation correlated with a complete loss of promoter DNA hypermethylation within two weeks, whereas the more weakly silenced Aprt promoter with less DNA methylation in D3 cells did not require demethylation for reactivation. Thus, the presence of DNA methylation in *Aprt* promoters of D3 reactivants after two weeks of AzA selection or longer demonstrates *Aprt* can be actively expressed from a methylated promoter. Active expression from a heavily methylated promoter is unexpected, but it has been observed after reactivating silenced alleles by inhibition of the class III HDAC Sirtuin 1 [72]. Additionally, in vitro experiments have shown Sp1 transcription factors can bind to recognition sequences that have been methylated at the internal CpG site[152]. Most experiments examining reactivation of silenced promoters use direct inhibitors of DNA methylation to induce reactivation [73, 123, 128], and therefore, those experiments would miss the active promoters with DNA methylation that we are reporting. Although some D3 reactivants express *Aprt* with significant DNA methylation in the *Aprt* promoter, the level of DNA methylation decreases as active expression is maintained for extended periods of time.

Reactivation of hypermethylated and silenced alleles is consistently observed after inhibiting DNA methylation, but the induced expression is unstable and re-silencing occurs without continued DNMT inhibition [68, 70, 73, 137]. It is unclear what mediates the memory of transcriptional silencing in induced reactivants; whether repressive epigenetic modifications are never completely removed or activating epigenetic modifications are never fully restored. One difference between those studies and the study described here is that we used continuous

selection for *Aprt* to ensure that the promoters were expressed, and thus cells with re-silenced promoters would be eliminated. Nonetheless, a propensity for very high frequency re-silencing of *Aprt* was exhibited by both D3 reactivants and D3S1 reactivants even after three months of continuous growth in AzA medium, which represents approximately 180 cell divisions under selection for *Aprt* expression. In comparison, the silencing frequency for *Aprt* in the parental P19H22 cells, approximately 10^{-5} to 10^{-6} [148], is orders of magnitude lower than the measured re-silencing frequencies. These results demonstrate that a silenced and reactivated *Aprt* allele is remarkably unstable. We recently reported a similar result for a reactivated *HPRT* transgene [136], which suggests that unstable reactivation is a hallmark of silenced promoters.

An interesting aspect of unstable *Aprt* reactivation in the D3 and D3S1 reactivants is that a memory of the differential silencing states was apparently retained because the D3 reactivants exhibited re-silencing at a ten-fold higher frequency (~10% for D3 versus ~ 1% for D3S1). While it is tempting to invoke retention of DNA methylation on reactivated promoters as an explanation for this difference, the difference in re-silencing frequencies persisted even at the three-month time point when *Aprt* promoters in D3 reactivants were unmethylated. In an attempt to identify the memory of silencing in reactivants and determine why the memory was different in the D3 versus D3S1 reactivants, methyl-H3K4, acetyl-H3K9, and dimethyl-H3K9 modifications were examined by ChIP. While this analysis failed to identify a specific histone modification that could explain the molecular memory that led to high frequency re-silencing, it did reveal that histone modifications were unstable in reactivants because the levels for a specific modification could change within a given reactivant clone as a function of time. Moreover, these changes could be in the opposite direction from what was expected, e.g. the repressive dimethyl-H3K9 modification was found to increase in some D3S1 reactivants from the one month to three

month time point despite continuous selection for *Aprt* expression. Thus, an intriguing possibility is that the instability of histone modifications in the reactivants reflects the inherent instability of epigenetic reactivation of aberrantly silenced alleles. Further work is required to identify the determinants of silencing memory, which could have important clinical implication because stable reactivation of silenced tumor suppressor promoters would improve cancer treatments [134].

In summary, this study has provided a detailed DNA methylation pattern present at the endogenous mouse *Aprt* gene and shown how this pattern can be altered upon epigenetic silencing of *Aprt*. Two distinct pathways of epigenetic silencing were identified. In D7 cells, *Aprt* silencing was characterized by increased dimethyl-H3K9 and occurred independent of DNA hypermethylation and loss of methyl-H3K4. In D3 and D3S1 cells, *Aprt* silencing was correlated with repressive histone modifications, loss of expressive modifications, and increased DNA methylation of the *Aprt* promoter. However, the extent of DNA methylation was different for the D3 and D3S1 cells and apparently resulted in markedly different kinetics of promoter reactivation. Most notably, reactivation was accompanied by rapid loss of promoter region methylation for the D3S1 reactivants, but initial retention of promoter region methylation for many reactivated promoters in D3 reactivations. Moreover, *Aprt* promoters in both D3 and D3S1 reactivants demonstrated a memory of having undergone silencing, as demonstrated by re-silencing frequencies as high as 1% for the D3S1 cells and 10% for the D3 cells. Neither DNA methylation nor a specific histone modification could explain the silencing memory, though shifting histone modifications suggested an inability of the reactivated promoter to permanently reset. In total, these results demonstrate that DNA methylation modulates but does not control epigenetic silencing and reactivation of mouse *Aprt*.

Materials & Methods

Cell Culture

The mouse embryonal cell lines P19H22, D3, D7, and D3S1 were cultured as described previously [112]. P19H22 contain a single copy of *Aprt* derived from the C3H mouse strain [148]. The D3, D7, and D3S1 cell lines were maintained in the presence of 80 μg/ml 2'-6-diaminopurine (DAP) unless otherwise indicated.

Bisulfite Sequencing Analysis

Bisulfite sequencing of the entire 1600 bp region containing the *Aprt* promoter and upstream sequence in P19H22 cells required three independent primer sets. The first reaction (B1) included CpGs between base positions –1599 and –1105, the second reaction (H1) included CpGs between –1135 and –500, and the third reaction (H2) included CpGs between –498 and +60. Genomic DNA was isolated from cell cultures using DNAzol (Molecular Research Center) according to the manufacturer's instructions. For each treatment, 2 - 4 μg of genomic DNA was digested by restriction enzyme, BsrI for the B1 and H2 region or MspI for the H1 region. Digested genomic DNA was modified in a solution of 6.24 M urea, 4 M sodium bisulfite, and 10 mM hydroquinone as described previously [95]. PCR amplification of modified DNA, cloning of PCR products, and sequence analysis were also described elsewhere [126], with the following exceptions. The primers used in the initial PCR reaction for the B1 region were the sense primer B1+S1 5'-TTT GAA GGT TTA TGG GAG TTG-3' and the antisense primer B1+AC 5'-ATC TAA CAC ACA ATC TCC CAT C-3'. PCR product from this initial reaction was used as input in a second reaction with the nested sense primer B1+S2 5'-ATT TGT GTA GTA ATT GTA GAG TTA AGG TTG-3' and the antisense primer B1+AC. For the H1 region, the primers used

in the initial PCR reaction were the sense primer H1+SC 5'-GAG ATT ATG ATG GGA GAT TGT GTG -3' and the antisense primer H1+A1 5'-ATA CCT TCT CTA AAA CCA CAA ACA-3'. PCR product from this initial reaction was used as input in a second reaction with the sense primer H1+SC and the nested antisense primer H1+A3 5'-CTA TAC ATT ACA ACA AAA TAT ACC CTC-3'. For the H2 region, the primers used in the initial PCR reaction were the sense primer H2+S 5'-GAG GAG GGT ATA TTT TGT TGT AAT G-3' and the antisense primer ACA+29 5'-AAA AAC AAA AAA AAA ATA AAT ATC AAC AC-3'. PCR product from this initial reaction was used as input in a second reaction with the nested sense primer H2+NS23 5'-AGT GTT TGT GGT TTT AGA GAA GG-3' and the antisense primer ACA+29. PCR products were cloned using Strataclone PCR cloning kit (Stratagene). Sequencing analysis showed all cytosine bases not present in the CpG dinucleotide context were converted to thymine indicating complete bisulfite modification of the genomic template occurred.

RNA Preparation and Analysis

Total RNA was isolated from cell cultures with the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Total RNA samples were converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen) with removal of genomic DNA contamination. 100 ng cDNA was used as input in subsequent quantitative-PCR analysis for either *Aprt* amplification across the exon 2-3 splice site with the sense primer qAprt-F 5'-CTC TTG GCC AGT CAC CTG AAG-3', the antisense primer qAprt-R 5'-TCT AGA CCT GCG ATG TAG TCG ATC T-3' and the TaqMan probe 5'-FAM-CAC GCA CAG CGG C-MGB-3' or *Gapdh* (Mouse TaqMan Endogenous Control, Applied Biosystems) with iQ Supermix (Bio-Rad) and a Bio-Rad iCycler.

Aprt results were normalized in relation to *Gapdh* mRNA levels and displayed relative to expression levels in P19H22 cells.

Reactivation and Re-silencing Cell Cloning Assays

To measure *Aprt* reactivation, cells were plated into 100 mm culture plates at densities ranging from $1x10^3$ to $1x10^5$ cells per plate. The next day the medium was removed, and medium containing 10 µg/ml azaserine and 10 µg/ml adenine (AzA) was added to select for active *Aprt* expression. The same protocol was used to measure *Aprt* re-silencing, but the selective media contained 80 µg/ml DAP instead of AzA. Cells were cultured for approximately two weeks in the appropriate selective media before staining live colonies with crystal violet solution. To estimate cloning efficiencies, additional cells were plated under identical conditions as selective plates but at lower densities, 250 to 1000 cells per plate, without selection for or against Aprt expression. Silencing or reactivation frequencies were calculated by dividing the number of clones growing under selection by the effective number of cells plated (as determined with the cloning efficiency plates).

Drug Treatments

Cells were treated overnight (~16 hours) with media containing 300 nM TSA (Wako) to inhibit histone deacetylation, 3 µM 5-aza-dC (Sigma) to inhibit DNA methylation, or the combination of 300nM TSA and 3µM 5-aza-dC. Cells were allowed to recover 24 hours in DMEM after drug treatment before harvesting for RNA purification or plating to measure *Aprt* reactivation.

Chromatin Immunoprecipitation

ChIP assays were carried out as described previously [136]. Protein-DNA complexes were immunoprecipitated with antibodies to acetyl-H3K9 (07-352, Millipore), mono/di/trimethyl-H3K4 (05-791, Millipore), dimethyl-H3K9 (ab1220, Abcam), and trimethyl-H3K27 (17-622, Millipore). Quantitative PCR using an Icycler and iQ Supermix (Bio-Rad) was used to analyze the immunoprecipitated DNA. The *Aprt* promoter was amplified and detected using the sense primer 5'-AAC GTA TGT CGA GGT AGG CGT GTA-3', the antisense primer 5'-ATC TCC TTC ATC ACA TCT CGA G-3', and the TaqMan probe 5'-FAM-TAC CTC CTC CCT GCC TCC TAC A-3'. The active *Gapdh* promoter was amplified using the sense primer 5'-TTG AGC TAG GAC TGG ATA AGC AGG-3', the antisense primer 5'-AAG AAG ATG CGG CCG TCT CTG GAA-3', and the TaqMan probe 5'-FAM-TAT AAA TAC GGA CTG CAG CCC TCC CT-3'. The silenced *Mage-a* promoter was amplified using the sense primer 5'-GTT CTA GTG TCC ATA TTG GTG-3' and the antisense 5'-AAC TGG CAC AGC ATG GAG AC-3', and amplification and quantitation was done using iQ SYBR Green Supermix (Bio-Rad). The specific signal from each immunoprecipitation relative to signal from input was calculated for the three promoters, *Aprt*, *Gapdh*, and *Mage*. For activating modifications, levels at *Aprt* are displayed relative to the *Gapdh* promoter; for the repressive modification, dimethyl-K9 H3, results are displayed relative to the *Mage* promoter.

Chapter 4

DISCUSSION AND CONCLUSIONS

Summary

A central goal of my thesis research was to examine the dynamic process of aberrant epigenetic silencing. Specifically, the aim was to discern the sequence of epigenetic changes that occur as an active, expressed allele transitions to an inactive, silenced state. Extensive research has characterized specific molecular modifications that are associated with either active expression or epigenetic silencing. Therefore, several epigenetic modifications that correlate with aberrant silencing are known, but the order in which they occur is not clear. In the past, research in cancer epigenetics focused almost exclusively on DNA methylation, but more current methods measure additional epigenetic modifications, covalent histone modifications and nucleosome positioning, to determine how these changes function in epigenetic silencing pathways.

Despite the strong emphasis initially placed on DNA methylation, recent results have suggested it is a late step in the silencing process [79, 101, 153] and even unnecessary in certain instances of aberrant silencing [103, 104, 132, 133, 154]. My results, which show that induction of epigenetic silencing is not reduced after inhibition of DNA methylation, functionally demonstrate that DNA methylation is not an early requirement during initiation of the silencing process. For these experiments I developed a novel application of the Tet-Off gene expression system designed to test the effect of reduced transcription on aberrant epigenetic silencing. Using this system, I was able to accurately model aberrant silencing pathways that contribute to tumorigenesis, as the epigenetic modifications associated with silencing induced in the Tet-Off/*HPRT* system were the same as those commonly observed at silenced tumor suppressor genes. Additionally, this system showed that transient reductions in gene expression induce aberrant epigenetic silencing.

Although induced silencing remained a rare event (< 1% of cells), the corresponding loss of *HPRT* expression allowed for selection of cells with silenced promoters to be analyzed separately from an overwhelming background of promoters that reactivate expression. Thus, the ability to isolate and analyze the silenced promoters quickly after epigenetic silencing allowed distinction between early (H3K9 deacetlyation) and late (DNA methylation) epigenetic changes. Similar selective strategies were used to examine epigenetic changes during reactivation of aberrantly silenced *Aprt* in mouse EC cells.

Three cell lines were isolated with variable levels of DNA methylation at the silenced endogenous *Aprt* promoter, ranging from no methylation in the D7 cell line to nearly complete methylation in the D3S1 cells. ChIP analysis showed that in the D7 cells *Aprt* silencing correlated with a bivalent histone modification pattern consisting of high levels of methyl-H3K4 and methyl-H3K9 at the promoter independent of DNA methylation. In contrast, the silenced *Aprt* promoters in the D3 and D3S1 cells contained DNA hypermethylation and were associated with decreased levels of methyl-H3K4. These silenced promoters were also characterized by increased methyl-H3K9 relative to the active promoter in P19H22 cells. However, absolute levels of methyl-H3K9 were variable, and higher methyl-H3K9 at *Aprt* in D3S1 cells were associated with increased promoter DNA methylation and resistance to spontaneous reactivation. Although the *Aprt* promoter was more methylated and stably silenced in the D3S1 cells than the D3 cells, reactivation in D3S1 cells correlated with a loss of DNA methylation from the *Aprt* promoter, whereas some reactivated *Aprt* promoters in D3 cells still contained DNA methylation. Maintaining active expression eventually led to demethylation of the *Aprt* promoter in D3 cells, but a memory of epigenetic silencing exists and destabilizes the reactivated expression as measured by high frequency re-silencing. The memory of epigenetic silencing was also

observed in reactivated *Aprt* alleles lacking DNA methylation in D3S1 cells and P_{TRE}-*HPRT* alleles in the Tet-Off system, which suggests memory of silenced expression is a common feature of reactivated promoters. Several reactivated promoters restored of mRNA expression and activating histone modifications to the same levels originally measured before silencing, but expression still re-silences at a high frequency indicating a persistence of the silencing memory.

Transcriptional activation & initiation of aberrant silencing

Epigenetic silencing is a molecular mechanism frequently responsible for inactivation of tumor suppressor genes in sporadic human cancers, but how this aberrant process initiates is not clearly understood. Identification of initiating factors would provide valuable molecular targets for cancer prevention and treatment. Experimental systems demonstrate that loss of transcription precedes DNA methylation during aberrant silencing [79, 80, 101, 118, 153], which suggests that reductions in gene expression could be an early initiating step. Examples of decreased gene activation leading to subsequent aberrant silencing have also been observed *in vivo*. Loss of *estrogen receptor-*α and *GATA6* in cancer cells have been shown to correlate with stable epigenetic silencing of downstream target genes [81, 82]. While both of these examples involve loss of activating factors, increased gene repression may also initiate aberrant silencing [87]. Genetic targets of polycomb repressor complexes are maintained at low levels of gene expression and appear to be highly prone to aberrant silencing and DNA hypermethylation in tumors [83-85]. Moreover, the Sp1 family of transcription factors has been shown to protect against epigenetic silencing by a mechanism that may be independent from promoting gene transcription and unrelated to gene expression levels [88, 89, 100, 142, 144]. At the silenced *Aprt* allele in D3 cells, high frequency DNA methylation spread to the upstream Sp1 binding

site, including the CpG site within the Sp1 consensus sequence, which suggests that this site is also significant in silencing of the endogenous *Aprt* allele (Fig. 3-5).

In order to directly test the hypothesis that reduced binding of transcriptional activators increases susceptibility to aberrant silencing, I used the Tet-Off gene expression system to control expression levels of an *HPRT* cDNA in *Hprt* -/- mouse cells. Dox reduces *HPRT* expression in the Tet-Off system by directly binding the Tet-transcriptional activator and preventing its localization to the P_{TRE}-*HPRT* promoter. The Tet-transcriptional activator contains the VP16 transcriptional activation domain that promotes gene expression by recruitment of the TATA-binding protein (TBP), TFIIB, and the SAGA histone acetyltransferase complex [117]. Therefore, reduced P_{TRE}-*HPRT* expression occurs by losing recruitment of these activating factors, and this is a significant difference from previous experimental systems that induced aberrant silencing by direct establishment of DNA methylation [78]. A consequence of inducing aberrant silencing by directly establishing DNA methylation is the inability to examine either repressive epigenetic changes that would normally precede DNA methylation or silencing pathways that function independent of DNA methylation. Although Dox treatment does not directly induce repressive epigenetic modifications, decreased recruitment of the Tet-transcriptional activator and the corresponding reduction in gene expression induced aberrant epigenetic silencing of the P_{TRE}-*HPRT* promoter (Fig. 2-2). The induced frequencies of aberrant silencing are low relative to the total number of alleles; less than 1% are epigenetically inactivated, but significantly, the silencing frequencies after Dox treatment were orders of magnitude greater than those of untreated controls. Additionally, the frequency of aberrant silencing increased as the reduced transcriptional state was maintained for longer time periods (Fig. 2-2). Because the silencing of tumor suppressor genes is an aberration as opposed to a

normal regulated function, it is unlikely that epigenetic silencing at extremely high frequencies would accurately mimic the occurrences in cancer. Instead, the relative levels of induction are what is important. Equivalent gene inactivation frequencies seen with mutational mechanisms or chemical carcinogens support the notion that the level of induction observed in these experiments would be relevant to tumorigenesis *in vivo*.

Although the reduction in gene expression increased epigenetic silencing frequencies, reactivants that restore gene expression are not necessarily stabilized. This observation showed that the unknown epigenetic changes must accompany active transcription in order to establish stable expression. However, this could indicate either the existence of an activating epigenetic modification that is not restored by active transcription or a repressive modification that persists after reactivation and destabilizes the promoter without inhibiting active expression.

Histone deacetylation

Histone deacetylation is a repressive epigenetic modification that is consistently observed at silenced alleles and is also detected as an early epigenetic change that occurs before DNA methylation [79, 101, 118]. Although histone deacetylation is known to be an early change during the silencing process, the Tet-Off/*HPRT* experimental system provided a functional demonstration that initiation of aberrant silencing is dependent on HDAC activity (Fig. 2-9). Several lysine residues on the histone amino-terminal tails are *in vivo* substrates for acetylation, and it has been shown previously that in general histone acetylation correlates with active transcription. However, the functional consequences of acetylation at the individual sites are not well understood. Results from the Tet-Off/*HPRT* system indicated that acetylation at H3K9 and H3K14 can be regulated independently as Dox treatment reduced gene expression and caused

deacetylation of H3K14, but not of H3K9 (Fig. 2-5). Therefore, maintenance of H3K9 acetylation did not require high levels of gene expression or localization of the transcriptional activator. Similar observations that acetylation of H3K9 can be maintained at a promoter without of active expression or continuous recruitment of transcriptional machinery have also been made in other experimental systems [118, 155]. Deacetylation of H3K14 may be an epigenetic change required for aberrant silencing, but this modification alone is insufficient to initiate epigenetic silencing. During reduced expression, acetylation at H3K14 is reduced in the entire cell population, but most cells reactivate expression after removal of Dox, thus indicating additional epigenetic changes are required for stable silencing. However, deacetylation at H3K14 may have contributed to the increased susceptibility to epigenetic silencing measured after reducing gene expression.

Deacetylation of H3K9 appeared to be a significant epigenetic modification in the initiation of silencing because decreased acetyl-H3K9 was measured at all silenced promoters, P_{TRE}-*HPRT* and *Aprt*, analyzed by ChIP (Fig. 2-5 & 3-9). Moreover, HDAC inhibition inhibited aberrant silencing in the Tet-Off/*HPRT* system during initial induction and re-silencing of reactivated promoters. As a consequence of occurring early in the silencing process, later modifications are dominant and must be reversed prior restoring histone acetylation in reactivation of silenced alleles. For example, DNA methylation has been considered dominant to histone deacetylation based on the observation that TSA treatment, which inhibits HDAC, usually produces no effect on reactivation of heavily methylated alleles, but acts in synergy when used together with DNA methylation inhibitors. In these studies, TSA was capable of inducing reactivation of silenced P_{TRE}-*HPRT* alleles presumably because they were tested before the epigenetic silencing had stabilized. This is consistent with the observation that the cell lines

most responsive to TSA treatment were also those with lower levels of DNA methylation at the promoter. The use of selection allowed for more sensitive measurement of reactivation frequencies. This showed TSA treatment alone did have a small but biologically significant effect on inducing reactivation of the heavily methylated *Aprt* promoter in D3S1 cells. The increased number of cells in AzA resistant-colonies induced by TSA treatment suggested that HDAC inhibition increased the expression levels of reactivated promoters. The failure of HDAC inhibition to induce reactivation of silenced *Aprt* promoter without DNA hypermethylation in D7 cells demonstrates that DNA methylation is not the only repressive epigenetic modification that is dominant to reversal of histone deacetylation. This could be a consequence H3K9 methylation that was highly enriched at the *Aprt* promoter in D7 cells.

Histone methylation

Methylation at H3K9 was another repressive epigenetic modification associated with transcriptional repression that was observed at all silenced promoters in these experiments. Loss of acetylation must precede this repressive modification because both modifications occur at the same residue. Therefore, deacetylation of H3K9 must occur before the lysine can be methylated. Although increased methylation was measured at silenced promoters relative to actively expressed controls, the levels of H3K9 methylation among the silenced promoters were variable. In general, high levels of H3K9 methylation correlated with increased resistance to reactivation and higher levels of promoter DNA methylation. The one exception was the silenced *Aprt* promoter in the D7 cell line, which only had enriched H3K9 methylation as a repressive epigenetic modification. In the absence of DNA methylation, this modification was sufficient to silence *Aprt* expression, although the D7 cell line had a high spontaneous reactivation frequency

and relatively high levels of *Aprt* mRNA compared to other silenced cell lines. Enrichment of dimethyl-H3K9 was also measured at silenced promoters containing DNA methylation. In the filamentous fungi, *Neuorspora crassa*, DNA methylation patterns are established by H3K9 methylation [16]. A relationship between the two repressive epigenetic modifications exists in mammals, but it is not as direct. In mammals, the H3K9 methyltransferase, G9a, is required for proper de novo DNA methylation, but neither the G9a catalytic activity nor H3K9 methylation is required [23]. Instead, the relationship may occur due to interactions between heterochromatin protein 1 (HP1) and the DNA methyltransferases.

DNA methylation

Conventional thinking has regarded the relationship between DNA methylation and epigenetic silencing as a simple cause and effect scenario where DNA methylation induces epigenetic silencing. This simplified interpretation seemed reasonable based on deceptively straightforward experimental results that showed methylation of promoter DNA silences transcription [100]. Conversely, silenced and hypermethylated promoters can be reactivated by inhibiting DNA methylation. However, there are caveats to each of these observations that complicate interpretation of the data. Although DNA methylation is sufficient to induce silencing that does not necessarily mean that aberrant silencing *in vivo* initiates with DNA methylation. Because of the self-reinforcing nature of the epigenetic state, it would be expected that a late modification can be experimentally established and mediate other repressive modifications that otherwise would have occurred earlier. The caveat regarding DNA methylation and reactivation is that although transcription is resumed, it is unstable and transcription will quickly re-silence. Therefore, inhibiting DNA methylation can induce a

temporary reactivation, but it does not entirely reverse the silenced epigenetic state. DNA methylation was not required for epigenetic silencing because inhibiting DNA methylation did not affect the initiation of silencing in the Tet-Off/*HPRT* system and *Aprt* in D7 cells is silenced without DNA methylation of the promoter. Additionally, DNA methylation must be unable to independently prevent transcription as evidenced by reactivated *Aprt* in D3 cells without loss of promoter DNA methylation.

Conclusions

Collectively, these experiments have provided significant details of the epigenetic silencing process. Transient reductions in expression were shown to increase promoter susceptibility to silencing. This represents a potential mechanism by which environmental factors known to repress transcription (hypoxia, disrupted hormone signaling, and exposure to toxins) can initiate epigenetic silencing and promote cancer. Additionally, I showed the transition from transiently reduced expression to silenced expression requires histone deacetylase activity. However, this transition does not require promoter region DNA methylation, but eventually silenced promoters do acquire DNA methylation as well as other epigenetic modifications associated with silenced tumor suppressor genes. Once alleles have been silenced, stable reactivation requires more than reversing the DNA methylation status. Using selection to maintain fully reactivated mRNA expression levels for three months, I found expression fails to completely stabilize. Alleles still re-silence at a high frequency although the promoters no longer contain DNA hypermethylation. In addition to these results, these experimental systems can be used further to continue to identify steps or specific enzyme requirements in the silencing pathway. Learning more about the dynamic process of silencing as opposed to the endpoint may

help extensively in cancer prevention strategies as well as providing details of endogenous pathways of epigenetic regulation.

Appendix 1

OUTLINED FUTURE EXPERIMENTS

Functional analysis of epigenetic silencing pathways using the Tet-Off/HPRT system

Examine epigenetic modifications at earlier time points after the isolation of silenced TG-resistant clones.

For the experiments presented in Chapter 2, TG-resistant clones had been growing continuously in TG media in the absence of Dox for more than one month before ChIP and bisulfite sequencing analysis, but adequate cell numbers for these assays are available at earlier time points. Even for extremely early time points, individual clones could be pooled to generate adequate numbers of cells for analysis and yield informative results. These experimental approaches could further distinguish the differential timing of epigenetic changes during aberrant silencing.

Measure binding of the Tet-transcriptional activator (Tet-TA) at silenced P_{TRE}-*HPRT* promoters by ChIP analysis.

This experiment is relatively straightforward but would provide important details regarding how repressive epigenetic modifications inhibit transcription. If the repressive epigenetic modifications that silence P_{TRE}-*HPRT* act by altering the chromatin structure and reducing accessibility to transcriptional machinery, then Tet-TA binding at the promoter would be reduced. However, it is also possible that the repressive epigenetic modifications are able to inhibit expression by mechanisms that do not prevent physical localization of transcription factors. A technical complication could be the lack of an antibody against the Tet-TA that binds effectively enough for immunoprecipitations. This problem could be avoided by addition of an epitope tag to Tet-TA.

Measure additional histone modifications at the distinct transcriptional states in the Tet-Off/*HPRT* system; active expression, reduced expression, early silencing, and late silencing.

With the ever-expanding number of histone modifications that may affect epigenetic regulation of transcription, it is exceedingly difficult to measure all potential histone modifications. However, certain modifications may warrant examination based on recent studies. Histone methylation at H3K27 or H4R3 have both been reported to promote subsequent DNA methylation and could be potential early changes measured in this system [25, 83, 85]. Additionally, multiple methyl groups can be added to histone residues and may reflect different functional consequences. In Chapter 2, the antibody used to measure H3K4 methylation recognizes all different forms (mono-, di-, and tri-) of methylation. After Dox treatment and reduced expression, levels of methyl-H3K4 increased (Fig. 2-5) and may reflect a shift in methylation status and preferential binding between the ChIP antibody and a specific methyl configuration. Antibodies specific to mono-, di-, or tri-methyl H3K4 would confirm this, if true.

Identify specific enzymes that mediate aberrant silencing.

This experimental aim would use the same methodology employed to show that HDAC inhibition by TSA treatment prevented initiation of aberrant silencing. Additional chemical inhibitors could be used following Dox treatment to determine if inhibition either decreases or increases the frequency of induced silencing. Although this approach is limited to commercially available inhibitors, the technique could also be adapted to inhibit function of a single specific protein by transfection of siRNAs directed against the protein of interest. For example, to determine if a specific histone deacetylase, HDAC2, is required for initiation of aberrant silencing, the cell line could first be screened to ensure HDAC2 is expressed. Next, siRNAs

targeted specifically against HDAC2 could be designed and conditions for effective knockdown could be verified and optimized. Finally, siRNAs could be transfected during the Dox treatment to measure any change in the frequency of induced silencing. Reciprocal experiments could be performed by transfection of an expression construct to show HDAC2 overexpression causes an opposite effect from that observed in knockdown experiments. These experiments could be used to extensively breakdown and identify protein requirements for the dynamic process of aberrant silencing.

Transfer the Tet-Off/*HPRT* system into other cell types to determine how cell type-specific differences affect epigenetic silencing pathways.

The general Tet-Off gene expression system has been used extensively across many diverse cell types and organisms, so the basic mechanics of the Tet-Off/*HPRT* system should work in nearly any cell culture model. Besides normal cell types, silencing pathways that are active in cancer cell lines could be compared to those in more normal primary cells to identify tumor-specific epigenetic regulation. Additionally, if induction of silencing was tested in cells that can be put into a reversible growth arrest during the Dox treatment, it could be determined if DNA replication is required for epigenetic modifications that initiate aberrant silencing. The main caveat to these experiments is that cells need to be *HPRT*-deficient. If *HPRT* null cells cannot be isolated, then a different selectable gene product can be substituted for the *HPRT* cDNA and expressed from the P_{TRE}. For example, ganciclovir could be used as a selective agent against expression of the viral *thymidine kinase* gene to isolate silenced promoters.

Determine how induced silencing is affected by loss of transcriptional activators, Tet-TA, and simultaneous recruitment of transcriptional repressors via fusion to reverse-Tet.

DNA binding activity of the reverse-Tet (rTet) protein has been engineered to be the opposite of the normal Tet-protein. That is, reverse-Tet binds to its DNA recognition sequence only when it is also bound by Dox. Therefore, upon Dox treatment Tet-TA disassociates from the promoter, while rTet simultaneously binds to the promoter. If rTet is fused to a specific protein repressor domain, the potential effects on induced silencing due to recruitment of the repressor domain could be measured. Alternatively, activation domains could be fused to rTet in an attempt to identify proteins that would protect against aberrant silencing.

Test for induced aberrant silencing of promoters that are regulated by environmental factors present *in vivo*.

The Tet-Off/HPRT system confirmed the hypothesis that reductions in gene expression would increase susceptibility to stable epigenetic silencing. If endogenous promoters are subject to the same regulatory mechanisms, environmental factors that decrease expression should induce epigenetic silencing. Hypoxia, altered estrogen levels, and inflammation are various environmental factors associated with cancer that have been shown to decrease expression of tumor suppressor genes. An experimental system similar to the Tet-Off/HPRT system can be designed by cloning specific promoter regions upstream of selectable gene products. For example, the promoter from human mismatch repair gene *hMLH1* (P_{hMLH1}) can be cloned upstream of the *HPRT* cDNA sequence and transfected into an *HPRT*-deficient human cell line. Hypoxia has been shown to repress transcription from the P_{hMLH1} [156], so silencing frequencies can be measured after cells are grown under hypoxic conditions to determine if hypoxia-

mediated repression induces epigenetic silencing of P_{hMLH1}. One unique aspect of this system is that epigenetic changes induced by hypoxia can be measured at the endogenous *hMLH1* locus as well as the P_{hMLH1}-*HRPT* transgene. These two distinct copies of can be distiguished by primers designed to the unique sequences flanking endogenous P_{hMLH1} or P_{hMLH1} within the transgene. This comparison would determine whether any epigenetic modifications or hypoxic effects are directed by DNA sequence. In other words, the comparison would identify any changes that occur specifically at promoter sequence elements within P_{hMLH1} independent of stochastic chance or differences in genomic context.

Epigenetic silencing of endogenous *Aprt*

Identify molecular requirements for DNA methylation-independent silencing of *Aprt* in D7 cells.

Aprt silencing in D7 cells was associated with increased methylation of H3K9 and did not require promoter DNA methylation. As a result, inhibition of DNA methylation by 5-aza-dC treatment had no effect on induced reactivation. HDAC inhibition by TSA treatment also failed to induce reactivation although histone deacetylation modification present at the silenced *Aprt* promoter in the D7 cells. To determine if H3K9 methylation is maintaining epigenetic silencing, the H3K9 methyltransferase G9a can be targeted for knockdown by transfected siRNAs. ChIP analysis will measure H3K9 methylation after G9a knockdown to verify a functional consequence at *Aprt*. If H3K9 methylation at Aprt is not decreased after G9a knockdown, other H3K9 methyltrasferases (Suv39H1, EuHMTase, etc.) could be targeted in attempt to identify the responsible enzyme. Another approach to test the dependence of *Aprt* silencing on H3K9 methylation would be to measure *Aprt* reactivation after overexpression of a Jumonji family H3K9 demethylase.

Attempt to induce DNA methylation at the *Aprt* promoter in D7 cells to identify factors responsible for the resistance to DNA hypermethylation.

The high levels of H3K4 methylation at the silenced Aprt promoter in D7 cells may be preventing DNA hypermethylation. Overexpression of the H3K4 demethylase LSD1 will decrease global levels of H3K4 and may potentially increase susceptibility of the Aprt promoter to DNA methylation. After LSD1 overexpression, D7 cells could be subcloned under stringent selection (FA media) against *Aprt* expression. Selection of FA-resistant D7 clones was

attempted previously and failed to isolate *Aprt* promoters with DNA methylation. However, a majority of D7 cells initially die in FA selection. If decreased H3K4 does allow DNA methylation and stronger epigenetic repression, these clones may be detected above background levels of FA-resistant D7 subclones. Binding of Sp1 transcription factors may also contribute to protection against DNA methylation. Binding of Sp1 or Sp3 transcription factors at *Aprt* could be measured by ChIP analysis. If the Sp1 or Sp3 transcription factors were protecting the promoter from DNA methylation, the silenced *Aprt* promoter in D7 cells should be associated with higher levels than the silenced and DNA methylated *Aprt* promoter in D3 cells.

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