

MECHANISMS UNDERLYING THE PHARMACOLOGIC REVERSAL OF
GENETIC AND EPIGENETIC COMPONENTS OF TUMOR SUPPRESSOR GENE
SILENCING IN HUMAN BREAST CANCER

by

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A Dissertation Submitted to the Faculty of the
COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2006

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

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ACKNOWLEDGEMENTS

The author would like to acknowledge the contributions of others to the research described in this dissertation. First, I would like to thank my parents for their steadfast support and unselfish sacrifices throughout my life, without which none of this would have been possible. I owe a debt of gratitude to my advisor, Dr. Bernard Futscher, for his patience, enthusiasm, professional advice and above all, a willingness to allow me the freedom of exploration throughout my time in his laboratory. To the members of my committee, I am especially thankful for their interest and thoughtful insight into my project. I would also like to thank Drs. Klimecki, Lau and Tsaprailis, as well as Yelitzia Rodriguez and Yelena Feinstein, who each made significant technical contributions to my sodium bisulfite sequencing and proteomics efforts. In the lab, I am grateful to have worked with a group of people that shared a true sense of camaraderie and with whom I've shared the many good times and wonderful memories of graduate school. Finally, I thank God for all he has given me in this life, but particularly for my best friend, greatest supporter, and the love of my life, Carrie.

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LIST OF ABBREVIATIONS

5-aza-CR	-	5-azacytidine
5-aza-CdR	-	5-aza-2'-deoxycytidine
Ad-GFP	-	adenovirus containing GFP
Ad-p53/GFP	-	adenovirus containing wt p53 and GFP
AP-1	-	activator protein-1
ATP	-	adenosine triphosphate
ATRA	-	all-<i>trans</i> retinoic acid
BAF	-	BRG1/BRM-associated factors
BRCA1	-	breast and ovarian cancer susceptibility protein 1
BRCA2	-	breast and ovarian cancer susceptibility protein 2
BRG1	-	brahma-related gene-1
BRM	-	brahma
°C	-	degrees Celsius
CAR	-	coxsackie adenovirus receptor
CBP	-	CREB-binding protein
cDNA	-	complementary DNA
ChIP	-	chromatin immunoprecipitation
CID	-	collision induced dissociation
cm	-	centimeter(s)
CP-31398	-	small molecule rehabilitator of mutant p53
CpG	-	cytidine-guanosine DNA dinucleotide
Ct	-	threshold cycle
dA	-	deoxyadenosine
dC	-	deoxycytidine
DCIS	-	ductal carcinoma <i>in situ</i>
dCTP	-	deoxycytidine tri-phosphate
dG	-	deoxyguanosine
DMEM	-	Dulbecco's modified Eagle's medium
DMSO	-	dimethylsulfoxide
DNA	-	deoxyribonucleic acid
DNMT	-	DNA methyltransferase
dT	-	deoxythymidine
DTT	-	dithiothreitol
ECL	-	electrochemiluminescence
EDTA	-	ethylenediaminetetraacetic acid
EED	-	embryonic ectoderm development protein
ESI	-	electrospray ionization
ESR	-	electron spin resonance
ETS	-	erythroblastosis virus E26 oncogene
EZH2	-	enhancer of zeste 2

eV	-	electron volt
FACS	-	fluorescence-activated cell sorter
FBS	-	fetal bovine serum
g	-	force of gravity (e.g., 10000 x g)
G418	-	geneticin, antibiotic
G9A	-	H3 K9-specific methyltransferase
GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase
GFP	-	green fluorescent protein
GLP	-	H3 K9-specific methyltransferase
H1	-	histone 1
H2A	-	histone 2A
H2B	-	histone 2B
H3	-	histone 3
H4	-	histone 4
HBSS	-	Hank's balanced salt solution
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer
HFF	-	human foreskin fibroblasts
HMEC	-	human mammary epithelial cells
HP1	-	heterochromatin protein 1
HPLC	-	high-performance liquid chromatography
hrs.	-	hours
IgG	-	immunoglobulin
IP	-	immunoprecipitate(d)
JNK	-	Jun-N-terminal kinase
K	-	lysine
kb	-	kilobase
K9	-	lysine residue 9 within histone H3
K27	-	lysine residue 27 within histone H3
kDa	-	kilodalton
KJ	-	kilojoules
kV	-	kilovolt
LC-MS	-	liquid chromatography-mass spectrometry
LCQ	-	ion trap mass spectrometer used in these studies
LCIS	-	lobular carcinoma <i>in situ</i>
LiCl	-	lithium chloride
mAb	-	monoclonal antibody
MAD1	-	mitosis arrest deficiency 1
MALDI	-	matrix assisted laser desorption ionization
MBD	-	methyl-CpG binding domain
MDA-MB	-	MD Anderson-metastatic breast cell lines
MDM2	-	mouse double minute 2 protein
MECP	-	methyl-CpG binding protein
MEGM	-	mammary epithelial growth medium
mg	-	milligram(s)

μg	-	microgram(s)
mJ	-	millijoules
min.	-	minute(s)
mL	-	milliliter(s)
μL	-	microliter(s)
mm	-	millimeter
μm	-	micrometer
mM	-	millimolar
μM	-	micromolar
MOI	-	multiplicity of infection
mRNA	-	messenger ribonucleic acid
MS/MS	-	multi-dimensional mass spectrometry
MW	-	molecular weight
m/z	-	mass-to-charge ratio
nm	-	nanometer(s)
nt	-	nucleotides
p300	-	300 kDa histone acetyltransferase protein
p53	-	tumor suppressor protein p53
pfu	-	plaque forming units
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
PBS-T	-	phosphate buffered saline-tween
PCAF	-	p300/CBP-associated factor
PCR	-	polymerase chain reaction
PIPES	-	piperazine-1,4-bis(2-ethanesulfonic acid) buffer
PMSF	-	phenylmethanesulfonyl fluoride
PRIMA-1	-	p53 reactivation/induction of massive apoptosis compound-1
PVDF	-	polyvinylidene fluoride
RACE	-	rapid amplification of cDNA ends
RIPA	-	radio-immunoprecipitation assay buffer
Rn	-	normalized fluorescence
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
RPMI	-	Roswell Park Memorial Institute (cell culture media)
RSB	-	nuclei isolation buffer
RT	-	reverse transcriptase
SAHA	-	suberoylanilide hydroxamic acid
SAM	-	S-adenosylmethionine
SDS	-	sodium dodecyl sulfate
sec.	-	second(s)
s.e.m.	-	standard error of the mean
SERPINB5	-	serine proteinase inhibitor, clade B, member 5
SIM	-	single ion monitoring
siRNA	-	small interfering ribonucleic acid

SUV39H1	-	suppressor of variegation 3-9 homolog 1
SUV39H2	-	suppressor of variegation 3-9 homolog 2
SUZ12	-	suppressor of zeste 12
SV40	-	simian virus 40
SWI/SNF	-	switching/sucrose non-fermenting (originally in yeast)
SYBR	-	fluorescent dye
TBE	-	tris-borate-EDTA buffer
TIC	-	total ion current
TSA	-	trichostatin A
TSQ	-	triple quadrupole mass spectrometer
U	-	units
UACC	-	University of Arizona Cancer Center
UV	-	ultraviolet light
V	-	volts
v/v	-	volume/volume percent
wt	-	wild type
yr	-	year(s)

ABSTRACT

In women, tumors of the breast remain the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths. One of the hallmarks of carcinogenesis is the abnormal silencing of tumor suppressor genes by both genetic and epigenetic alterations, leading to defects in cell-cycle control, DNA repair, apoptosis and cell adhesion. This dissertation focuses on the elucidation of the genetic and epigenetic mechanisms associated with tumor suppressor gene silencing in human epithelial breast tumor cells, and the development of pharmacologic strategies aimed at reversing these types of repression through gene therapy and chromatin remodeling. Desmocollin 3 (DSC3) and MASPIN are anti-metastatic tumor suppressor genes that are silenced in a large percentage of breast tumors via aberrant DNA hypermethylation and histone hypoacetylation of their promoters. DSC3 and MASPIN are also p53-target genes, requiring its transcriptional activation to promote normal expression levels, yet a significant fraction of breast tumor cell lines express mutant forms of p53. Adenoviral-mediated re-introduction of wild type (wt) p53 into mutant p53-expressing breast tumor cells resulted in significant up-regulation of DSC3 and MASPIN expression, although not to the levels seen in normal breast epithelial cells. Mechanistically, the addition of wt p53 to these tumor cells resulted in increased histone acetylation and enhanced chromatin accessibility of the DSC3 and MASPIN promoters, despite continued cytosine hypermethylation. Pre-treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-CdR) prior to wt p53 addition produced synergistic reactivation of both DSC3 and MASPIN in breast cancer cells, approaching their levels in normal

mammary cells. However, 5-aza-CdR did not significantly reduce DNA methylation in many cases as originally theorized. Therefore, follow-up studies focused on the identification of alternative, novel mechanisms of 5-aza-CdR-mediated induction of epigenetically silenced genes, finding that it consistently reduced transcriptionally repressive histone H3 lysine 9 (K9) methylation levels in the promoter regions of both DSC3 and MASPIN in breast tumor cells, by mediating global decreases in the histone H3 K9 methyltransferase, G9A. In summary, these results clearly show that cancer treatments targeting both genetic and epigenetic facets of gene regulation may be a useful strategy towards the therapeutic transcriptional reprogramming of cancer cells.

INTRODUCTION

Breast Cancer Overview

Types of breast cancer

Breast cancer is a complex, multistage disease process with multiple endpoints. Breast tumors typically begin formation in the epithelial cells that line the ducts or lobules of the breast (1) (**Fig. 1**). If the cancer cells spread outside the ducts or lobules of the breast into the surrounding tissue, the cancer is termed invasive or malignant (2). Pre-invasive breast cancer is the name for abnormal cells or cancer cells that stay inside the milk ducts or milk sacs (lobules) of the breast. Ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) are types of pre-invasive breast cancer, with DCIS accounting for ~85% of localized breast tumors (1). If not treated in this early stage, pre-invasive cancers of the breast can progress to the invasive type (3), decreasing the likelihood for successful treatment and underscoring the importance of early detection by mammography (1).

Epidemiology of breast cancer

Breast cancer is the most frequently diagnosed cancer in women, with 212,920 new cases of invasive and 61,980 cases of *in situ* breast cancer expected in the United States in 2006 (1). Despite increased early detection by mammography since the 1980s, breast tumors are still the second leading cause of cancer-related deaths in women, with 40,970 predicted to die this year from this disease (1). Important risk factors for breast

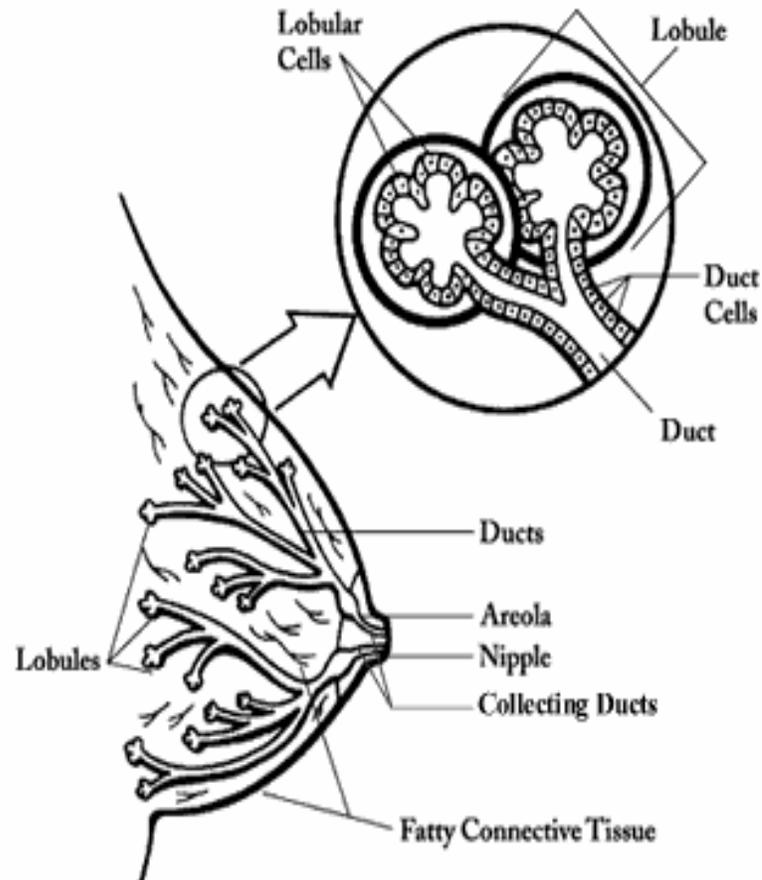


Figure 1. Breast tissue is made up of milk glands, supportive fibrous tissue, fatty tissue, arteries, veins, lymph vessels and nerves. The milk glands consist of lobules, where milk is made, and ducts, which take the milk to the nipple. There are several types of breast cancer, with nearly all beginning in either the milk ducts or milk lobules (see diagram above). Some breast cancers are found *in situ*, meaning they have not spread outside the duct or lobule where they began. However, most breast cancers are found when they are invasive, having grown beyond the duct or lobule into other breast tissue or spread out of the breast to distant sites such as the lymph nodes. (Diagram taken from http://www.cancer.org/docroot/CRI/content/CRI_2_2_1X_What_is_breast_cancer_5.asp)

cancer include: sex (female), age (over 50), race (caucasian), obesity, excessive alcohol consumption, use of post-menopausal hormone replacement therapy, familial or personal history of breast cancer and exposure to previous breast radiation to treat other types of cancer (e.g., Hodgkin's disease or non-Hodgkin's lymphoma) at a young age (4).

Although only 5-10% of breast cancer cases are linked to genetic predisposition, a few important genetic risk factors have been identified for this disease. Most notably, women with inherited mutations in the BRCA1 or BRCA2 tumor suppressor genes have been cited to have up to an 80% chance of developing breast cancer during their lifetime (5). In addition, rare cases of inherited germline mutations in the p53 tumor suppressor gene (a condition known as Li-Fraumeni syndrome) have also been shown to greatly increase breast cancer risk in women (6). These genetic predispositions are especially troublesome, as those inheriting them are likely to acquire breast cancer at a much earlier age than those developing this disease due to the other risk factors noted above.

Genetic Mechanisms of Carcinogenesis

Alterations to DNA during carcinogenesis

The development of cancer is a complicated process in which a host of factors interact to disrupt normal cell growth and division. These can include both internal factors such as heredity, immunology and hormones, as well as external factors such as chemicals, viruses and radiation, which are collectively referred to as carcinogens or mutagens. Carcinogens can act to chemically alter DNA bases so that they are replaced by an incorrect base during replication, resulting in a substitution or point mutation. If

these erroneous substitutions are not corrected by DNA repair enzymes, this mutation becomes irreversible and is passed on to all future progeny. Additionally, defects in DNA replication and/or repair can result in the insertion or deletion of bases, causing a frameshift that alters the read-out of all subsequent codons. Of relevance to cancer, mutations, deletions or insertions in the coding regions of a gene can lead to the functional activation of proto-oncogenes or the inactivation of tumor suppressor genes, enhancing the opportunity for tumor development (7, 8). Additionally, during the latter stages of cancer progression, many types of larger-scale chromosomal aberrations can also occur as a result of mutagen-mediated DNA damage and errors in chromosomal segregation during meiosis. The most common chromosomal alterations in cancer include structural changes in the form of deletions, amplifications or translocations of large stretches of DNA, or in some cases, the gain or loss of whole chromosomes, leading to increases or decreases in gene copy number or the creation of distinct oncogenic fusion gene products. Although individuals can be predisposed to develop cancer by inheriting mutations in genes that regulate cell growth, proliferation and differentiation (as noted earlier), most cancers, including those of the breast, arise from multiple exposure-related defects to these pathways. These acquired defects take time to accumulate, resulting in a latency period of years or decades between carcinogenic exposures and the appearance of cancer. However, because of this latency period, it is especially difficult to detect tumors at their earliest and most vulnerable stages.

Genetic alterations and the stages of carcinogenesis

Carcinogenesis proceeds in an ordered manner that is comprised of three distinct phases: initiation, promotion and progression (9). While abnormal alterations to both the genome and epigenome can contribute to each stage of tumorigenesis, the roles played by epigenetic events throughout tumor development will be discussed later. With regard to genetic abnormalities, normal cells can become initiated when a single somatic cell undergoes a non-lethal, but heritable mutation in a gene that alters its normal function. This initiating mutation is irreversible and may confer a growth advantage upon the initiated cell necessary for the second stage of promotion, allowing it to escape normal cellular regulatory mechanisms that prevent uncontrolled cell division. Tumor promotion occurs only to initiated cells following exposure to a promoting agent, causing these cells to undergo phenotypic clonal expansion and increasing the likelihood of propagating a genetic error (10, 11). Tumor promoters can be either external (e.g., ethanol, phorbol esters, UVA radiation) or internal (e.g., hormones) stimuli (11), and the signal to expand clonally can result from a direct effect of the tumor promoter or be due to an indirect effect on adjacent cells (12). Nonetheless, tumor initiation and promotion together produce relatively benign growths. It is the third step in which these growths undergo malignant conversion, following further deregulation of cell growth to a more uncontrolled state that allows for metastasis to distant sites in the body. Progression, like initiation, also often requires genetic alteration, most notably at the chromosome level in the form of somatic aneuploidy and progressive karyotypic instability (11). This is also the most complex of the three stages, because both acquired genetic and phenotypic

changes occur, and cellular expansion is rapid. As the tumor progresses, sensitivity to dietary compounds, inhibitors of growth, and enhancers of differentiation gradually disappears until the tumor becomes progressively more autonomous and non-localized, requiring more drastic intervention (13).

Tumor Suppressor Protein p53

History and function of tumor suppressor protein p53

The first tumor suppressor gene to be identified, p53, was thought of as an oncogene when it was originally described in 1979, since it was found in the extracts of transformed cells and reacted with antiserum from tumors induced by simian virus 40 (SV40) (14-16). Subsequent studies also showed the ability of p53 cDNA clones isolated from tumor cell lines to cooperate with the RAS oncogene in the transformation of primary rat embryo fibroblasts (17, 18). However, the initial identification of p53 as an oncogene was later rescinded as the p53 cDNA clones used in these earlier studies were all found to encode mutated forms of the p53 gene, and wt cDNAs from normal cells did not induce transformation (19, 20). Further evidence began to shed light on the anti-tumor effects of p53 and led to it being dubbed the “guardian of the genome” due to the ability of p53 to induce G1 arrest in response to DNA damage, demonstrating that it was instead a tumor suppressor gene in its native form (21, 22). It is now well-known that p53 is a potent transcriptional activator of downstream genes involved in the initiation of multiple tumor suppressor functions, including the surveillance of genomic integrity/DNA repair, cell-cycle arrest, inhibition of angiogenesis/metastasis and

apoptosis (**Fig. 2**) (23, 24). Consistent with the functions of wt p53 in normal cells, levels of this protein have been shown to be elevated in response to a variety of cellular stresses, including DNA damage, hypoxia and the expression of mitogenic oncogenes (25). In the face of cell stress, p53 is activated via post-translational phosphorylation, leading to disruption of its basal interaction with MDM2 (26), a protein that keeps p53 levels low in unstressed cells by targeting it for ubiquitin-mediated proteolysis (27, 28). In its inactive state, p53 can homotetramerize and bind to its response elements in the promoters of p53-target genes, however, post-translational activation is required to induce transcription of these genes (29, 30). The activation of p53 allows it to recruit transcriptional co-activators such as the histone acetyltransferases p300, CREB-binding protein (CBP) and PCAF to p53-responsive promoters, proteins that act to maintain an open chromatin conformation that is transcriptionally permissive (29-34).

Genetic alterations to p53 during tumorigenesis

Due to its many roles in protecting cells from aberrant growth, it is not surprising that the dysregulation of the p53 protein is a frequent event in cancer. In fact, genetic mutation of p53 and loss of function occurs in approximately 50% of all cancers (35, 36) and 20-40% of human breast cancers (37-39). Moreover, mutation of p53 is the most common genetic defect related to a single gene in human cancer (24). Functionally, p53 is almost exclusively subject to single missense mutations in its centrally-located DNA binding domain (amino acids 92-293) (**Fig. 3**), often leading to deleterious conformational changes or the loss of important DNA contact points (38). Thus, most

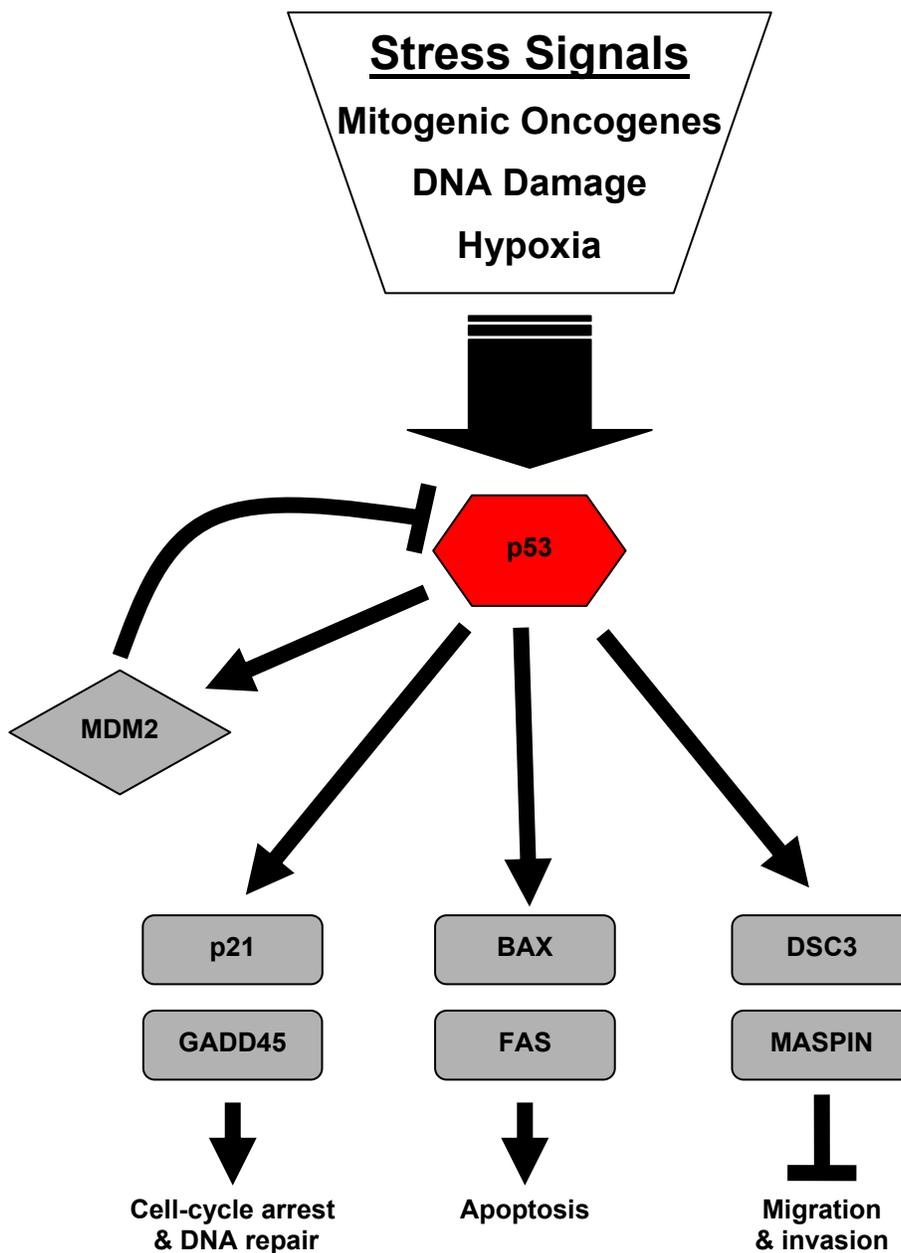


Figure 2. Summary of the mechanisms of action for the tumor suppressor protein p53 in normal cells. In response to various stress signals, p53 is upregulated resulting in the transcriptional activation of genes involved in cell cycle arrest (p21), DNA repair (GADD45) or the inhibition of migration and invasion (DSC3 and MASPIN). However, in cases of irreversible DNA damage, p53 has also been shown to enhance expression of the pro-apoptotic genes BAX and FAS. Increased p53 activation also induces transcription of MDM2, which targets p53 for ubiquitin-mediated degradation to keep p53 levels in check.

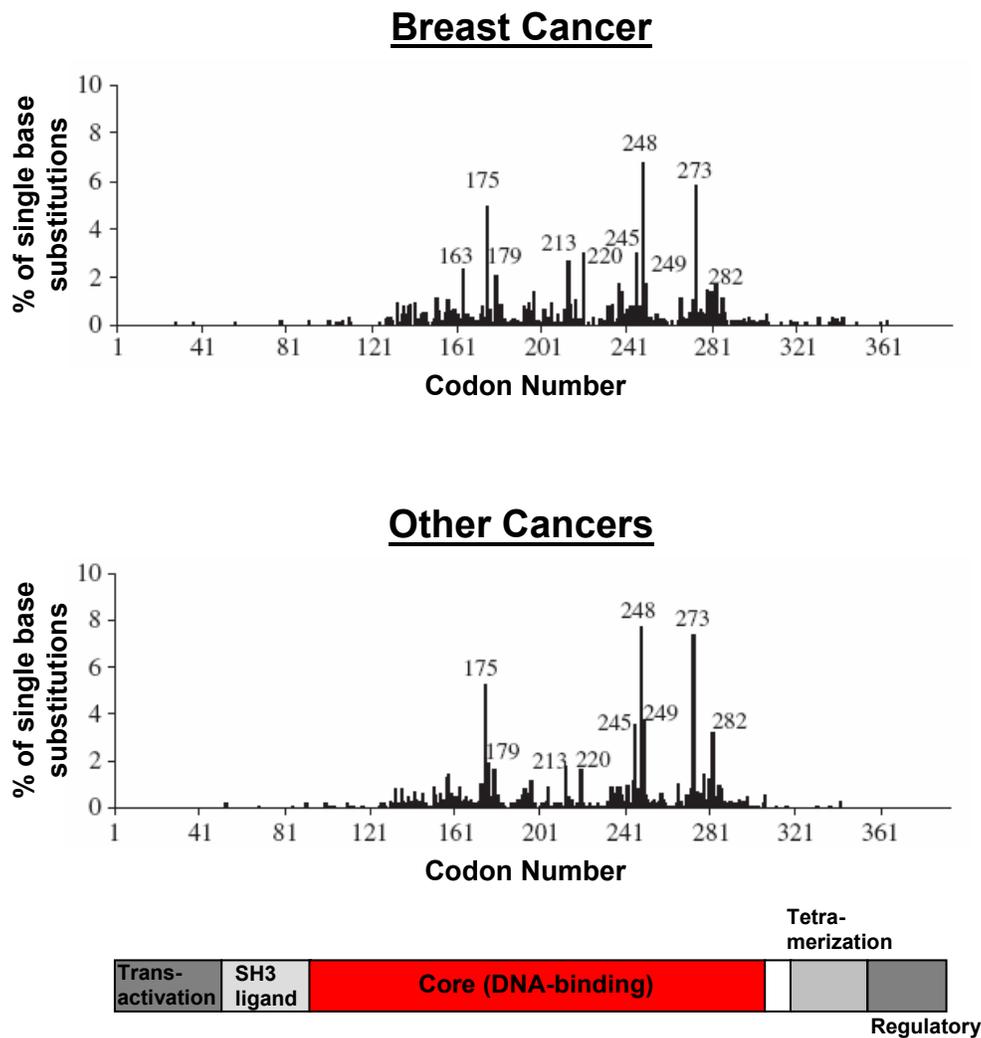


Figure 3. The p53 protein consists of 393 residues and contains an amino-terminal transactivation domain, a proline-rich SH3 ligand, a core DNA-binding domain, a tetramerization domain and a carboxy-terminal regulatory domain. Histograms are shown summarizing the type and position of p53 mutations in breast cancer and other cancers. Somatic mutations in p53 were extracted from the IARC p53 Mutation Database and graphed to show the distribution and frequency of single base substitutions along the p53 coding sequence. Approximately 95% of p53 mutations occur in the core DNA binding domain, with the labeled codons being hotspots for mutation.

studies to date have focused on how mutation of p53 affects DNA binding and transactivation of its target genes (40, 41). Seminal research in this area demonstrated that while wt p53 bound a p53 consensus site to activate the expression of downstream genes in a non-chromatin context, various mutant forms of p53 could not bind the same site to activate transcription (42). Other studies, however, have shown that many p53 point mutants are still capable of binding to certain p53 responsive elements, but are likewise unable to initiate transcription (41, 43, 44). Additionally, in cells transfected with both wt and mutant p53, it was concluded that mutant forms can associate with wt p53 to create a heteromeric complex that abolishes the ability of wt p53 to bind DNA through a dominant-negative effect, preventing transcriptional activation of target genes (42). These results provide a basis for the selection of p53 mutants during tumorigenesis.

Prognostic significance of p53 mutation in breast cancer

The fact that p53 is often mutated during tumorigenesis led researchers to study the clinical implications of p53 mutation in breast cancer. These studies have investigated the association between alterations in the p53 gene and clinical outcome of breast cancer, with most investigators reporting poorer overall and disease-free survival in breast cancer cases with somatic mutations in p53 (37, 45, 46). Efforts to differentiate the significance of mutations in different regions of the p53 protein have also demonstrated that patients with alterations in the DNA binding domain, or in the zinc-binding regions have the worst overall survival (47, 48), consistent with its function as a transcription factor. Further work found that p53 mutation also correlates with more

aggressive breast tumors (49-51), as well as resistance to traditional anticancer therapies and relapse (52-55). Therefore, mutation of p53 is a clinically significant event in breast cancer and an important prognostic indicator in patients with this disease.

Pharmacologic Approaches to Overcoming Mutant p53 in Tumors

Reintroduction of wt p53

As p53 mutation is a common event in cancer and predicts decreased survival and increased resistance to traditional cancer therapies, many researchers have sought more specific means to attack this target therapeutically in mutant p53-expressing tumors. A number of investigators have attempted to simply reintroduce wt p53 in tumor cells that express mutant forms of the p53 protein, using stable transfection and adenoviral-based infection methods. These studies found that forced over-expression of the native protein can overcome the dominant-negative effects of mutant p53 in these cells to reactivate p53-target genes, leading to suppression of tumor growth and the potentiation of DNA damaging drugs (56-59). Clinical trials using intratumoral injection of wt p53-expressing adenovirus have also been conducted, with objective antitumor activity seen in several patients in a manner that was well-tolerated (60-63). However, there have also been failures with this approach, with the main obstacle being effective delivery of wt p53 to tumor cells. Adenoviral uptake is mediated by the coxsackie adenovirus receptor (CAR) present on the cell surface, and several primary human cancers express low levels of CAR and are thus refractory to adenovirus infection (64-66). Therefore, new approaches aimed at improving infection efficiency in CAR-deficient tumor cells are currently being

developed to redirect adenoviral entry via CAR-independent pathways (67-70). Another reason for clinical inactivity in some cases has been due to insufficient gene delivery and viral transduction after intratumoral injection of adenoviral p53 expression vectors, since most clinically applied adenoviruses are engineered to be incapable of replication for safety reasons. To increase the tissue penetrance of p53 adenoviral treatments, researchers have developed a conditionally replicative adenovirus (ONYX-015) that can only produce viral progeny in tumor cells harboring p53 mutations, allowing infected tumor cells to specifically amplify the oncolytic effect via lateral spread through solid tumors carrying this particular mutation (71, 72). In initial clinical trials, the safety and anti-tumor activity of ONYX-015 in a number of patients with a variety of solid tumor types has been encouraging (73-81), especially in combination with other chemotherapeutics (cisplatin and 5-fluorouracil), making p53 gene therapy a potentially promising option in the future. However, while some degree of tumor-selective replicaton has been demonstrated in patients, further improvement in the design of p53 recombinant vectors will be needed in order to achieve better clinical response as a single agent.

Reactivation of mutant p53

In addition to reintroduction of wt p53 in tumor cells expressing mutant p53, much work has focused on methods to reactivate or rehabilitate these mutant p53 proteins. Most of these efforts have been aimed at identifying peptide chaperones or small molecules capable of interacting with conformationally inept mutant forms of the

p53 protein to help stabilize and restore their native structure and DNA binding affinities. These strategies are especially attractive since mutant p53 proteins evade MDM2-mediated degradation, resulting in a large pool of p53 in tumor cells that has been previously sensitized to apoptosis due to oncogene activation (82). Moreover, such treatments should be specific for tumor cells, since normal cells express very low levels of wt p53 that are already capable of target gene transactivation in their present state. Thus, effective pharmacologic reactivation of mutant p53 should efficiently eliminate tumor cells by inducing apoptosis with minor undesirable side-effects.

Early attempts at mutant p53 reactivation focused on the development of synthetic peptides derived from the p53 C-terminus that could bind the core and C-terminal domains of endogenous p53 mutants to induce stabilization of the wild type conformation. A number of these peptides demonstrated the ability to restore the specific DNA binding/transactivation function to mutant p53 proteins, leading to the suppression of tumor growth by inducing rapid, p53-dependent apoptosis in cancer cells (83-85). Following these studies, another flurry of intense effort went into the screening of chemical libraries, with the goal of uncovering small molecules capable of inducing pharmacologic rescue of mutant p53 conformation and function. Two of the most widely studied small molecules in this regard include CP-31398 and PRIMA-1. Similar to the results seen with synthetic peptides, both of these compounds have been shown to stabilize the DNA binding domain of mutant p53 proteins in tumor cells to maintain an active conformation that promotes transcriptional activation and tumor growth suppression in mice inoculated with human tumor cells (86, 87). In yet another attempt

to reactivate mutant p53, Dobbelstein and colleagues created an adenovirus expressing an adaptor protein composed of the DNA binding and tetramerization portions of a splice variant of the p53-homologue p73, which efficiently activates transcription of several p53-responsive promoters, fused to the oligomerization domain of wt p53 (**Fig. 4**) (88). The oligomerization domain of wt p53 in this chimera is necessary for the recruitment of mutant p53 proteins that are still thought capable of interacting with their cadre of co-activator proteins, but simply lack the ability to bind DNA in most cases. Upon infection of tumor cells harboring p53 mutations with this p53/p73 chimera construct, these researchers have reported re-expression of p53-target genes and the suppression of tumor cell growth/colony formation in culture via increased apoptosis, effects that were limited to cells expressing mutant p53 (88).

Although the above attempts to rehabilitate mutant p53 have shown promise, some limitations of these treatments have also been uncovered. One such obstacle resides in the fact that there are a wide spectrum of mutations in the core domain of p53, with each resulting in differing degrees of destabilization and loss of DNA binding affinity (89). In fact, it has been found that some mutants with extensive structural changes are indeed refractory to reactivation by the strategies cited above, most notably those with mutations in the zinc binding site (86, 88, 89). Moreover, although studies with small molecule rehabilitators have proven efficacious in inhibiting tumor growth, the cellular concentrations of drugs such as CP-31398 are not currently maintained at high enough levels for practical treatment (87). Therefore, it is important to expand upon these initial attempts to reactivate mutant p53 in cancer cells, by synthesizing or

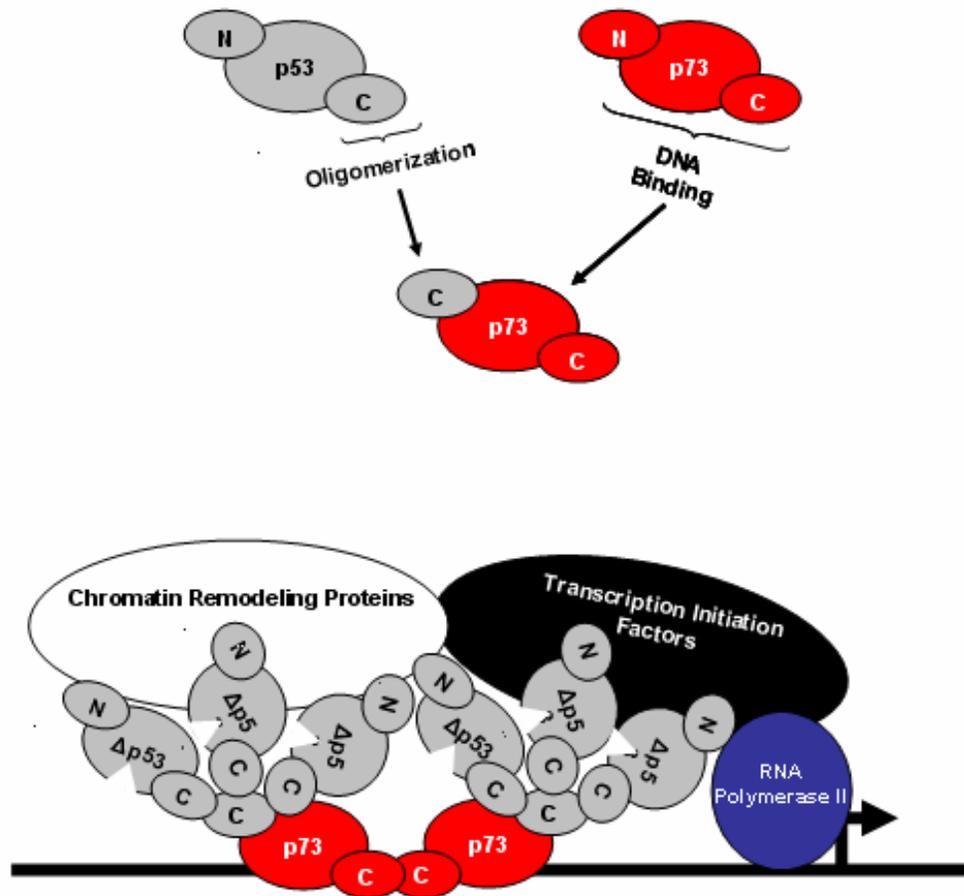


Figure 4. Diagram of the p53/p73 fusion chimera, designed to bind to p53 response elements and recruit point mutants of p53 incapable of binding DNA by themselves. This adaptor protein was created by fusing the DNA binding and tetramerizing portions of the p53-homologue p73 to the oligomerization domain of p53. This chimera binds to the DNA of p53-responsive promoters through the p73-derived portions, and it binds to mutant p53 by the p53-derived oligomerization domain. Through this one-hybrid system, mutant p53 is re-enabled to activate transcription by recruiting its normal co-activator proteins involved in chromatin remodeling and transcriptional initiation.

identifying derivatives of these compounds that increase potency and allow for reduced dosages that are more tolerable to cancer patients with tumors expressing a wide array of p53 mutants.

Epigenetic Mechanisms of Carcinogenesis

Epigenetic regulation of gene transcription and its role in carcinogenesis

The human genome contains information in two forms, genetic and epigenetic. The genetic information provides the blueprint for the manufacture of all the proteins necessary for cell survival, while the epigenetic information supplies instructions on how, when and where the genetic information should be utilized, ensuring that genes are turned on at the proper time and turned off when not needed. More specifically, epigenetic regulation results in heritable but reversible phenotypic states that are not mediated by changes in DNA sequence, but rather by modifications to DNA and histones that alter chromatin structure, among other mechanisms. The reversible nature of epigenetic modifications allows them to serve as a tractable set of switches crucial to normal cellular differentiation and proper organism development via transcriptional regulation, X chromosome inactivation, genomic imprinting and enhanced chromosomal stability (90). Over the years, a multitude of epigenetic modifications have been identified and found responsible for the direction of chromatin states that are either transcriptionally permissive or repressive (91-94). The promoter regions of transcriptionally active genes contain an abundance of permissive epigenetic modifications, which create open, accessible chromatin architectures. Conversely, the promoters of inactive genes are

associated with high levels of repressive modifications, leading to the formation of closed, condensed chromatin domains that prevent transcription factor access and binding. Because of the importance of epigenetic gene regulation in normal cells, it is not surprising that abnormal alterations to the epigenome have been implicated in a number of human diseases such as cancer, autoimmune syndromes and psychiatric and behavioral disorders (95). In fact, epigenetic dysregulation is now recognized as an additional contributor to nearly every stage of tumor development, from the silencing of critical tumor suppressor genes to the enhancement of chromosomal instability (91, 96). Moreover, there is increasing evidence that the epigenetic disruption of progenitor cells may be a common early event in human cancer (97).

DNA methylation

DNA methylation at the C5 position of cytosine residues within cytosine-guanine dinucleotides (CpG) is the only covalently modified base in the mammalian genome not associated with DNA damage, and was the first epigenetic mark to be correctly identified (98). In normal human somatic cells, 5-methylcytosine accounts for ~1% of total DNA bases and therefore affects 70%-80% of all CpG dinucleotides in the genome (99). The cytosine methylation machinery in mammals is primarily composed of the DNA methyltransferase (DNMT) enzymes DNMT1, DNMT3A and DNMT3B. DNMT1 is the most abundant methyltransferase and functions to preserve methylation patterns during DNA replication by preferentially methylating hemi-methylated DNA (100, 101), whereas the DNMT3 family members are largely responsible for establishing *de novo*

methylation patterns (102, 103). All DNMTs utilize S-adenosylmethionine (SAM) as the methyl-donor in these reactions (**Fig. 5**). Functionally, researchers have found that 5-methylcytosine is necessary for the maintenance of genomic integrity and is also strongly associated with transcriptional repression when elevated levels of this modified base are present in CpG-dense islands that encompass upstream gene regulatory regions (104, 105). Cytosine methylation can directly interfere with the binding of certain transcription factors (106), but this is unlikely to be a widespread mechanism for transcriptional silencing as most transcription factors do not have CpG dinucleotides within their binding sites. Instead, the principal method by which DNA hypermethylation within these regions suppresses gene expression is through an indirect mechanism, by virtue of its ability to attract proteins that alter the modification profile of nearby histones to drive the formation of transcriptionally incompetent chromatin domains (107-109). Evidence for this indirect silencing effect comes from the identification of a number of proteins that contain methyl-CpG binding domains (MBDs) capable of selectively binding to methylated DNA. The first such protein to be discovered was methyl-CpG binding protein 2 (MECP2) (110), which was later found to be one member of a family of MBDs that now include MBD1-4 (111). Further characterization of these MBDs found that they also contain other domains involved in the recruitment of repressive histone modifying enzymes to aid in the transcriptional silencing of gene promoter regions by inducing localized chromatin condensation (112-114). In addition, the DNMTs are also now known to interact with many of the same transcriptionally suppressive histone modifiers

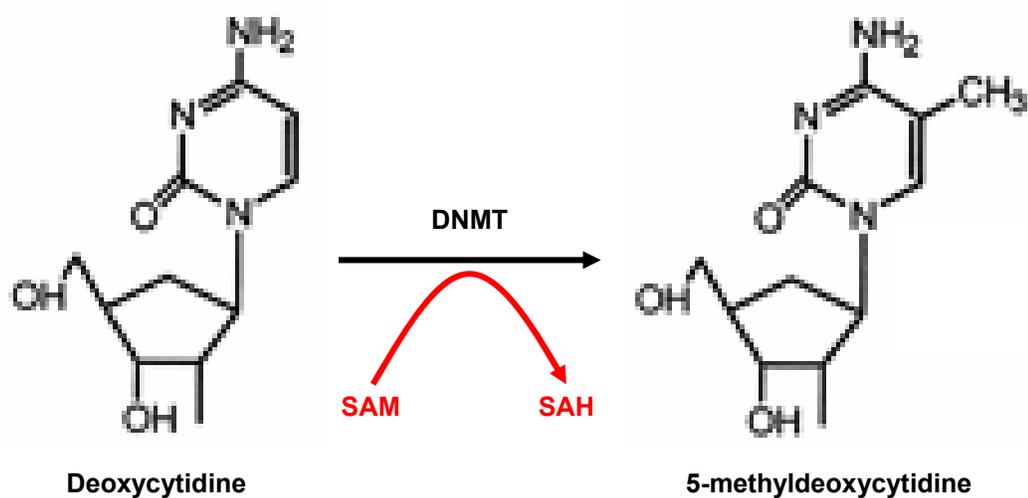


Figure 5. Structures of the deoxycytidine residue and its methylated derivative, 5-methyldeoxycytidine, which is methylated at the 5 position of the carbon ring of the cytosine base. Cytosine methylation is catalyzed by the DNMT enzymes, which utilize the methyl-donor SAM.

as MBDs (109, 115-117), giving further validation for the indirect silencing of gene expression by DNA methylation.

Alterations to DNA methylation in cancer

It has long been recognized that DNA methylation patterns in tumor cells are altered relative to those of normal cells. Tumor cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation events (118-121). In cancer, most of the hypomethylation events appear to occur in repetitive and parasitic elements, which are normally heavily methylated, whereas the promoter CpG islands associated with tumor suppressor genes are known to become aberrantly hypermethylated (122, 123). Hypomethylation of repetitive DNA during tumorigenesis can lead to chromosomal instability and increased mutation rates (124, 125), while the hypermethylation and inactivation of tumor suppressor genes has its own obvious deleterious consequences. In human cancers, it has been estimated that an average of 600 out of 45,000 CpG islands in the genome become aberrantly hypermethylated, in a manner that is non-random (126). With ~50% of human genes containing CpG islands within their promoters (127), the list of tumor suppressor genes found transcriptionally inactivated by hypermethylation in cancer is long and steadily growing, and includes genes that are part of every cancer-related pathway (91). The aberrant hypermethylation of CpG islands in cancer could be explained by the observation that all of the DNMTs exhibit over-expression in many cancers, relative to their normal counterparts, especially DNMT3B which has *de novo* methylation activity (128-130). In total, these results

highlight the negative consequences of altering normal DNA methylation patterns with regard to tumor development. Hence, aberrant DNA methylation is now accepted as a critical mechanism of gene silencing, in addition to mutation and deletion.

Histone modifications

The basic repeat element of chromatin is the nucleosome, which is comprised of a histone octamer containing two copies each of the core histones H2A, H2B, H3, H4 around which 146 bp of DNA are wrapped. With the help of additional proteins, including histone H1, the nucleosomes are packed into higher order structures. The N-terminal tails of core histones are exposed on the nucleosomal surface and are subject to several covalent modifications. In recent years, an ever-growing number of these histone modifications have been identified and characterized (92, 93) (**Fig. 6**). Serine residues can be phosphorylated, arginine residues methylated and lysine (K) residues acetylated, methylated or ubiquitinated. These modifications affect chromatin structure/accessibility to either potentiate or inhibit gene expression. The large number of modification sites and their huge combinatorial potential allows a wealth of epigenetic information to be stored within the histone tails. Recently, it has been suggested that these modifications constitute a "histone code" and function as master on/off switches to determine whether particular genes are active or inactive, or perhaps more appropriate, as a rheostat to finely tune gene expression levels (93). According to the "histone code" hypothesis the modification patterns on histone tails may also be bound by adaptor proteins, which specifically recognize distinct modifications and then assist in the maintenance of open or



Figure 6. Common sites of post-synthetic modifications on the amino-termini of histone tails. These modifications primarily include the methylation (Me), acetylation (Ac) and ubiquitination (Ub) of lysine residues, as well as the phosphorylation (P) of serine residues. The acetylation of histones H3 and H4 have been closely linked with transcriptional activity, while the methylation of lysines 9 and 27 on histone H3 is tightly associated with gene inactivation. Diagram adapted from reference (92).

closed chromatin states. It is now well established that changes in histone modification profiles are involved in the regulation of many, if not all, genes in eukaryotic cells (131).

Histone modifications are typically classified as to whether they induce an open chromatin state (euchromatin) or a closed chromatin state (heterochromatin). The most commonly studied group of euchromatic histone modifications are the various acetylated lysine residues on histones H3 and H4, which are associated with transcriptionally active promoter regions (132-134). Histone acetyltransferases (HATs) such as p300, CBP and PCAF transfer acetyl groups to the tails of histones H3 and H4 to neutralize the positive charge on lysine residues and disrupt their electrostatic interaction with the negatively charged phosphate backbone of nucleosomal DNA, creating a more open chromatin structure (135, 136). These effects are augmented by a class of bromo-domain containing proteins that specifically attach to acetylated lysines and act as transcriptional co-activators (137, 138). However, members of the histone deacetylase (HDAC) family remove acetyl groups from histone tails, causing a condensation of the chromatin in that region that occludes transcription factor binding and effectively silences gene transcription (139). Thus, there is a dynamic tug-of-war between HATs and HDACs to mediate either open or closed chromatin states, respectively.

In contrast to histone acetylation, the most well-characterized heterochromatin inducing histone modifications include methylation of the lysine 9 (K9) and lysine 27 (K27) residues on histone H3, which are associated with transcriptional inactivation (140-142). The enzymes explicitly responsible for H3 K9 methylation include G9A, GLP, SUV39H1 and SUV39H2 (143-146). Notably, H3 K9 can be mono-, di- or tri-

methylated, with various degrees of methylation being associated with discrete, condensed regions of the genome. Specifically, it appears that H3 K9 mono- and di-methylation are reserved for transcriptionally suppressed regions of euchromatin and are deposited by the histone methyltransferase G9A, while tri-methylation is prevalent in pericentric heterochromatin domains, primarily from the activity of the SUV39H1 and SUV39H2 histone methyltransferases (147, 148). H3 K9 methylation is also known to be selectively bound by the chromo-domain of heterochromatin protein 1 (HP1), which aids in chromatin condensation (149, 150). Meanwhile, the methylation of H3 K27 is mediated by a protein complex consisting of EZH2, EED and SUZ12 (151), which specifically attracts Polycomb proteins involved in heterochromatin formation (141). Importantly, there appears to be increasing evidence for numerous interactions between many key enzymes and adaptor proteins involved in epigenetic repression and heterochromatin formation (i.e., DNMTs 1 and 3B, MECP2, SUV39H1, HP1, EZH2 and HDACs 1 and 2) (116, 152-155). These results provide compelling testimony for the presence of large repressor complexes at silenced promoters, helping to explain the fact that histone H3 K9 and K27 methylation often co-exist with cytosine hypermethylation and histone deacetylation in these regions (156-158).

Alterations to histone modification profiles in cancer

The divergent activities of HATs and HDACs allow gene expression to be exquisitely regulated through their ability to mediate chromatin remodeling. However, the dysregulation of these enzymes can lead to a host of harmful effects on normal

cellular function. In fact, the initiation of abnormal transcriptional states via the altered expression or function (via mutation) of genes that encode HATs, HDACs or their binding partners is a key event in the onset and progression of cancer (159, 160). Additionally, changes in HDAC specificity are also associated with neoplastic transformation (96). For example, in some types of leukemia, commonly occurring gene translocations can create fusion products between transcription factors and other proteins with HDAC-interacting domains. These fusion proteins then form repressor complexes that bind to their usual target promoters to inappropriately silence genes involved in such processes as differentiation (161-163). Histone deacetylation and DNA hypermethylation have also been shown to be associated with enriched H3 K9 methylation levels in the promoter regions of inactivated tumor suppressor genes in cancer (164, 165). Although little is currently known about the mechanisms of aberrant H3 K9 methylation-mediated tumor suppressor gene silencing in tumorigenesis, it appears this process may be due to an indirect effect initiated by prior histone deacetylation, as deacetylation has been reported to precede H3 K9 methylation in gene inactivation (166). In this way, the abnormal targeting of tumor suppressor gene promoters by HDACs during tumorigenesis may then be followed secondarily by the recruitment of histone H3 K9 methyltransferases to further enhance gene silencing. Moreover, evidence in this dissertation demonstrates that G9A levels are frequently elevated in breast tumors, which may further aid in the aberrant silencing of genes by H3 K9 hypermethylation. Finally, the up-regulation of EZH2 and SUZ12 has also been reported in a number of human tumor types and is strongly correlated with increased invasiveness (167-170), indicating

that dysregulation of the H3 K27 methylation machinery also contributes to aberrant gene silencing in cancer.

Interplay between genetic and epigenetic silencing of tumor suppressor genes

Loss or inactivation of tumor suppressor genes may result in tumor initiation or progression, as these genes produce protein products that normally function to mediate cell-cycle arrest, DNA repair, apoptosis or cell adhesion (171). According to Knudson's two-hit hypothesis, a tumor suppressor gene may become nonfunctional only when both alleles are inactivated (172). In the case of individuals with inherited mutations, the first allele is already mutated and thus they simply need an acquired inactivation of the second allele to result in loss of heterozygosity and permanent dysfunction of a tumor suppressor gene. For the development of sporadic cancers, both alleles must be inactivated during ones lifetime for complete tumor suppressor gene loss-of-function to occur (172).

Mechanisms of gene inactivation were initially thought be due to either gene mutation or deletion, however, recent advances in our understanding of epigenetic mechanisms of transcriptional gene silencing have added another layer of complexity to the two-hit hypothesis posited by Knudson some 25 years ago (173-175). As stated earlier, the aberrant deposition of repressive epigenetic modifications to DNA and histones in the regulatory regions of genes during tumorigenesis constitutes another mechanism of tumor suppressor gene inactivation by inhibiting their transcriptional potential. Thus, it is possible for tumor suppressor genes to be inactivated on both chromosomes by either two genetic or epigenetic events, or by a combination of the two (**Fig. 7**) (118). Moreover, it

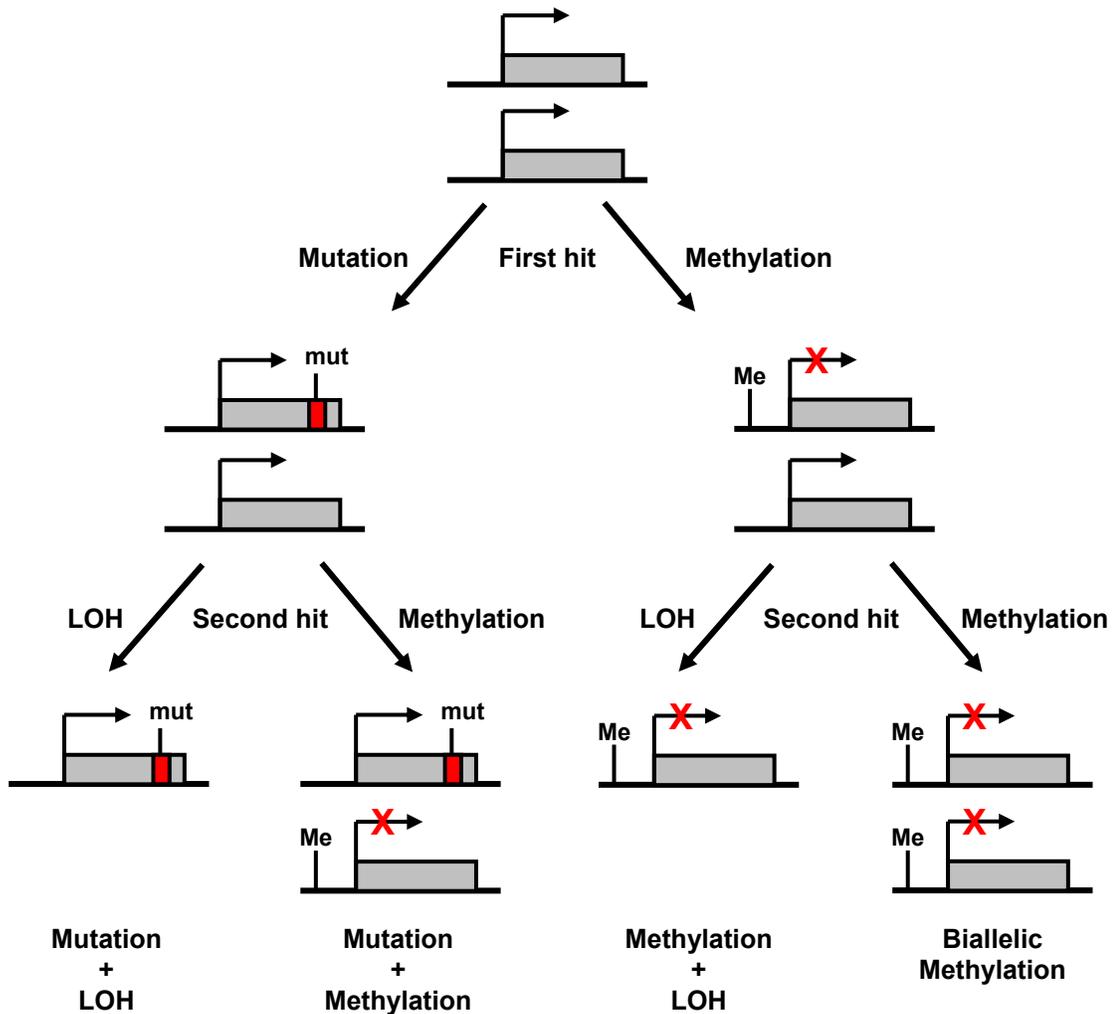


Figure 7. This figure details revisions in Knudson's two-hit hypothesis of tumor suppressor gene inactivation to include the silencing of alleles by DNA methylation. Two active alleles of a tumor suppressor gene are indicated by the two gray boxes shown at the top. The first step of gene inactivation is shown as a localized mutation on the left or by transcriptional repression via DNA methylation on the right. The second hit then occurs by either LOH or methylation-induced transcriptional silencing. Diagram adapted from reference (118).

is also likely that other forms of secondary interplay exist between these gene inactivating mechanisms in cancer. For example, it has been theorized that loss-of-function mutations in important transcription factors can lead to their inability to occupy the promoters of target genes with tumor suppressor functions, leading to their epigenetic deregulation (176). However, potential mechanisms such as this “loss-of-protection” theory are poorly understood, making investigation of the complex interplay between genetic and epigenetic silencing events during neoplastic formation an important area of future research.

Pharmacologic Reversal of Epigenetic Tumor Suppressor Gene Silencing

Approaches toward epigenetic therapy in cancer

The fact that cancer is now known to possess an epigenetic etiology has encouraged the development of new therapeutic options that aim to take advantage of the reversibility of epigenetic modifications to reactivate abnormally silenced tumor suppressor genes. This reversibility, however, is induced by both passive and active mechanisms, thereby requiring different therapeutic approaches. While histone deacetylation can be actively targeted by HDAC inhibition, there are no known DNA or histone H3 K9 demethylases present in cells, so efforts to deplete these repressive marks must rely on their passive loss over time via replication, following inhibition of the enzymes responsible for maintaining their deposition patterns in daughter cells. Therefore, efforts to reduce 5-methylcytosine levels to reactivate silenced tumor suppressor genes in cancer have focused on the development of DNA methyltransferase

inhibitors. Importantly, since these drugs require cell proliferation for their optimal activity, it is noteworthy that their DNA demethylation capabilities have been found to be most effective at low doses that are non-cytotoxic (177).

In contrast to the long-recognized role of DNA methylation in transcriptional repression, histone H3 K9 methylation is a relative newcomer in this regard. Owing to its newfound importance as an epigenetic silencing mechanism, specific inhibitors of the histone H3 K9 methyltransferases are not currently available. However, one of the most widely-used DNA methyltransferase inhibitors has also been reported to mediate H3 K9 demethylation by a presently unknown mechanism of action (164, 178, 179). In the case of histone acetylation, HDACs are capable of actively removing these transcriptionally permissive marks, leading to gene inactivation. Attempts to disrupt the cellular equilibrium between the antagonizing effects of HATs and HDACs on histone acetylation levels have made this another important target of epigenetic therapy. With this in mind, many HDAC inhibitors have been developed over the years that work by disrupting the ability of HDACs to actively deacetylate histones, leading to the re-acetylation and re-expression of aberrantly silenced genes in cancer (180, 181). In addition to DNMT inhibitors, the efficacy of HDAC inhibitors to reactivate tumor suppressor genes provides great promise to both researchers and clinicians alike in their efforts to reprogram tumor cells to more phenotypically normal or benign states.

DNA methylation inhibitors

The earliest and most commonly used DNMT inhibitors include the nucleoside anti-metabolite 5-aza-CdR and its ribonucleoside analogue 5-azacytidine (5-aza-CR), both of which were originally synthesized over 40 years ago (182, 183). Both drugs require metabolic activation via the sequential phosphorylation of their attached sugars, yielding the DNA precursor 5-aza-2'-dCTP and the RNA precursor 5-aza-CTP (184). Following tri-phosphorylation, 5-aza-CdR and 5-aza-CR are incorporated into the DNA and RNA of proliferating cells, respectively. However, only the DNA precursor is capable of irreversibly binding DNMTs to inhibit them from catalyzing further cytosine methylation. Interconversion of 5-aza-CDP into 5-aza-5'-dCMP by ribonucleotide reductase allows a small portion (10-20%) of the administered 5-aza-CR to be incorporated into DNA (185), explaining the fact that 5-aza-CdR is 10- to 30-fold more potent than 5-aza-CR at inhibiting DNMTs at the same cellular concentrations (186, 187). Moreover, the incorporation of 5-aza-CR into RNA leads to the inhibition of protein synthesis and enhanced cytotoxicity, preventing replication and DNA hypomethylation following DNMT inhibition (188, 189). For these reasons, 5-aza-CdR has gained greater acceptance and more widespread use as a DNMT inhibitor and cytosine hypomethylating agent in recent years.

Since its origination, numerous mechanistic studies revealed that 5-aza-CdR is a potent inhibitor of DNA methyltransferase (DNMT) activity, through irreversible binding of DNMTs to 5-aza-CdR-substituted DNA (186, 190). The ability of 5-aza-CdR to sequester DNMTs and inhibit their function appears to have differential effects on tumor

cells *in vitro*, in a manner that is dose-dependent. At high doses, 5-aza-CdR is cytotoxic to cancer cells, via the formation of covalent DNA adducts and obstruction of DNA synthesis (191, 192). Meanwhile, lower, non-cytotoxic doses of 5-aza-CdR have been shown to initiate reductions in 5-methylcytosine levels both globally (186), as well as locally, in the CpG-dense regulatory regions of a number of epigenetically silenced tumor suppressor genes, leading to their reactivation (193-196). The non-cytotoxic, DNA hypomethylating effects of 5-aza-CdR have also been associated with differentiation and growth inhibition of tumor cells through cell-cycle arrest and/or apoptosis (197-199), enhancing interest in this drug as an anti-cancer agent.

Although the cytotoxic and DNA demethylation capabilities of 5-aza-CdR have been well characterized both *in vitro* and *in vivo*, there is also reason to believe that this drug may exhibit alternative mechanisms of action with regard to transcriptional reactivation. For example, 5-aza-CdR can induce expression of genes lacking CpG methylation, such as p21WAF1, CDKN2D and APAF-1 (200-202), and also in an organism lacking DNA methylation by its ribose analog, 5-aza-CR (203, 204). Consistent with the idea that 5-aza-CdR has additional modes of action, recent work indicates that 5-aza-CdR treatment is associated with histone H3 K9 demethylation in the regulatory regions of silenced genes (164, 178, 179). Nonetheless, a detailed comparison of the extent and relative importance of DNA demethylation and H3 K9 demethylation mediated by 5-aza-CdR has not been conducted for genes harboring high levels of both modifications. In fact, none of the studies listed above reporting 5-aza-CdR-mediated decreases in H3 K9 methylation performed a concurrent, rigorous analysis of DNA

methylation levels across the same region following 5-aza-CdR treatment (164, 178, 179). Furthermore, the mechanism by which 5-aza-CdR potentiates reductions in H3 K9 methylation has not been previously identified. Therefore, it will be important in future studies with this drug to thoroughly analyze the relative contributions of DNA and histone H3 K9 methylation reversal to the reactivation of epigenetically silenced genes in breast tumor cells, and also to elucidate the mechanism by which 5-aza-CdR alters H3 K9 methylation levels.

Clinical use of DNA methylation inhibitors in cancer

Early clinical studies with 5-aza-CdR sought to take advantage of its cytotoxic properties at high-doses in an attempt to treat various neoplastic diseases in humans, with some encouraging results in hematologic malignancies, however, solid tumors have been largely refractory to such treatments (205). In light of growing support for the role that epigenetic gene inactivation plays in tumorigenesis, recent trials have focused on lower-dose, longer duration treatment schedules, in an effort to exploit the DNA demethylation and tumor differentiation effects of 5-aza-CdR in cancer patients (206, 207). These new dosing regimens have been generally well tolerated and have produced favorable results in patients with various hematopoietic cancers, with many seeing significant clinical improvement and/or complete remission (208-211). Of interest, these studies also reported that patient responses were slow to develop, typically requiring 3 cycles of treatment for best results, implying that a more prolonged, subtoxic 5-aza-CdR treatment schedule induces its anti-proliferative effects via an alternative, non-cytotoxic mechanism

of action. This notion has garnered further support from other clinical investigations demonstrating that low-dose 5-aza-CdR treatment results in both global and promoter-specific decreases in DNA methylation in patients with myelodysplasia, and also in those with intractable solid tumors (212-214). In total, these results using low-dose, more frequent 5-aza-CdR administration provide compelling evidence for its continued optimization and use as a DNA hypomethylating agent in the treatment of a wide range of tumor types.

HDAC inhibitors

HDAC inhibitors are divided chemically into three main groups: short-chain fatty acids, hydroxamic acids and cyclic tetrapeptides. Within these classes, the most widely used HDAC inhibitors for research and clinical application include butyrate and valproic acid (short-chain fatty acids), suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) (hydroxamic acids), and depsipeptide (cyclic tetrapeptide) (180). These compounds primarily act by binding to the catalytic regions of HDACs to impede their ability to deacetylate histones (215, 216), resulting in the accumulation of acetylated histones and the activation of epigenetically silenced tumor suppressor genes involved in such processes as apoptosis, cell-cycle arrest and the inhibition of angiogenesis and metastasis (217-220). Like the inhibitors of DNMT enzymes, HDAC inhibitors can also induce *in vitro* growth arrest, differentiation and/or apoptotic cell death in a wide variety of transformed cells, including those from the brain, breast, colon, lung, ovary, prostate

and skin (160). Additionally, these compounds have been shown to inhibit tumor growth in animal models (221, 222), spurring interest in their use in humans.

Clinical use of HDAC inhibitors in cancer

Following confirmation of their *in vitro* anti-tumor effects, a number of HDAC inhibitors have been evaluated in various phase I and phase II clinical trials (180). Phase I clinical trials with SAHA, administered intravenously and orally, have demonstrated its anti-tumor effects in a subset of patients with solid and hematological malignancies in a manner that was well tolerated (223, 224). Moreover, post-therapy tumor biopsies from respondents showed an accumulation of acetylated histones by immunostaining, confirming the ability of SAHA to inhibit the correct biological target *in vivo* (223, 224). Likewise, depsipeptide has also shown encouraging anti-cancer effects as a single agent against leukemias and lymphomas, with many patients showing partial or complete clinical responses with minimal side effects (225-227). This drug has largely been tested in phase I trials, with many of the same results as SAHA, and a phase II trial is currently underway for the treatment of T-cell lymphoma (228). Finally, although HDAC inhibitors have shown promise as anti-tumor drugs, the optimal dose, timing and duration of therapy are still to be defined

Coupling epigenetic and genetic therapies in cancer

Despite the encouraging effects of DNMT and HDAC inhibitors in cancer patients, it is unlikely that monotherapies have the potential to cure malignant disease due

to the rapid development of drug resistance to single drug treatments. Moreover, greater anti-tumor effects are typically seen with therapeutic regimens that combine multiple drugs to attack multiple mechanisms of carcinogenesis. Therefore, many researchers have sought to utilize combination therapies with multiple epigenetic drugs administered together or singly in conjunction with more traditional chemotherapeutics and immune modifiers. For instance, the HDAC inhibitor valproic acid has been evaluated in combination with 5-aza-CdR in the treatment of leukemic cells lines, with promising, synergistic effects in terms of growth inhibition, induction of apoptosis and gene reactivation (229). Similarly, phenylbutyrate and 5-aza-CdR co-treatment was able to significantly reduce mouse lung tumor development more effectively than with either treatment alone (230). Further studies revealed that pre-treatment of mice inoculated with human ovarian and colon tumor xenografts with 5-aza-CdR enhanced their sensitization to the DNA-damaging agents cisplatin, carboplatin, temozolomide and epirubicin (231). Likewise, treatment of colon adenocarcinoma cells with 5-Aza-CdR sensitized them to growth inhibition by exogenous interferon (232). Valproic acid has also been successfully used in combination with the retinoic acid receptor ligand, all-*trans* retinoic acid (ATRA), in elderly patients with acute myelogenous leukaemia to induce tumor differentiation and remission (233).

In addition to the combination therapies noted above, the future development of treatment regimens that attempt to overcome both genetic and epigenetic defects in various tumor types will be essential to permit more targeted and effective anti-cancer interventions. For example, the p53 tumor suppressor gene is a common target of

inactivating mutations in many cancers, as discussed earlier. This fact has led to the pursuit of strategies aimed at restoring wt p53 expression in tumors harboring this particular genetic defect. To this end, researchers have been able to successfully reactivate p53 responsive genes following adenoviral-mediated reintroduction of wt p53, or the use of small molecule/peptide chaperones that can stabilize mutant forms of p53 to a more native conformation. However, even with restoration of wt p53 function in tumor cells, this transcription factor must also be able to effectively access its response elements in the promoters of target genes to induce their expression. Interestingly, a number of p53-target genes with tumor suppressor functions also undergo epigenetic silencing in many tumor types (234-238), making the use of epigenetic modifiers coupled with the restoration of wt p53 activity an attractive option for future treatment in these cases. Further identification of the full range of tumor suppressor genes dually silenced in cancer via the mutation of critical transcription factors and the adoption of repressive epigenetic states, therefore, remains an important goal, as specialized cancer treatments that target both genetic and epigenetic facets of gene dysregulation represent a potentially useful strategy towards the therapeutic transcriptional reprogramming of cancer cells.

DSC3 and MASPIN

Protein functions

DSC3 and MASPIN (or SERPINB5) are tumor suppressor proteins that function primarily at the extracellular membrane. DSC3 is a member of the cadherin superfamily of calcium-dependent cell adhesion molecules and a principle component of

desmosomes. Desmosomal proteins are integral to the maintenance of epithelial tissue architecture (239) and the loss of these components leads to a lack of adhesion and a gain of cellular mobility (240, 241). Like DSC3, MASPIN also functions to inhibit cell migration and invasion (242), and has additionally been shown to inhibit angiogenesis (243) and to sensitize cells to apoptosis (244), making it a more functionally complex protein. However, the precise mechanisms by which MASPIN induces its various effects remain largely unknown at this time.

Expression patterns and regulation in normal and cancer cells

DSC3 and MASPIN are uniquely expressed in epithelial cells (245, 246), with p53 being an important transcriptional activator of both genes (247, 248). However, in a large percentage of epithelial breast cancers, expression of both DSC3 and MASPIN is significantly down-regulated or completely silenced (238, 249), increasing the metastatic potential of these tumors (240, 249-251). Earlier work found that the loss of these genes in breast cancer is not due to deletion or gross chromosomal rearrangement (249, 252), suggesting that other factors which normally regulate DSC3 and MASPIN expression are disrupted during cancer progression. In fact, further investigation in our lab revealed a strong link between DNA hypermethylation and histone hypoacetylation of the DSC3 and MASPIN promoter regions and their transcriptional inactivation in human breast cancers (235, 238, 248, 253), providing proof of a strong epigenetic component to their silencing in these cells. Moreover, the p53 protein is often mutated in many breast cancers and breast cancer cell lines (254, 255), making DSC3 and MASPIN class II

tumor suppressors as genes that are regulated by a different suppressor gene that has lost its function via mutation or deletion (p53; class I) (256). Given the importance of the DSC3 and MASPIN proteins in restricting epithelial cell motility/invasion and maintaining proper tissue architecture, these genes represent ideal candidates for pharmacologic interventions aimed at transcriptional reactivation in tumors and anti-metastases effects. Moreover, as both genes share striking similarities with regard to their function(s) and expression patterns/regulation, DSC3 and MASPIN may portend the existence of a larger class of p53-responsive, anti-metastatic tumor suppressor genes that also undergo aberrant epigenetic silencing in breast tumors to promote their spread to distant sites.

Dissertation Aims

Breast cancer remains the most frequently diagnosed cancer in women (1), making attempts to further understand its molecular etiology vital to the development of more effective therapies against this disease. Since cancer is fundamentally a disease of gene dysregulation, elucidating the genetic and epigenetic aberrations that contribute to the inactivation or silencing of critical tumor suppressor genes in human breast cancer is of utmost importance. As the p53 gene is a critical tumor suppressor that is frequently mutated in breast cancer, a greater understanding of the downstream effects of functional loss of this transcriptional activator is likely to provide significant insight into the

molecular determinants of this disease. With that said, the primary aims of my doctoral research were 3-fold:

1. To identify p53 target genes with tumor suppressor functions that are down-regulated in human breast cancers that express mutant forms of p53.
2. To elucidate the mechanisms that cooperate with or are driven by the loss of functional p53 to control long-term target gene inactivation.
3. To develop pharmacologic strategies to reverse the inappropriate silencing of p53 target genes and determine the mechanisms associated with their reactivation.

MATERIALS AND METHODS

Materials

Drugs

PRIMA-1 was purchased from Calbiochem (Calbiochem, San Diego, CA) and resuspended in H₂O, while a sample of CP-31398 was provided by Pfizer (Pfizer, Groton, CT) and dissolved in DMSO. Doxorubicin, 5-aza-CR and 5-aza-CdR were purchased from Sigma (Sigma, St. Louis, MO) and dissolved in H₂O and 1X PBS, respectively. See **Figure 8** for structures of PRIMA-1, CP-31398, 5-aza-CR and 5-aza-CdR.

Adenoviruses

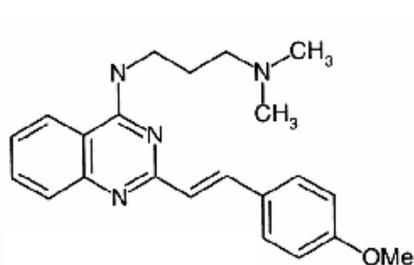
Adenoviral stocks containing gene inserts capable of expressing both wt p53 and green fluorescent protein (GFP) were a kind gift from Dr. Bert Vogelstein's lab at The Johns Hopkins University, while the constructs necessary for creating the p73-p53 chimera were supplied by Dr. Matthias Dobbelstein at Philipps-Universität Marburg. Both adenoviruses, along with a control adenovirus containing only the GFP insert were packaged and propagated by the Gene Transfer Vector Core at the University of Iowa.

Methods

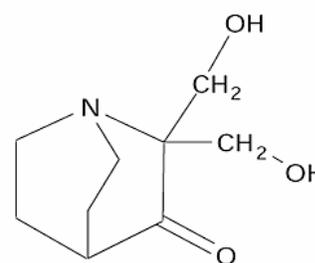
Cell culture and treatments

Normal human mammary epithelial cells (HMECs) were acquired from Clonetics (Cambrex, Walkersville, MD). Human foreskin fibroblasts (HFFs) and the early passage

Small molecule mutant p53

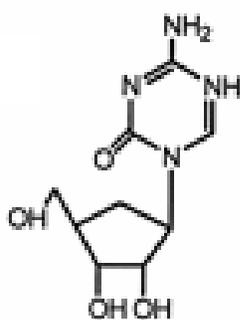


CP-31398

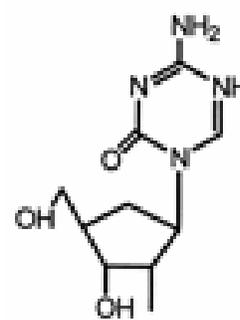


PRIMA-1

Nucleotide analogues



5-azacytidine



5-aza-2'-deoxycytidine

Figure 8. Chemical structures of the small molecule mutant p53 rehabilitators CP-31398 and PRIMA-1 used in this dissertation, as well as the nucleotide analogue and epigenetic modifier drugs, 5-azacytidine and 5-aza-2'-deoxycytidine.

sporadic breast cancer cell line UACC 1179, derived from pleural effusion (257), were purchased from and maintained by the Arizona Cancer Center Cell Culture Shared Service. MDA-MB-231 breast tumor cells and the immortalized normal breast cell line MCF-10A were obtained from the American Type Culture Collection (Rockville, MD). HMEC and MCF-10A cells were cultured in mammary epithelial growth media (MEGM) (Cell Applications, Inc., San Diego, CA). MDA-MB-231 cells were maintained in RPMI 1640 containing 5% fetal bovine serum supplemented with 50 $\mu\text{g}/\text{mL}$ Penicillin/Streptomycin and 292 $\mu\text{g}/\text{mL}$ L-glutamine. UACC 1179 and HFF cells were grown in M15 media containing 5% fetal bovine serum. All cell lines were cultured in 95% air/5% CO_2 atmosphere at 37°C.

The duration of treatment for the various drugs used in these studies, other than 5-aza-CdR, were as follows: doxorubicin for 18 hrs., CP-31398 for 20 hrs. and PRIMA-1 for 24 hrs. For all adenoviral infections, lysates were harvested 24 hrs. post-infection, unless otherwise stated. For 5-aza-CR and 5-aza-CdR treatments, cells were grown at low density in six-well plates or T-150 dishes and the indicated dose of each drug was administered on day 0 and also on day 2, for a total duration of 4 days. Media was changed immediately prior to drug addition on both day 0 and day 2. RNA, DNA, protein or chromatin immunoprecipitation lysates were isolated from each of the samples on day 4. Adenoviral infections and CP-31398 treatments in conjunction with 5-aza-CdR were administered 24 or 20 hrs. prior to cell lysis, respectively.

Nucleic acid isolation

Total RNA was isolated from cells using an RNeasy[®] mini kit (QIAGEN, Valencia, CA), and genomic DNA was isolated using the QIAamp[®] DNA Blood Mini Kit (QIAGEN). RNA and DNA samples were quantitated and purity estimated by UV absorbance measurements at 260 and 280 nm, respectively.

p53 genotyping

The cell lines UACC 893, UACC 1179, and UACC 2087 were grown to near confluence, harvested by trypsinization and DNA was isolated. Genomic DNA (50 µg) from each of the cell lines was sent to the Molecular Diagnostic Laboratory, Institute for Molecular and Human Genetics, Georgetown University Medical Center for analysis. Exons 2-10 and approximately 20 bp of flanking intron regions of the p53 gene were PCR amplified followed by Big Dye[™] (Applied Biosystems, Foster City, CA) sequencing to determine the status of the p53 gene in these cell lines.

cDNA microarray analysis

cDNAs were printed onto amino-alkyl silane glass slides (Telechem International, San Jose, CA) using four quill-type pins (Telechem International) mounted onto an OmniGrid robot (GeneMachines, San Carlos, CA) (258). In addition to the 5184 sequence-verified IMAGE consortium clones (Research Genetics, Carlsbad, CA), a set of 88 human housekeeping genes (Research Genetics) and Cy3/Cy5 end-labelled oligonucleotides were placed strategically into the array to aid in data normalization,

measurement of nonspecific hybridization and identification of the corners of the array. Additionally, a set of 104 IMAGE consortium clones representing known genes of interest, but not found in gf200, were purchased from Research Genetics and included in the microarray. In all, 5% of the Research Genetics clones were resequenced following their purchase to further verify the library. The library was found to have an error rate below the 2% Research Genetics specification; in fact no errors were found. Following printing, slides were rehydrated, snap-dried and UV-cross-linked with 65 mJ of 254 nm light using a Stratalinker. Slides were then washed in 1% SDS for 2 min., rinsed in double-distilled water three times, spun dry, and stored at room temperature in a desiccator until use.

Total RNA (40 μ g) was converted to fluorescent first-strand cDNA using the Micromax Direct cDNA Microarray System (NEN Life Sciences, Boston, MA) following the manufacturer's protocols. Labeled cDNA from the two reactions was combined and purified using the Qiaquick PCR Purification Kit (QIAGEN) following the manufacturer's protocols. After elution from the purification column, probe was lyophilized to dryness and resuspended in 15 μ L hybridization buffer, denatured by boiling for 2.5 min., and added to the cDNA microarray manufactured at the Arizona Cancer Center Microarray Core facility. A coverslip (22 x 22 mm²) was applied, and the array placed in a hybridization chamber (GeneMachines) at 62°C for 18 hrs. Following hybridization, arrays were washed by placing them into 50 ml conical tubes containing 2X SSC, 0.1% SDS for 5 min.; 0.06X SSC, 0.1% SDS for 5 min.; and 0.06X SSC for 2 min., all at room temperature. Arrays were scanned for Cy3 and Cy5 fluorescence using

an Axon GenePix 4000 microarray reader (Axon Instruments, Foster City, CA) and quantitated using GenePix software (Axon Instruments).

Multidimensional scaling was performed using BRB-ArrayTools software (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). Genes were entered into the multidimensional scaling analysis that had a p-value < 0.05 and contained data in at least 24 of 33 hybridizations (1118 genes). Multidimensional scaling analysis using the Euclidean distance metric identified eight p53/GFP-infected cell lines that clustered together; these eight cell lines were further analysed using GeneSpring software (Silicon Genetics, Redwood City, CA) to identify genes whose expression was induced by p53/GFP infection. Global error modeling was used to estimate p-values, and a Bonferroni-corrected p-value of < 0.00001 was used to select genes that displayed statistically significant increases in expression across the eight selected cell lines.

RT-PCR analysis

For real-time RT-PCR analysis of DSC3, MASPIN, G9A, DNMT1 and GAPDH gene expression, a reverse transcription step was performed using TaqMan[®] Reverse Transcription Reagents (Roche Molecular Systems, Branchburg, NJ), 125 ng of total RNA in a 50 μ L reaction. The reverse transcription reaction was primed with random hexamers and incubated at 25°C for 10 min. followed by 48°C for 30 min., 95°C for 5 min. and a chill at 4°C. Each 25 μ L PCR reaction consisted of 4 μ L of cDNA added to 12.5 μ L of TaqMan[®] Universal PCR Master Mix (Roche Molecular Systems), 1.25 μ L of gene-specific primer/probe mix (Assays-on-Demand, Applied Biosystems), and 7.25 μ L

of PCR water. The PCR conditions were 95°C for 10 min., followed by 40 cycles of 95°C for 15 sec. alternating with 60°C for 1 min. DSC3-, MASPIN-, G9A-, DNMT1- and GAPDH-specific PCR was performed and the data collected using the ABI Prism 7000 real-time sequence detection system (Applied Biosystems). Differences in target gene expression in our normal and tumor cell lines, before and after treatment/transfection were determined using the equation $2^{\Delta Ct}$ for normalized relative expression, or $2^{\Delta\Delta Ct}$ to calculate the normalized expression of samples in relation to normal HMEC or untransfected control levels (259). For these calculations, the target gene Ct value for each sample is subtracted from its corresponding GAPDH Ct value for normalization (ΔCt value), followed by subtraction of the normalized HMEC or untransfected control Ct values from the normalized Ct values of our tumor cell lines before and after treatment/transfection to yield a $\Delta\Delta Ct$ value. This value was then inserted into the equation $2^{\Delta\Delta Ct}$ and multiplied by 100, to yield % HMEC or untransfected control expression.

FACS analysis of GFP-positive cells

MDA-MB-231 cells were grown in 6-well dishes and infected with wt p53/GFP adenovirus (200 pfu/cell) for 0, 4, 8, 12, 18, 24 or 36 hrs. Post-infection, cells were trypsinized, pelleted and resuspended in 1mL of PBS. Resuspended cells were then analyzed by the Arizona Cancer Center Flow Cytometry Shared Service for GFP-positivity using a BD FACSAria™ Cell Sorter (Becton Dickinson, Franklin Lakes, NJ) to

measure GFP fluorescence. Untreated cells were used to gate the GFP-negative cell population and to determine the percentage of infected cells expressing detectable GFP.

Sodium bisulfite genomic sequencing

Genomic DNA (5 µg) was modified with sodium bisulfite under conditions previously described (238), with minor adjustments. Following sodium bisulfite modification, the DNA was bound to QIAGEN DNeasy spin columns, followed by on-column desalting and desulfonation. Regions of the DSC3 and MASPIN promoters were amplified from the bisulfite-modified DNA by two rounds of PCR using the following nested primer sets specific to the bisulfite-modified sequences of the DSC3 and MASPIN

CpG islands:

DSC3 Promoter

1st Rd. Forward: 5'-GATTGGGGTTTTGTATTGAGA-3'

1st Rd. Reverse: 5'-TTAACCTCTCTCAAACCTACC-3'

2nd Rd. Forward: 5'-ATTTGGGTTGTTAGGGTTTTTTT-3'

2nd Rd. Reverse: 5'-AAAACAACCTCACTTCTAAAACC-3'

MASPIN Promoter

1st Rd. Forward: 5'-AAAAGAATGGAGATTAGAGTATTTTTTTGTG-3'

1st Rd Reverse: 5'-CCTAAAATCACAATTATCCTAAAAAATA-3'

2nd Rd. Forward: 5'-GAAATTTGTAGTGTTATTATTATTATA-3'

2nd Rd. Reverse: 5'-AAAAACACAAAAACCTAAATATAAAAA-3'

First-round PCR amplification was performed using 2.5 μ L bisulfite-modified DNA in a 25 μ L reaction, under the following conditions: 95°C for 5 min. followed by 35 cycles of 92°C for 1 min., 56°C for 3 min., 72°C for 2 min., and ending with a final extension of 72°C for 5 min. Second-round PCR amplification utilized 1% of the first round PCR product as the template for the second round of PCR and the same thermocycler conditions as the first round, except that the annealing temperature was raised to 58°C.

For earlier studies, the resultant PCR product was cloned into a TA vector according to the manufacturer's instructions (pGEM-T-Easy cloning kit (Promega, Madison, WI)). In all, 10 positive recombinants were isolated using a Qiaprep Spin Plasmid Miniprep kit (QIAGEN) according to the manufacturer's instructions and sequenced on an ABI-automated DNA sequencer. The methylation status of individual CpG sites was determined by comparison of the sequence obtained with the known DSC3 and MASPIN sequences. The number of methylated CpGs at a specific site was divided by the number of clones analysed (minimum of 10 in all cases) to yield a per cent methylation for each site.

For later studies, the resultant PCR product was run on a 3% agarose gel, gel purified using a QIAquick[®] Gel Extraction Kit (QIAGEN), and cloned into a pCR[®]2.1-TOPO[®] vector supplied with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The inserts of positive recombinants (from white colonies) were PCR-amplified using M13 primers and evaluated on a 1.5% agarose gel. For each sample, PCR-amplified inserts from 94 clones were chosen for further

sequence analysis. The cycle-sequence reaction was carried out using Big Dye[®] Terminator v3.1 chemistry (Applied Biosystems) on an MJ Thermocycler (MJ Research, Inc., Watertown, MA). These reactions were further processed by running a clean-up procedure with CleanSEQ beads (Agencourt Bioscience Corporation, Beverly, MA) on a Biomek FX automated workstation (Beckman-Coulter, Inc., Fullerton, CA). Finally, the products were sequenced on an Applied Biosystems 3730xl DNA Analyzer.

The methylation status of individual CpG sites was determined by comparison of the sequences obtained for each clone with the known DSC3 and MASPIN sequences. The total number of methylated CpGs at all sites in the amplified region for all clones analyzed was summed and divided by the number of total CpG sites in all analyzed clones for the entire region to yield a percent regional methylation for each gene. Only CpG sites with good quality, decipherable sequence data were included in this calculation. A two-sample test of proportions was then performed using STATA 9.1 (StataCorp LP, College Station, TX) to calculate a p-value to determine whether or not statistically significant differences in regional methylation existed between treated and untreated samples at the 95% confidence level for the DSC3 and MASPIN regions analyzed in both MDA-MB-231 and UACC 1179 breast tumor cell line.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitations (ChIPs) using anti-H3 K9 di-methyl, anti-H3 K27 tri-methyl, anti-acetyl H3 and anti-acetyl H4 (Upstate Biotech, Lake Placid, NY) were performed according to the manufacturer's instructions with slight modifications

(260). Cells were rinsed in 1X HBSS with 0.1% EDTA and treated with 1% formaldehyde for 10 min. at 37°C to form DNA-protein crosslinks. The cells were rinsed in ice-cold 1X HBSS with 0.1% EDTA containing protease inhibitors (1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL pepstatin A), scraped and collected by centrifugation at 4°C. Cells were then resuspended in PIPES buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP-40) containing protease inhibitors, and incubated for 10 min. on ice. Cells were collected by centrifugation and resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)) containing protease inhibitors and incubated on ice for 10 min. The DNA-protein complexes were sonicated to lengths between 500 and 1000 bp, as determined by gel electrophoresis. Samples were centrifuged at 14,000 rpm at 4°C to spin out cell debris; then the supernatant was diluted 10-fold with ChIP dilution buffer containing protease inhibitors. One-tenth of the sample was set aside for input control, and the remaining sample was pre-cleared with Protein A Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hr. on a nutator at 4°C.

Following pre-clearing, the samples were split into thirds: two of the three samples were incubated with the appropriate antibody (5 µL anti-di-methyl H3 K9 or anti-tri-methyl H3 K27; 7.5 µL anti-acetyl H3 or H4), while the third sample was left as a minus Ab control. All samples were rotated overnight at 4°C. The chromatin-antibody complexes were collected using Protein A Sepharose and then sequentially washed with the manufacturer's low salt, high salt, and LiCl buffers, followed by two washes with Tris-EDTA.

p53 ChIPs was performed as stated above with the following modifications. At the preclearing step protein, A/G PLUS Agarose (Santa Cruz Biotech, Santa Cruz, CA) was used in place of Protein A Sepharose in order to reduce the -Ab background. Following the preclearing step, the sample was split into half and one portion was incubated with 30 μ L of anti-p53 antibody conjugated to agarose beads (clone DO1 Oncogene, Boston, MA) or 30 μ L of protein A/G PLUS agarose. Following an overnight incubation at 4°C, the beads were washed with low salt, high salt and LiCl wash buffers, and then twice with Tris-EDTA.

The chromatin-antibody complexes were eluted and for all samples (including input DNA), DNA-protein crosslinks were reversed with 5 M NaCl at 65°C for 4 hrs. All samples were treated with proteinase K, and genomic DNA was recovered and purified by phenol-chloroform extractions and ethanol precipitations. This DNA was then quantitated using a BioPhotometer (Eppendorf Scientific, Westbury, NY). PCR amplification was performed using Taqman primer/probes specific for the DSC3, MASPIN and GAPDH promoters. Primer/probe sequences were designed by our lab, and synthesized by Applied Biosystems' Assays-by-Design Service.

Real-time PCR was used to analyze ChIP DNA, using the ABI Prism 7000 sequence detector following PE Applied Biosystems' TaqMan® Universal PCR Master Mix protocol. Each 25 μ L PCR reaction consisted of 10 μ L of DNA (at 1 ng/ μ L) added to 12.5 μ L of TaqMan® Universal PCR Master Mix (Roche Molecular Systems), 1.25 μ L of gene promoter-specific primer/probe mix (Assays-by-Design, Applied Biosystems), and 1.25 μ L of PCR water. The PCR conditions were 95°C for 10 min., followed by 40

cycles of 95°C for 15 sec. alternating with 60°C for 1 min. DSC3-, MASPIN- and GAPDH-promoter-specific PCR was performed and the data collected using the ABI Prism 7000 real-time sequence detection system (Applied Biosystems).

For each experiment the threshold bar was set within the linear range of PCR amplification. The resulting Ct and Rn files were exported to Microsoft Excel for data and graphical analysis. Quantification was determined by applying the comparative Ct method, as described in the ABI 7000 sequence detection user guide and others (259). Briefly, the percent respective input was calculated by subtracting the Ct value of the ChIP DNA sample from the Ct value of its respective input fraction and using this value as the power that 2 is raised to (i.e. $2^{\text{Ct}(\text{input})-\text{Ct}(\text{ChIP})}$) for fold-enrichment, or followed by multiplication by 100 for % relative input. Fold differences between immunoprecipitation samples were calculated using the equation $2^{\text{Ct}(\text{IP A})-\text{Ct}(\text{IP B})}$.

Chromatin accessibility assay

Chromatin accessibility assays were performed as previously described (261) with minor modifications. Ten million cells were washed twice with ice-cold 1X PBS, gently scraped and collected by centrifugation. Nuclei were extracted by resuspension of cells in ice-cold 1X RSB (10 mM Tris-HCl pH 8, 3 mM MgCl₂, 10 mM NaCl, 0.05% NP-40). The nuclei were collected by centrifugation, resuspended in appropriate 1X restriction endonuclease buffer, and divided into two aliquots of 200 µL/aliquot. Zero or 75 units of *MspI* (Gibco BRL, Bethesda, MD) was added to the nuclei and incubated at 37°C for 15 min. Genomic DNA was isolated and ligated to linkers specific for the *MspI* ends.

Linker: 5'-CGAGTACTGCACCAGCAATCC-3'
3'-TCATGACGTGGTCGTTTAGG-5'

The linker 'marks' accessible sites of chromatin, and acts as the primer sequence for PCR along with the MASPIN promoter-specific primer. To increase specific amplification of our band of interest, a second round of PCR is subsequently performed using a 1:1000 dilution of the first-round PCR product and adding a second, nested primer that is specific for the genomic region being analysed and internal to the first region-specific primer.

Linker-specific primer (1st and 2nd Rd.): 5'-GGATTTGCTGGTGCAGTACT-3'

Gene-specific primer (1st Rd.): 5'-CGTTGTAAGGCTGAGATTTAGGC-3'

Gene-specific primer (2nd Rd.): 5'-TGCCAGGCTATTCCAGAGAC-3'

First-round PCR was performed using PuReTaq Ready-To-Go PCR beads (Amersham Pharmacia Biotech) to amplify 100 ng of linkered DNA. The initial step in the first-round PCR reaction is a 15 min. incubation at 72°C, followed by a denaturation at 95°C for 2 min. and then 25 cycles of 95°C for 30 sec., 55°C for 1 min., 72°C for 2 sec., and a final extension at 72°C for 5 min. The second round of PCR was performed using the ABI Prism 7000 real-time sequence detection system (Applied Biosystems). For this nested PCR step, 25 pmol (1 µL) of internal MASPIN-specific primer was added to 5 µL of diluted first-round product (1:1000), 19 µL of PCR water, and 25 µL of 2X SYBR[®] Green PCR Master Mix (Applied Biosystems). The PCR conditions for this second round of PCR are as follows: a 10 min. denaturation at 95°C and 40 cycles of 94°C for 1 min., 56°C for 40 sec. and 72°C for 30 sec. SYBR[®] Green dye is incorporated into double-stranded DNA, allowing real-time monitoring of PCR amplification

reactions. Relative levels of chromatin accessibility were assessed by comparing the cycles at which each sample reached the threshold cycle (Ct). For each experiment, the threshold bar was set within the linear range of the PCR amplification. Fold differences in accessibility were then determined using the equation $2^{-\Delta Ct}$. Real-time PCR products were also separated on a 3% TBE agarose gel to verify the presence of a single PCR product of the appropriate size (241 bp).

5' RACE analysis

The basal and 5-aza-CdR-activated MASPIN transcriptional start site was determined via 5' RACE analysis, using Invitrogen's 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen), according to manufacturer's instructions. Briefly, total RNA (0 or 3 μ g) was reverse transcribed to convert the 5' end of the predicted MASPIN transcript (UCSC human genome assembly: May 2004) into cDNA, using a gene-specific primer (5'-CGCTTGATTAGTTTCAG-3'). RNA was digested and removed from the newly created cDNA using S.N.A.P. column purification (Invitrogen). Purified cDNA was then subject to a 5' poly-dC-tailing reaction using terminal deoxynucleotidyl transferase and dCTP, followed by PCR amplification using Invitrogen's Abridged Anchor Primer (Invitrogen) and another gene-specific primer (5'-GGTGGAGAGACAGATTGGAGAGAA-3') internal to that used for cDNA synthesis. PCR amplification was performed using 2.5X Eppendorf PCR MasterMix (Eppendorf Scientific) and 5 μ L dC-tailed cDNA in a 50 μ L reaction, under the following conditions: 94°C for 3 min., followed by 35 cycles of 94°C for 1 min., 55°C for 30 sec., 72°C for 1

min., and ending with a final extension of 72°C for 5 min. 5' RACE PCR products were run on a 3% agarose gel for size determination and then cloned into a pGEM T-Easy vector (Promega) for propagation and sequencing analysis of the inserted PCR product.

Global analysis of 5-methylcytosine levels via LC-MS

Global levels of 5-methylcytosine were measured according to the protocol used by Selhub and colleagues (262), with minor modifications. Genomic DNA (8 µg) isolated from MDA-MB-231 and UACC 1179 cells treated with 0 and 10 µM 5-aza-CdR was brought up to 50 µL in 1 mM EDTA and denatured at 99°C for 10 min., followed by snap freezing on ice. To this denatured genomic DNA, 62 µL of 2 mM sodium acetate (pH 4.5), 10 µL of 0.1 mM zinc chloride and 8 µL of Nuclease P1 (1 unit/µL) (Sigma) were added prior to incubation at 37°C for 1 hour to digest samples to 5'-mononucleotides. The mononucleotides were then dephosphorylated by adding 10 µL of 80 mM Tris and 10 µL of Shrimp Alkaline Phosphatase (0.1 unit/µL) (Fermentas Inc., Hanover, MD), followed by a 30 minute incubation at 37°C to yield mononucleosides.

Individual nucleosides were separated via HPLC, using an HP/Agilent 1050 pumping system (Hewlett Packard/Agilent Technologies, Germany) equipped with a Zorbax SB-C18, 5 µm, 2.1 x 250 mm column (Agilent Technologies, Palo Alto, CA), onto which 30 µL of each digested DNA sample was injected via an HP 1050 autosampler. The isocratic mobile phase consisted of 7 mM ammonium acetate pH 6.7 / methanol (95:5 v/v) delivered at a flow rate of 0.3 mL/min. for 60 min., which allowed for separation of all five nucleosides. UV detection was performed at 254 nm using an

HP 1050 Variable Wavelength Detector (Hewlett Packard/Agilent). LC separation was coupled to MS analysis using a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (ThermoElectron, San Jose, CA). Briefly, analytes were ionized using electrospray ionization (ESI) at an ESI source spray voltage of +4.5 kV. Ions were introduced into the mass spectrometer through a heated metal capillary maintained at 250°C. Initial screening of the samples was performed in positive mode in the first quadrupole based on full MS measurements between 50-550 m/z only (Q1 MS mode). Following the Q1 MS scan, single ion monitoring (SIM) was performed. The center masses in SIM mode were as follows: 227.90 for dC, 455.00 for dC dimer, 241.90 for 5-methyl-dC, 267.88 for dG, 242.90 for dT and 252.00 for dA. Pure dC, dG, dT, and dA obtained from Sigma were also used as external standards. Since pure 5-methyldeoxycytidine is not commercially available to use as a standard, its identification in biological samples was achieved by MS/MS analysis using the parent mass of 241.90. MS/MS was performed using 10 eV of energy (CID), yielding 5-methylcytosine as the major fragment ion (m/z 126.19). Finally, the percentage of methylated cytosines in each sample was determined using the following ratio of MS peak areas: $[\text{methyl-dC} / (\text{methyl-dC} + \text{dC})] \times 100$.

Acid extraction of proteins and immunoblot analysis of modified histones

For analysis of global di-methyl H3 K9 levels, MDA-MB-231 and UACC 1179 cells were grown to near confluence in T-150 dishes and were washed with ice-cold PBS. Cells were then scraped from the plate in ice-cold PBS supplemented with 1 mM PMSF

and pelleted by centrifugation at 200 x g for 10 min. at 4°C. The cell pellet was resuspended in 5-10 volumes of lysis buffer (1.5 mM MgCl₂, 10 mM KCl, 10 mM HEPES (pH 7.9)), supplemented with PMSF (1.5 mM) and DTT (0.5 mM). Hydrochloric acid was then added to the resuspended cells to a final concentration of 0.2 M and allowed to incubate on ice for 30 min., with periodic vortexing. Following cell lysis, samples were centrifuged at 11,000 x g for 10 min. at 4°C and the acid soluble supernatant fraction was retained, while the acid-insoluble pellet was discarded. Finally, using 10,000 MWCO Slide-A-Lyzer Dialysis Cassettes (Pierce, Rockford, IL), the acid soluble protein supernatant was dialyzed against 200 mL 0.1 M acetic acid, twice for 2 hrs. each, and then three times against 200 mL H₂O for 1 hour, 3 hrs. and overnight, respectively.

For each protein sample, concentrations were determined using Bio-Rad Protein Assay dye reagent (Bio-Rad Laboratories, Hercules, CA), and equal amounts of dialyzed, acid-extracted protein (20 µg) were size-fractionated via SDS-polyacrylamide gel electrophoresis on a 15% Ready Gel Tris-HCL Gel (Bio-Rad Laboratories) and transferred to a PVDF membrane using 1X Towbin transfer buffer (190 mM glycine, 25 mM Tris base, 1.5 mM SDS, 10% methanol). The membrane was blocked overnight at 4°C in 5% milk-PBS-T (5% nonfat milk, 140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.05% TWEEN 20). The blocked membrane was then incubated with either H3 K9 di-methyl (1:500; Upstate Biotech) or actin (1:2000; Sigma-Aldrich, St. Louis, MO) primary antibodies in 5% milk-PBS-T for 1 hour at room temperature and then washed three times with PBS-T. This was followed by another 1 hour room

temperature incubation with the appropriate peroxidase-conjugated secondary antibody diluted 1:10,000 in 5% milk-PBS-T, donkey anti-rabbit (Amersham Pharmacia Biotech) for anti-H3 K9 di-methyl and goat anti-mouse (Santa Cruz Biotechnology) for anti-actin. After three more washes in PBS-T, the blots were visualized via chemiluminescence using Amersham ECL detection reagents (Amersham Pharmacia Biotech) and Kodak BioMax XAR Film (Eastman Kodak Company, Rochester, NY). Protein bands were quantitated via integrated optical density measurements on a Kodak Gel Logic 200 Imaging System (Eastman Kodak Company) and normalized to each sample's respective actin loading control density.

Standard whole-cell protein isolation and immunoblot analysis

For analysis of global G9A levels, untreated and 5-aza-CdR-treated MDA-MB-231 and UACC 1179 cells were grown to near confluence in T-150 dishes and washed with ice-cold PBS. Cells were then scraped from the plate in ice-cold PBS supplemented with 1 mM PMSF, 10 µg/mL aprotinin and 25 µg/mL leupeptin, followed by centrifugation at 500 x g for 5 min. at 4°C. The cell pellet was lysed in ice-cold RIPA buffer (1% NP-40, 0.25% Na-deoxycholate, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA) with protease inhibitors (same as above) and placed on ice for 15 min., with periodic vortexing. Lysed cells were then centrifuged at 14,000 rpm for 10 min. at 4°C to remove cellular debris, and the solubilized protein supernatant was reserved. Concentrations of whole-cell lysates were determined as described above.

Equal amounts of whole-cell protein (30 μ g) were size-fractionated via SDS-polyacrylamide gel electrophoresis on a 4-15% Ready Gel Tris-HCL Gel (Bio-Rad Laboratories) and transferred to a PVDF membrane using 1X Transfer Buffer (40 mM glycine, 50 mM Tris base, 20% methanol). The membrane was blocked overnight as previously described, and was then incubated with either G9A (1:667; Upstate Biotech) or actin (1:2000; Sigma-Aldrich) primary antibodies in 5% milk-PBS-T for 1 hour at room temperature, followed by three washes with PBS-T. This was followed by another 1 hour room temperature incubation with the appropriate peroxidase-conjugated secondary antibody diluted 1:10,000 in 5% milk-PBS-T, donkey anti-rabbit (Amersham Pharmacia Biotech) for anti-G9A and goat anti-mouse (Santa Cruz Biotechnology) for anti-actin. Blots were visualized and quantitated as described for histone Western blot analyses.

Procurement of breast tumor specimens and isolation of RNA from tissues

Flash frozen breast cancer tissue specimens were obtained from patients who underwent surgery for breast cancer, either lumpectomy or mastectomy, at the University Medical Center in Tucson, AZ, from 2003 to 2004. All patients signed surgical and clinical research consents for tissue collection in accordance with the University of Arizona Institutional Review Board and HIPAA regulations. At the time of surgery, a 1-3 cm section of the tumor was immediately snap frozen in liquid nitrogen and stored in our prospective breast tissue bank at -80°C . From each tissue block, a series of 5 micron sections were cut and stained with hematoxylin and eosin (H&E) for pathological

evaluation. All of the H&E slides were reviewed by one breast pathologist to determine the integrity of the tumor specimen and this was correlated with the clinical pathologic review performed by an independent pathologist.

Isolation of RNA from frozen breast tumor specimens was performed as follows: 30–50 µg of tissue was disrupted in a 1.5 mL RNAase free tube with an RNAase-free Pellet Pestle (Kimble-Kontes, Vineland, New Jersey) then passed through a 21-gauge needle to homogenize the sample. Following homogenization, RNA was isolated using an RNeasy® Mini or Midi kit (QIAGEN). All breast tumor specimen RNAs were run out on an Agilent RNA Labchip (Agilent Technologies, Waldbronn, Germany) for quantitative and qualitative assessment of each RNA sample. Real-time RT-PCR was then performed to assess G9A expression levels in each normal breast and breast tumor patient sample. A Wilcoxon Rank-Sum Test was used to determine whether G9A expression is significantly elevated in breast tumor samples with respect to its expression in normal breast tissue samples at the 95% confidence level by calculating a p-value (i.e., p-value < 0.05).

siRNA transfections

MDA-MB-231 cells were plated in 6-well dishes and grown to ~50% confluence. Cells were then transfected using Lipofectamine 2000 (5 µL for single and 10 µL for dual transfections) (Invitrogen) and 6 µL G9A(BAT8)_2 (catalog #: 8100091196) and/or DNMT1_1 (catalog #: 8100300062) siRNA duplexes (QIAGEN) in a total volume of 1 mL antibiotic- and serum-free RPMI 1640 for 5 hours at 37°C. Following transfection, 1

mL RPMI 1640 supplemented with 5% fetal bovine serum was added to each well. The following day, media was removed and cells were covered with 2 mL of complete media (RPMI 1640 containing 5% fetal bovine serum supplemented with 50 $\mu\text{g}/\text{mL}$ Penicillin/Streptomycin and 292 $\mu\text{g}/\text{mL}$ L-glutamine). Three days post-transfection, the media was changed again and total RNA or protein was isolated a day later (four days after initial transfection). Real-time RT-PCR was then performed to assess mRNA expression levels following siRNA transfection.

RESULTS

The Role of Mutant p53 in Human Breast Cancer Cells

p53 mutation status of breast tumor cell lines

Mutations in the DNA binding domain of the p53 gene are a common occurrence in breast cancers, leading to inhibition of its transcriptional transactivation function and the suppression of p53-target genes (255). Therefore, we sought to assess the p53 mutational spectrum in a number of human breast cancer cell lines to determine the extent and types of mutations present in these cells. DNA sequencing of the p53 gene was performed to identify its mutation status in three previously uncharacterized UACC breast cancer cell lines for comparison to the known p53 status of other commonly used breast tumor cell strains found in the literature (**Table 1**) (248, 254). This compilation revealed that all but one of the breast tumor cell lines investigated express mutant forms of the p53 gene, with the mutations being found almost exclusively in the DNA binding domain of p53 in these cells. These results provide further evidence of loss of functional p53 in a wide range of breast tumor cell lines and the importance of p53 mutation in tumorigenesis.

Pharmacologic reactivation of silenced p53-target genes in human breast cancer

As many breast cancer cell lines produce mutant forms of the p53 protein, we were interested in identifying p53-target genes with tumor suppressor activities that are subject to down-regulation following functional loss of this important transcription factor

Cell Line	p53 Mutation	p53 Status
* MCF-10A	--	wt
UACC 893	--	wt
UACC 1179	R213X	mutant
UACC 2087	V216M	mutant
MDA-MB-157	Del. Exon 2-4	mutant
MDA-MB-231	R280K	mutant
MDA-MB-453	Del. Codon 368	mutant
MDA-MB-468	R273H	mutant
BT 549	R249S	mutant
HS 578T	V157F	mutant

* **Immortalized normal breast cell line**

Table 1. Table showing the p53 mutation status of commonly used breast tumor cell lines. DNA sequencing of the p53 gene determined the p53 mutation status of the UACC cell lines, while the p53 mutation status of the remaining cell lines was taken from the literature.

(i.e., class II tumor suppressors (256)). To identify such targets, two-color fluorescence cDNA microarray experiments were performed to profile the expression patterns of approximately 5376 genes in 10 breast cancer cell lines that were either untreated, infected with an adenovirus containing an insert for the wt p53 gene (Ad-p53/GFP) or infected with an empty vector control adenovirus (Ad-GFP) (263, 264). MCF-10A, an immortalized, non-tumorigenic, mammary epithelial cell line, was used as a common reference. Genes with expression that varied across the cell lines and treatments were identified at the $p < 0.05$ level and used in a multidimensional scaling analysis (265), revealing a set of genes that were induced specifically by wt p53 in eight of the breast cancer cell lines that clustered together following Ad-p53/GFP infection (248). This group of genes contained a number of previously identified p53-target genes, including MASPIN (**Table 2**), a tumor suppressor gene frequently lost in breast cancer (247, 250-252, 266). DSC3 was another p53-responsive, anti-metastatic tumor-suppressor gene identified by these microarray studies (**Table 2**) that is similar to MASPIN in that it is downregulated in breast cancer (249), and also has a CpG island promoter that contains a p53-binding site. The microarray results for MASPIN were confirmed by RT-PCR and Western blot analysis in two breast tumor cell lines (MDA-MB-231 and UACC 1179) that express mutant p53 and have epigenetically silenced MASPIN promoters, showing that only the wt p53/GFP adenovirus induced its expression, whereas the control adenovirus containing only the GFP gene insert had no effect (**Fig. 9**). It is important to note, however, that wt p53 reintroduction was only able to partially restore MASPIN

Cell Line	MASPIN Induction	DSC3 Induction	p53 Status
* MCF-10A	3.1	2.2	wt
UACC 893	14.5	0.2	wt
UACC 1179	7.5	13.2	mutant
UACC 2087	6.5	9.8	mutant
MDA-MB-157	55.7	58.0	mutant
MDA-MB-231	12.1	0.5	mutant
MDA-MB-453	70.3	28.0	mutant
MDA-MB-468	38.9	4.4	mutant
BT 549	7.2	17.3	mutant
HS 578T	2.4	3.9	mutant

* **Immortalized normal breast cell line**

Table 2. Table showing the cell lines studied and the fold induction of MASPIN and DSC3 expression by wt p53 relative to vector-only-infected cells, as determined by two-color cDNA microarray analysis. Also shown is the status of the p53 gene (wt or mutant) in these cell lines.

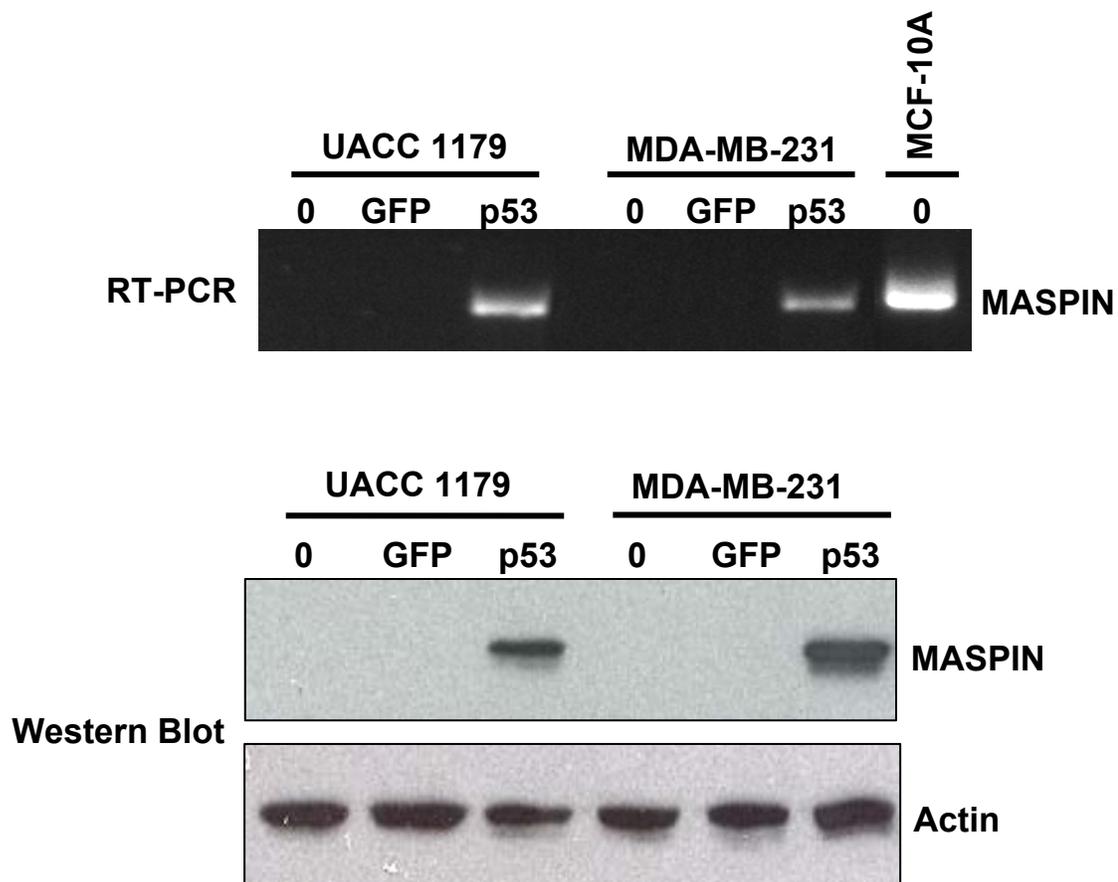


Figure 9. The top panel shows RT-PCR analysis of MASPIN gene expression in untreated (0), vector control-infected (GFP), and wt p53-infected (p53) MDA-MB-231 and UACC 1179 breast tumor cells, in addition to MCF-10A immortalized normal breast epithelial cells. The bottom panel is a western blot analysis of the same breast tumor cells, probed for MASPIN and Actin.

expression in these breast tumor cells, relative to the basal levels seen in MCF-10A normal breast cells.

These initial results were encouraging and prompted further optimization our wt p53 adenoviral dosing regimen and a comparison of the utility of wt p53 reintroduction with various mutant p53 reactivation strategies in breast tumor cells harboring inactivated p53. A 36-hour time-course experiment assessing MASPIN expression via RT-PCR after treatment of MDA-MB-231 cells with Ad-p53/GFP (200 pfu/cell) revealed peak MASPIN re-expression beginning at 24 hrs. post-infection (**Fig. 10**). A similar level of reactivation was seen 36 hrs. after infection, but this time-point also resulted in considerable cell death, making 24 hrs. the optimal infection duration for Ad-p53/GFP-mediated gene reactivation at this dose. In addition, MASPIN re-expression was closely correlated with the percentage of cells expressing GFP, the detection marker of infection, as measured by flow cytometry (**Fig. 10**). Further dose titering experiments after 24 hrs. treatment demonstrated maximal MASPIN re-expression in MDA-MB-231 cells following Ad-p53/GFP infection at 200 pfu/cell, with higher doses leading to extensive cell death (**Fig. 11**). Therefore, Ad-p53/GFP infection of breast tumor cells with 200 pfu/cell for 24 hrs. (the same treatment strategy used for the microarray studies discussed above) led to the greatest reactivation of MASPIN expression without inducing massive cell loss, making this the most favorable dosing regimen for further experiments.

A number of researchers have demonstrated the efficacy of various pharmacologic strategies aimed at reactivating mutant p53 in tumor cells, leading to p53-target gene reactivation and the suppression of tumor cell growth (86-88). Therefore, we

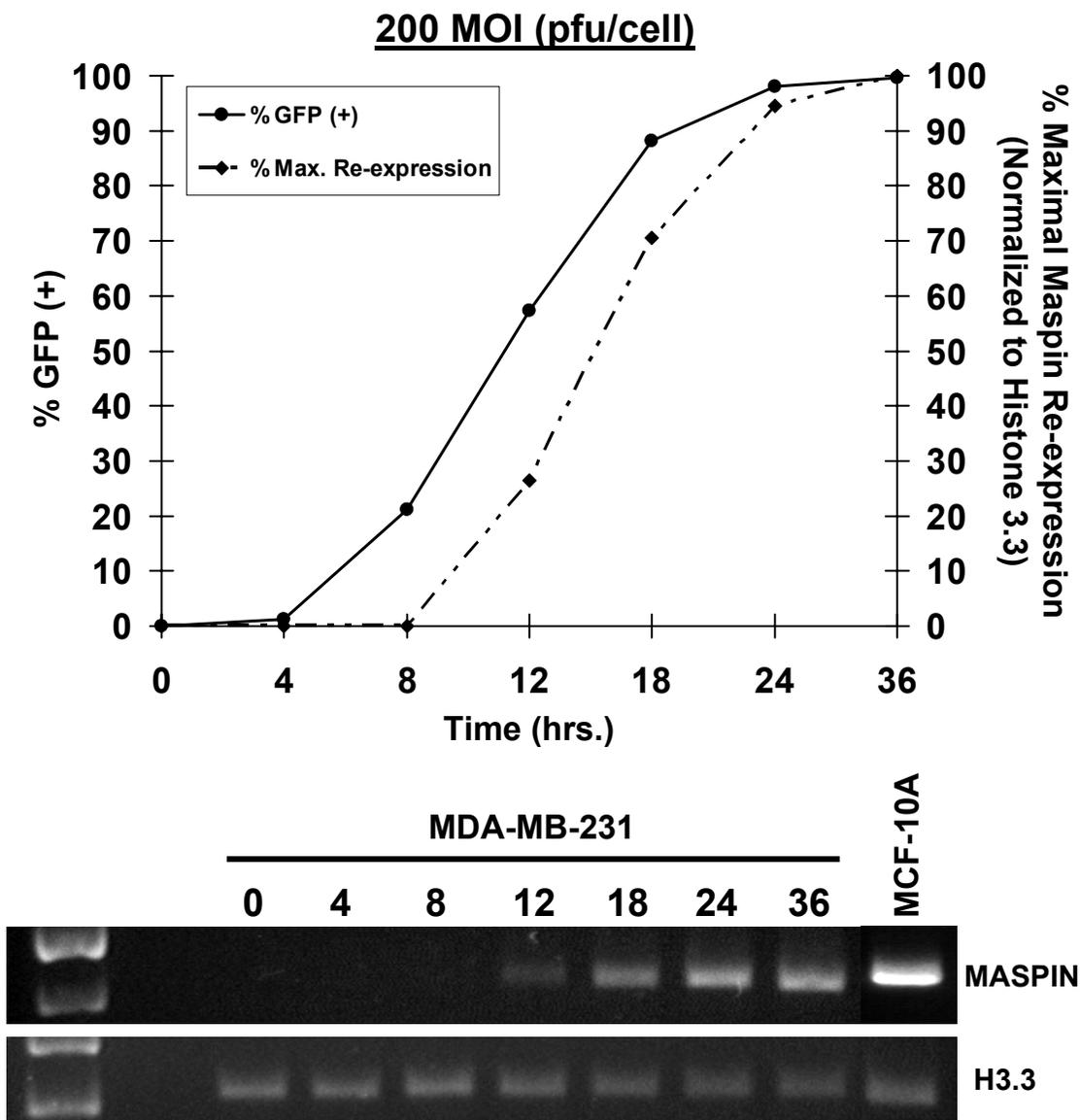


Figure 10. The graph on top illustrates the percentage of MDA-MB-231 cells expressing the detection marker of adenoviral infection (GFP) after various durations of infection with 200 pfu/cell Ad-p53/GFP, as measured by flow cytometry, as well as the percentage of maximal MASPIN expression (36 hrs.) achieved at the same time-points as analyzed via RT-PCR, after normalization to histone H3.3 expression. Below are agarose gel pictures of the MASPIN and histone H3.3 RT-PCR products used to quantitate expression of these genes using integrated densitometry in MDA-MB-231 breast tumor cells. Also shown for comparison is the basal expression of MASPIN and H3.3 in the normal breast cell line MCF-10A.

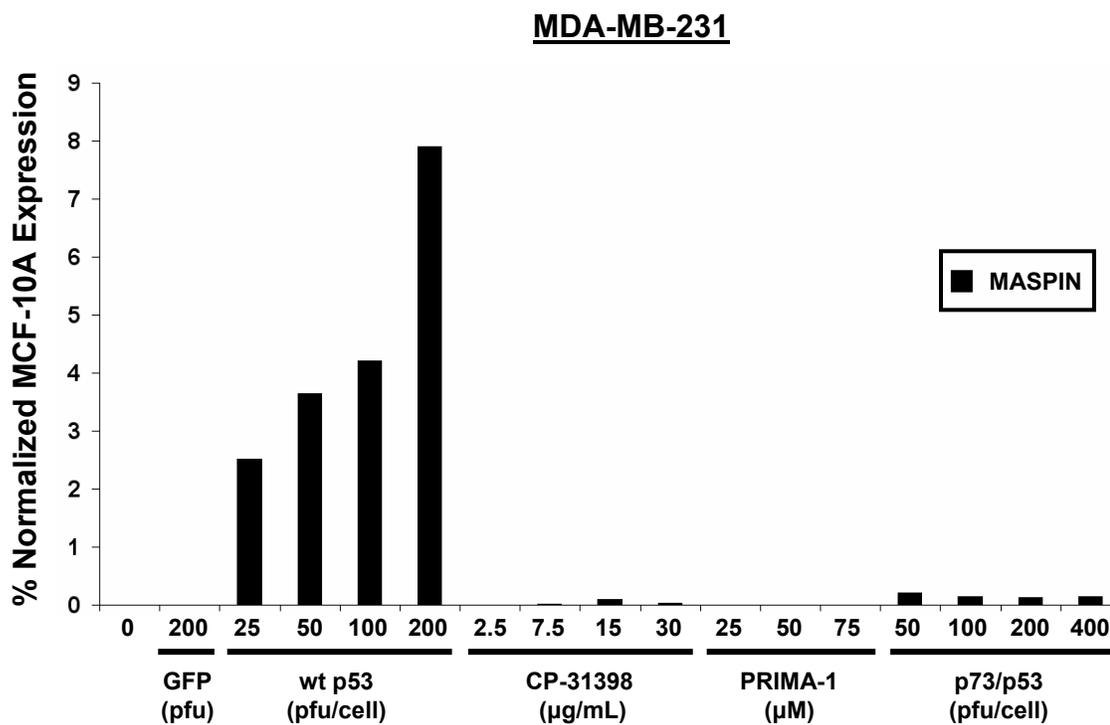


Figure 11. This graph compares the levels of MASPIN re-expression in MDA-MB-231 breast tumor cells following wt p53 reintroduction using a range of Ad-p53/GFP doses, with various doses of three mutant p53 rehabilitators (the small molecule chaperones CP-31398 and PRIMA-1, as well as the adaptor protein construct p73/p53) via RT-PCR. MASPIN expression for all samples was normalized to GAPDH and reported as a percentage of MCF-10A basal expression.

compared the ability of two small molecule chaperones (CP-31398 and PRIMA-1), as well as a p73/p53 adaptor protein to reactivate mutant p53 and induce MASPIN re-expression in MDA-MB-231 cells in relation to the reintroduction of wt p53 using Ad-p53/GFP. A range of doses of each drug or adenovirus were administered based upon previous reports in the literature, with Ad-p53/GFP treatment producing significantly higher levels of MASPIN re-expression than any of the mutant p53 rehabilitators, regardless of dose (**Fig. 11**). Although the rehabilitation of mutant p53 is a more tumor-specific approach to treating cancers, the relatively meager reactivation levels achieved by these currently available approaches, in comparison to wt p53 addition, make the latter method more attractive at this date. Moreover, mutant p53 rehabilitators typically only work on select mutants that are not severely destabilized, making their widespread use problematic, whereas reintroduction of wt p53 is a viable option in all tumors expressing mutant p53.

Mechanism of wt p53-Mediated Gene Reactivation

Confirmation of MASPIN and DSC3 as p53-target genes in vivo

Although MASPIN had already been described as a p53-target gene in prostate tissue, we were somewhat surprised to see MASPIN and DSC3 reactivation by wt p53 in breast cancer cells, since we and others have shown these genes to be silenced in association with aberrant DNA methylation and histone hypoacetylation of their CpG-rich promoters, leading to the adoption of inaccessible chromatin architectures (238, 248, 267). Chromatin immunoprecipitation experiments using an antibody against wt p53

demonstrated increased p53 binding to its consensus sites in the promoters of both MASPIN and DSC3 in MDA-MB-231 and UACC 1179 breast tumor cells following Ad-p53/GFP infection, confirming these genes as *in vivo* targets of wt p53 (**Fig. 12**). Consequently, MASPIN and DSC3 were used as models of endogenous p53-target genes to study the mechanism by which p53 reactivates the expression of genes whose inappropriate silencing is associated with aberrant epigenetic repression of their promoters. These characteristics are highlighted in **Figure 13**, showing that both promoters contain p53 response elements within CpG dense regions, making them potential targets of inactivation by both genetic and epigenetic dysregulation during tumorigenesis.

Addition of wt p53 does not decrease DNA methylation of target gene promoters

Aberrant cytosine methylation of the MASPIN and DSC3 promoters is an important facet of gene regulation associated with their inappropriate silencing. As the reactivation of MASPIN and DSC3 occurs within the first 24 hrs. after addition of wt p53, not enough time had elapsed to allow for a passive demethylation of these promoters; however, it is possible that p53 was invoking an active demethylation process. To determine if p53-mediated gene reactivation was associated with an active demethylation of the MASPIN and DSC3 promoters, we performed bisulfite sequence analysis on DNA isolated from untreated MDA-MB-231 and UACC 1179 breast tumor cells and those infected with Ad-p53/GFP for 24 hrs. The MASPIN promoter of untreated MDA-MB-231 and UACC 1179 cells are heavily methylated, and following the

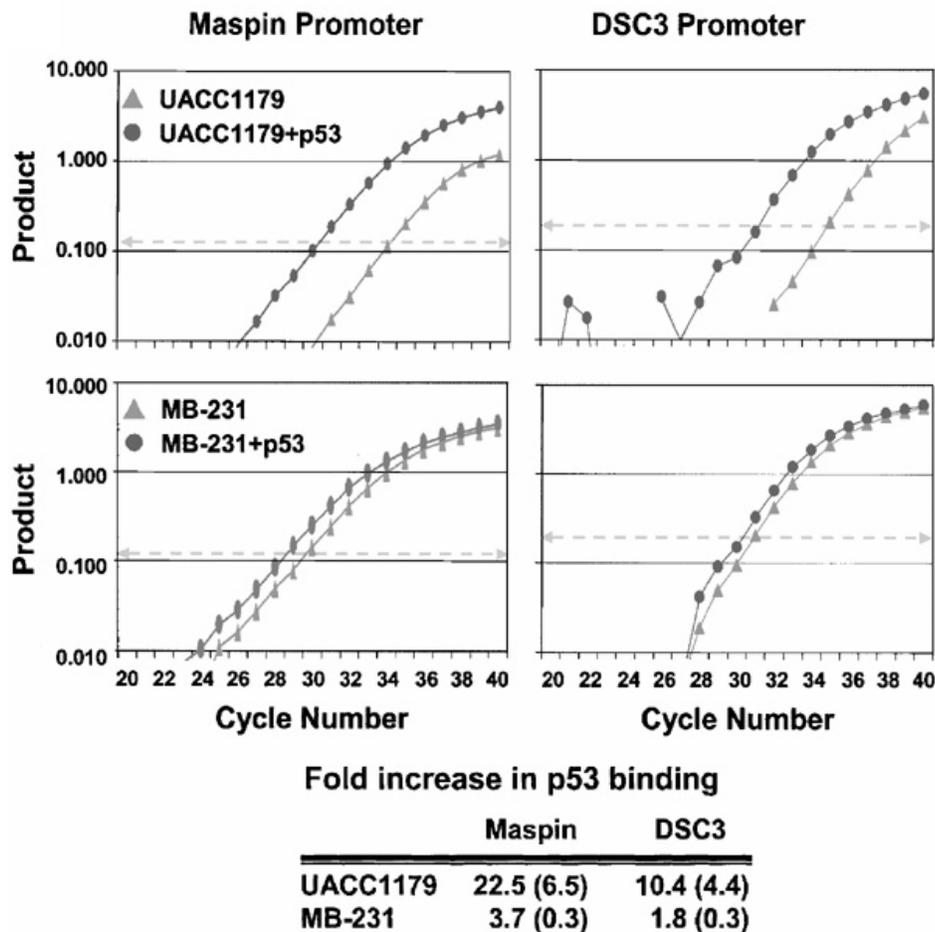
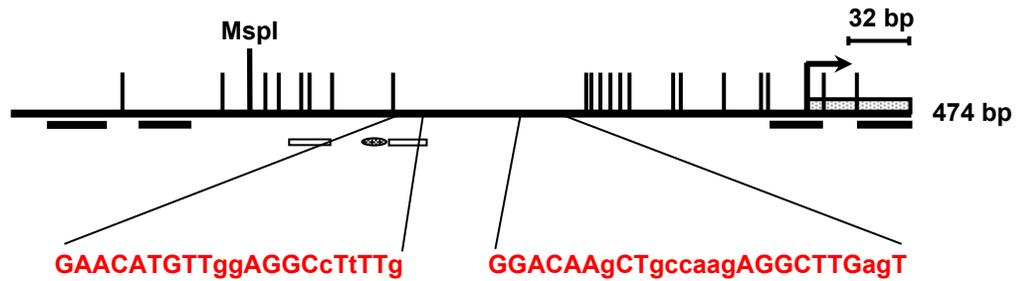
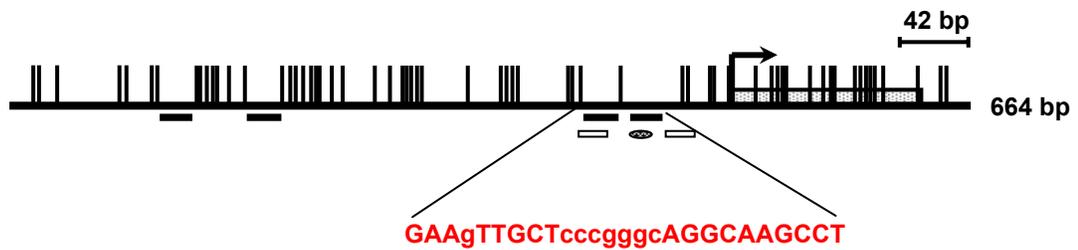


Figure 12. Binding of p53 to the MASPIN and DSC3 promoters was measured in UACC 1179 and MDA-MB-231 breast tumor cells basally and 24 hrs. post-infection with wt p53 adenovirus using a chromatin immunoprecipitation assay with a p53 antibody (clone DO1), coupled to real-time PCR. MASPIN and DSC3 promoter-specific real-time PCR was performed on DNA from the immunoprecipitated chromatin, and the threshold bar for Ct determination was set within the linear range of the PCR amplification. Fold increases in p53 binding following wt p53 adenoviral infection, in comparison to uninfected cells, were calculated using the comparative Ct method. A representative real-time PCR graph is shown above and the average fold enrichment of p53 binding (\pm s.e.m.) from three independent experiments is shown below the graphs.

MASPIN Promoter



DSC3 Promoter



| CpG Site **█** NaBS Primers **▬** ABI Primers **◉** ABI Probe **▨** 5' UTR / Exon 1

Figure 13. Diagrams of the MASPIN and DSC3 promoter regions analyzed, showing locations of primers and primer/probe sets used in our sodium bisulfite sequencing and chromatin immunoprecipitation analyses. The nucleotide sequences below (in red) show the p53 consensus sequences and their positions, while MspI indicates the position of the restriction site analyzed by the chromatin accessibility assay.

forced over-expression of wt p53 neither MDA-MB-231 nor UACC 1179 cells displayed significant changes in the cytosine methylation status of their MASPIN promoters (**Fig. 14**). Similarly, the DSC3 promoter does not become demethylated following Ad-p53/GFP infection in UACC 1179 cells; MDA-MB-231 cells were not assayed. (**Fig. 15**). These results indicate that wt p53 does not reactivate gene expression by inducing promoter demethylation.

Addition of wt p53 increases histone acetylation of target gene promoters

Histone hypoacetylation is another facet of epigenetic regulation that has previously been linked with aberrant cytosine methylation in the silencing of MASPIN and DSC3 in breast tumor cells (238, 248, 267). In addition, wt p53 is known to associate with histone acetyltransferases to stimulate histone acetylation of target genes (29, 32, 268). Thus, we sought to determine the acetylation state of histones H3 and H4 at the MASPIN and DSC3 promoters in untreated MDA-MB-231 and UACC 1179 cells and those infected with either Ad-p53/GFP or the empty vector control Ad-GFP (**Fig. 16**). In both MDA-MB-231 and UACC 1179, wt p53 produced 5- to 27-fold increases in H3 and H4 acetylation within the MASPIN promoter region, whereas infection with the Ad-GFP adenovirus had no effect on their acetylation state. Similarly, wt p53-dependent increases in promoter H3 and H4 acetylation were also seen in the DSC3 promoter in the same cell lines. However, the histone acetylation state of the GAPDH promoter did not change significantly following wt p53 expression in MDA-MB-231 cells, and acetylation of the GAPDH promoter decreased approximately 2-fold for both histones H3 and H4 in

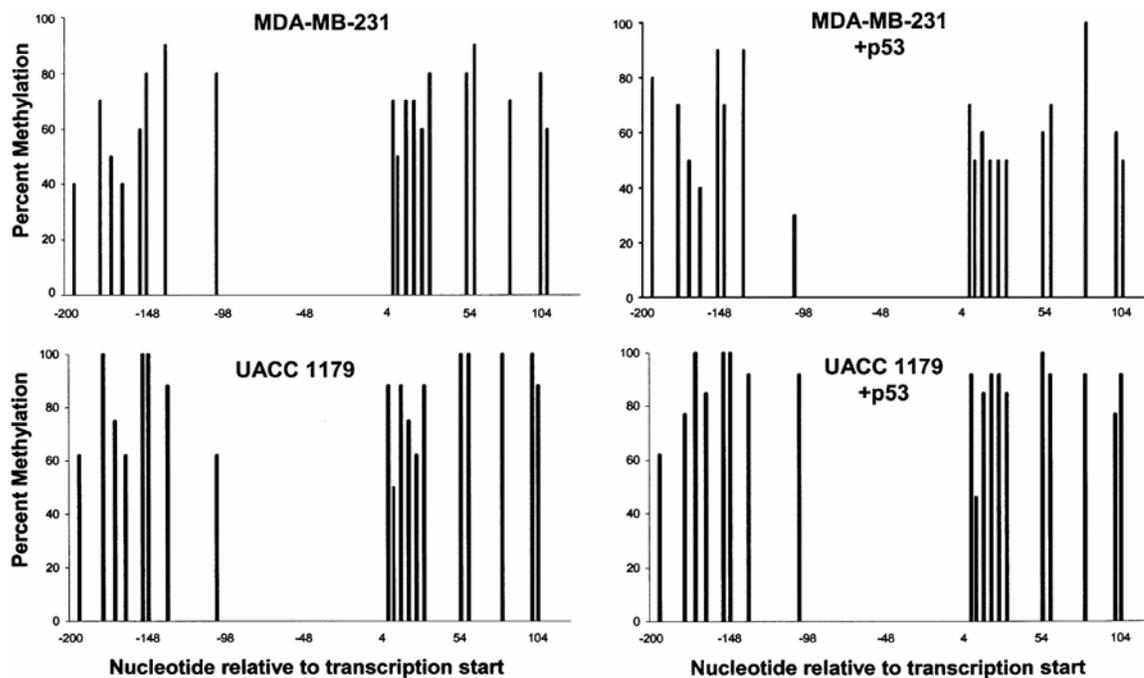


Figure 14. Summary of 5-methylcytosine levels obtained by sodium bisulfite sequencing of the Maspin promoter in uninfected MDA-MB-231 and UACC 1179 breast cancer cells and the same cells 24 hrs. after infection with wt p53. In all, 10 cloned PCR products from each sample were sequenced to determine the percent methylation of the 19 CpG sites in the region analyzed.

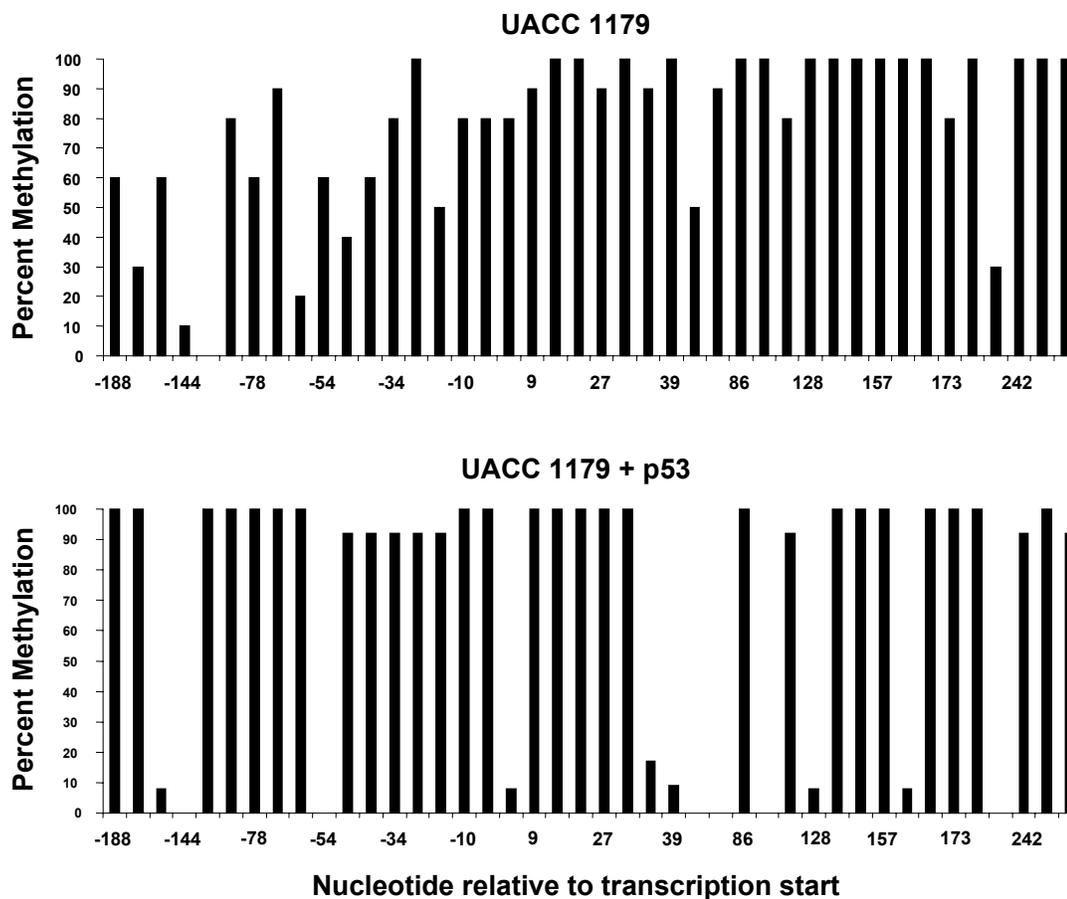


Figure 15. Summary of 5-methylcytosine levels obtained by sodium bisulfite sequencing of the DSC3 promoter in uninfected UACC 1179 breast cancer cells for comparison to the same cells 24 hrs. after infection with wt p53. Ten cloned PCR products were sequenced to determine the percent methylation of the 42 CpG sites in the region analyzed.

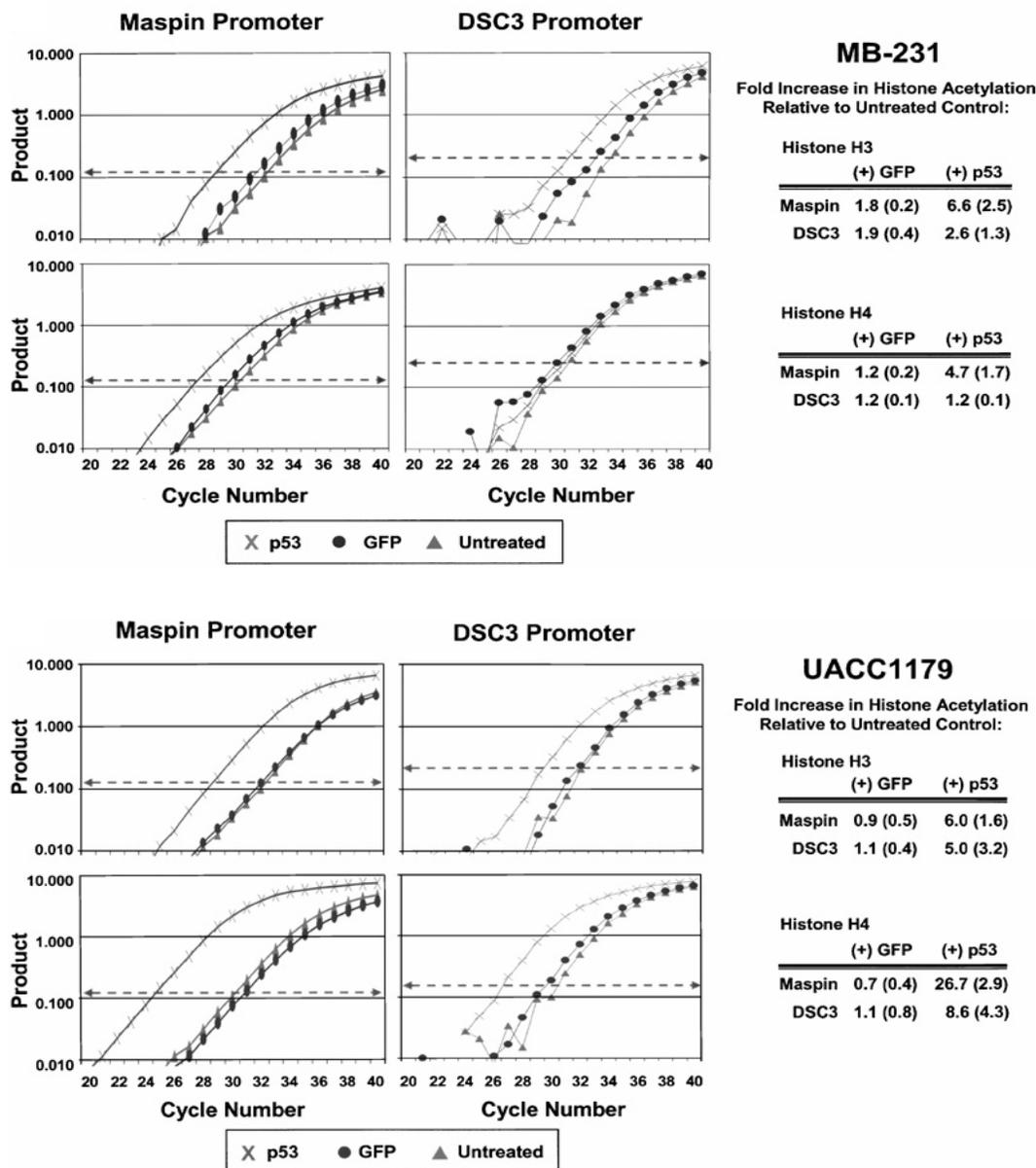
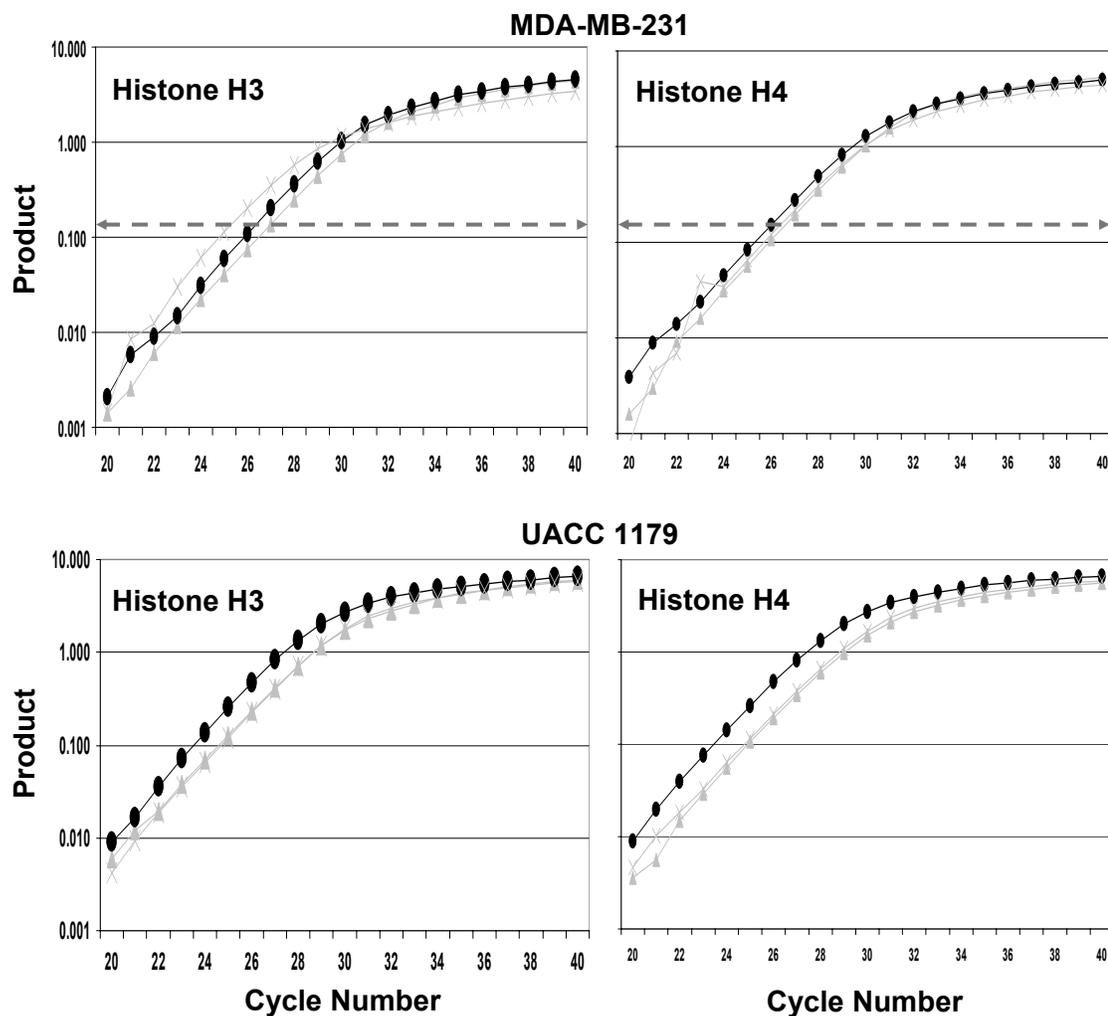


Figure 16. Histone acetylation was analyzed using a chromatin immunoprecipitation assay coupled to real-time PCR analysis. Chromatin was immunoprecipitated from untreated MDA-MB-231 and UACC 1179 breast cancer cells as well as cells 24 hrs. after infection with wt p53 or vector control. Gene promoter-specific real-time PCR was carried out on DNA from the immunoprecipitated chromatin as well as input DNA. A representative real-time PCR graph is shown and the average fold enrichment of histone acetylation relative to untreated control (\pm s.e.m.) from three independent experiments is shown to the right of the graphs.



Fold Enrichment		Ctrl ▲	GFP ●	p53 X
MDA-MB-231	H3	14.7	19.7	37.5
	H4	18.9	28.4	22.6
UACC 1179	H3	44.0	40.4	24.2
	H4	18.9	28.4	15.9

Figure 17. Analysis of histone H3 and H4 acetylation state in the GAPDH promoter of MDA-MB-231 and UACC 1179 using a real-time PCR chromatin immunoprecipitation assay. Chromatin was immunoprecipitated from these cells with either an antibody specific for acetyl H3 or H4. GAPDH promoter-specific PCR was carried out and fold enrichment of acetylated histones was calculated using the ΔC_t method. The fold enrichment values obtained are shown below the real time PCR plots.

UACC 1179 (**Fig. 17**). Taken together, these results indicate that the observed increases in histone acetylation mediated by wt p53 were selective for the MASPIN and DSC3 promoters, and that while histone hypoacetylation and DNA hypermethylation often co-exist in the same domains, increases in histone acetylation do not drive DNA demethylation.

Addition of wt p53 increases chromatin accessibility of target gene promoters

During transcriptional reactivation, changes in histone modification are likely to result in other localized changes in the chromatin architecture. For example, many researchers have demonstrated that increases in histone acetylation are closely linked to increases in chromatin accessibility and the creation of a transcriptionally permissive state (269, 270). As histone acetylation levels were significantly elevated in the MASPIN and DSC3 promoters in breast tumor cells following Ad-p53/GFP treatment, we sought to test the effects of wt p53 reintroduction on the *in vivo* chromatin structure of the p53-target gene MASPIN. To this end, we measured the accessibility of the restriction enzyme MspI to its cognate site in the MASPIN promoter in uninfected, vector-control-infected and wt p53-infected MDA-MB-231 and UACC 1179 cells, using a real-time linker-mediated PCR approach. Infection with wt p53 induced a 21-fold increase in MASPIN promoter accessibility in MDA-MB-231 cells, and a 14-fold increase in UACC 1179 cells, whereas no significant changes were seen after infection with the vector control adenovirus (**Fig. 18**). However, despite significant increases in the accessibility of the MASPIN 5' CpG island following wt p53 infection of these tumor

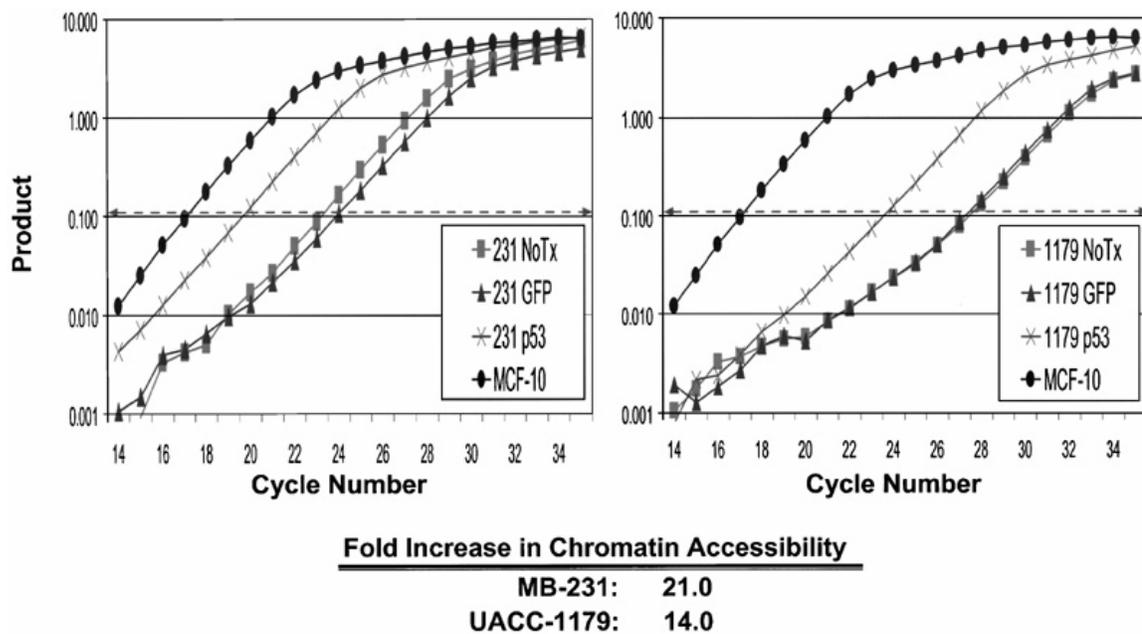


Figure 18. Intact nuclei were isolated from MDA-MB-231 and UACC 1179 after no treatment or 24 hrs. post-infection with wt p53. These nuclei were digested *in vivo* with MspI, for which there is a single site in the MASPIN promoter. Production of a PCR product following amplification was monitored and quantitated by real-time PCR. Increased amounts of PCR product reveal the presence of accessible chromatin. These experiments were repeated independently three times with similar results.

cells, it is important to note that the MASPIN promoter did not return to the basal accessibility seen in MCF-10A normal breast cells (**Fig. 18**). Taken together, these data suggest that wt p53 reactivates MASPIN and DSC3, at least in part, by directing an increase in the acetylation of histones associated with the MASPIN promoter to induce a more accessible chromatin architecture. The incomplete reactivation of MASPIN and DSC3 to normal levels suggested that wt p53 is not sufficient to completely overcome aberrant promoter methylation and its associated repressive effects. If this were the case, then inhibition of DNA methylation should diminish this additional layer of repression and allow for increased gene reactivation by wt p53.

Dual Treatment with 5-aza-CdR and wt p53

5-aza-CdR and wt p53 cooperate to synergistically reactivate MASPIN and DSC3

In a strategy to reverse the genetic (mutant p53) and epigenetic (promoter methylation) aberrations associated with the loss of MASPIN and DSC3 gene expression, we treated MDA-MB-231 and UACC 1179 cells with the DNA methyltransferase inhibitor 5-aza-CdR (10 μ M) for 96 hrs., in conjunction with Ad-p53/GFP infection during the final 24 hrs. Following this dual treatment, MASPIN and DSC3 expression were analysed by quantitative real-time PCR using TaqMan primer/probes. While 5-aza-CdR alone or p53 alone had a relatively small effect on MASPIN and DSC3 expression, when the two treatments were combined they produced a synergistic reactivation of both genes in both cell lines, with levels of expression approaching the basal levels seen in MCF-10A (**Fig. 19**). These results imply that mutant p53 and aberrant DNA methylation

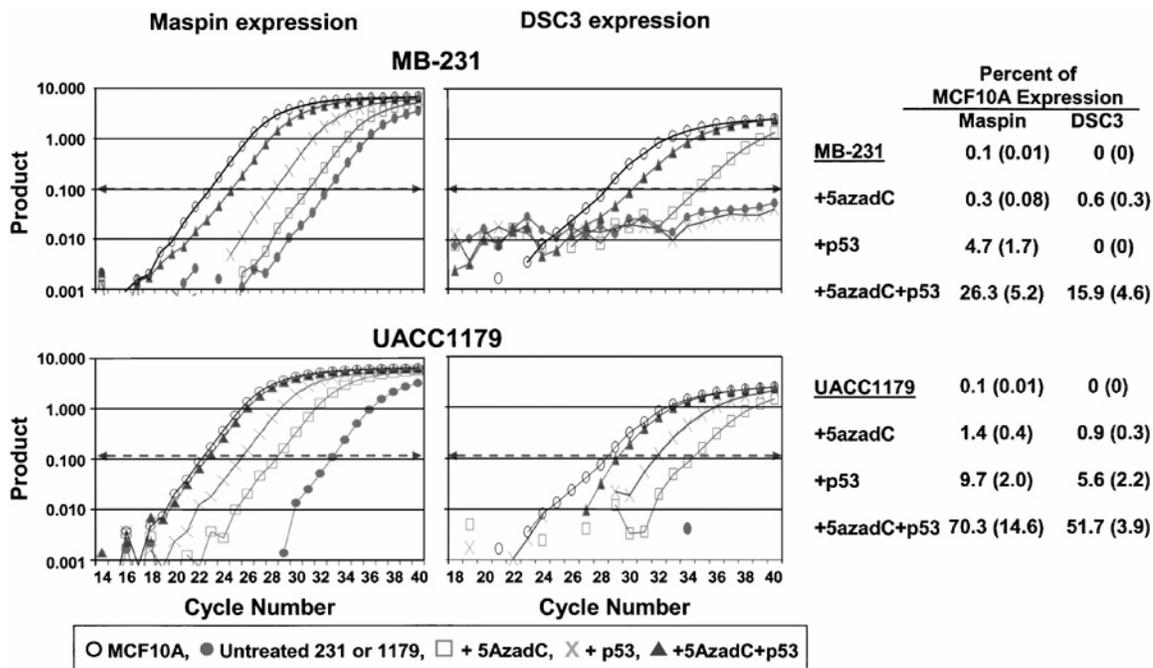


Figure 19. Total RNA from untreated, 5-aza-CdR-treated, wt p53-treated and 5-aza-CdR + wt p53-treated MDA-MB-231 and UACC 1179 breast cancer cells were reverse-transcribed and amplified by real-time PCR in the presence of MASPIN- or DSC3-specific TaqMan expression primer/probes. Results were quantitated using the comparative Ct method. A representative real-time PCR graph is shown and the average reactivation of MASPIN and DSC3 expression (\pm s.e.m.) from three independent experiments is shown to the right of the graphs.

participate in the silencing of gene expression through independent, but interrelated, mechanisms of transcriptional control.

Pre-treatment with 5-aza-CdR allows for the use of lower doses of Ad-p53/GFP

Since the forced over-expression of wt p53 in breast cancer cells lines (200 pfu/cell) expressing mutant p53 can initiate re-expression of epigenetically silenced genes, and its effects are potentiated by 5-aza-CdR pre-treatment, we also tested the effects of lower, more physiologically relevant doses of Ad-p53/GFP in combination with 5-aza-CdR on target gene reactivation. MDA-MB-231 cells were treated with 5-aza-CdR, followed by infection with 10, 15, 50 and 200 pfu/cell Ad-p53/GFP for the assessment of MASPIN reactivation levels via RT-PCR. Interestingly, 5-aza-CdR pre-treatment lead to the same level of synergistic MASPIN reactivation regardless of the dose of Ad-p53/GFP administered, with the lowest, most physiological relevant dose having the same effect as the highest (**Fig. 20**). These results yield further physiological weight to the idea that both genetic and epigenetic mechanisms of gene silencing cooperate in human breast cancers. Moreover, the ability to use lower doses of Ad-p53/GFP in combination with 5-aza-CdR makes this dual treatment regimen more clinically promising.

Pre-treatment with 5-aza-CdR does not enhance the effects of CP-31398

Small molecule rehabilitation of mutant p53 was not effective as a single treatment in the reactivation of MASPIN. However, since pre-treatment with 5-aza-CdR

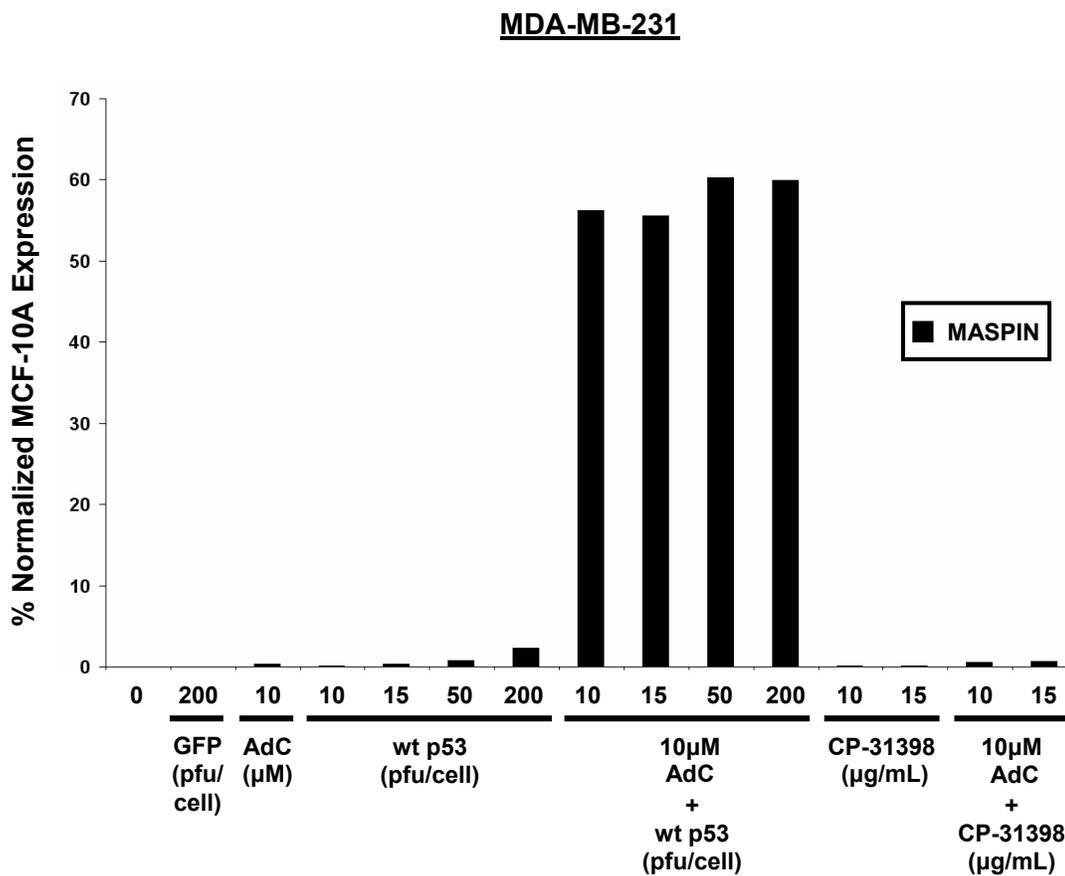


Figure 20. Total RNA from untreated MDA-MB-231 breast tumor cells and those treated with 10 µM 5-aza-CdR, with or without additional treatment with various doses of wt p53 or CP-31398, were reverse-transcribed and amplified by real-time PCR in the presence of a MASPIN-specific TaqMan expression primer/probe. MASPIN expression for all samples was normalized to GAPDH and reported as a percentage of MCF-10A basal expression.

allowed for the use of much lower doses of wt p53, we combined 5-aza-CdR with the most effective of these small molecule chaperones (CP-31398) to see if its limited effects on MASPIN reactivation were also enhanced significantly by 5-aza-CdR. Nevertheless, this dual treatment in MDA-MB-231 cells produced no synergism, with MASPIN re-expression remaining nominal even after 5-aza-CdR and CP-31398 combination (**Fig. 20**). These results further illustrate the ineffectiveness of currently available mutant p53 rehabilitators in the reactivation of MASPIN expression and the superiority of wt p53 reintroduction as a means of p53-target gene reactivation in tumors.

Mechanism of 5-aza-CdR-mediated Tumor Suppressor Gene Reactivation

5-aza-CdR and 5-aza-CR treatment dose-responses

To better understand the mechanism by which 5-aza-CdR potentiates wt p53-mediated induction of DSC3 and MASPIN in breast tumor cells, we performed dose-response studies to determine the optimal dose for further analyses of 5-aza-CdR in the reversal of epigenetic silencing. The breast epithelial tumor cell lines MDA-MB-231 and UACC 1179 were treated with increasing doses of the DNA methyltransferase inhibitor, 5-aza-CdR, in the non-cytotoxic range (0-10 μ M). Real-time RT-PCR analysis of DSC3 and MASPIN expression levels confirmed that treatment with 5-aza-CdR did reactivate transcription of both genes in both tumor cell lines, in a dose-dependent manner (**Fig. 21**). However, while the degree of DSC3 and MASPIN re-expression in MDA-MB-231 cells peaked at the highest dose of 5-aza-CdR administered (10 μ M), in UACC 1179 cells, the greatest levels of transcriptional reactivation occurred following a dose of 3 μ M

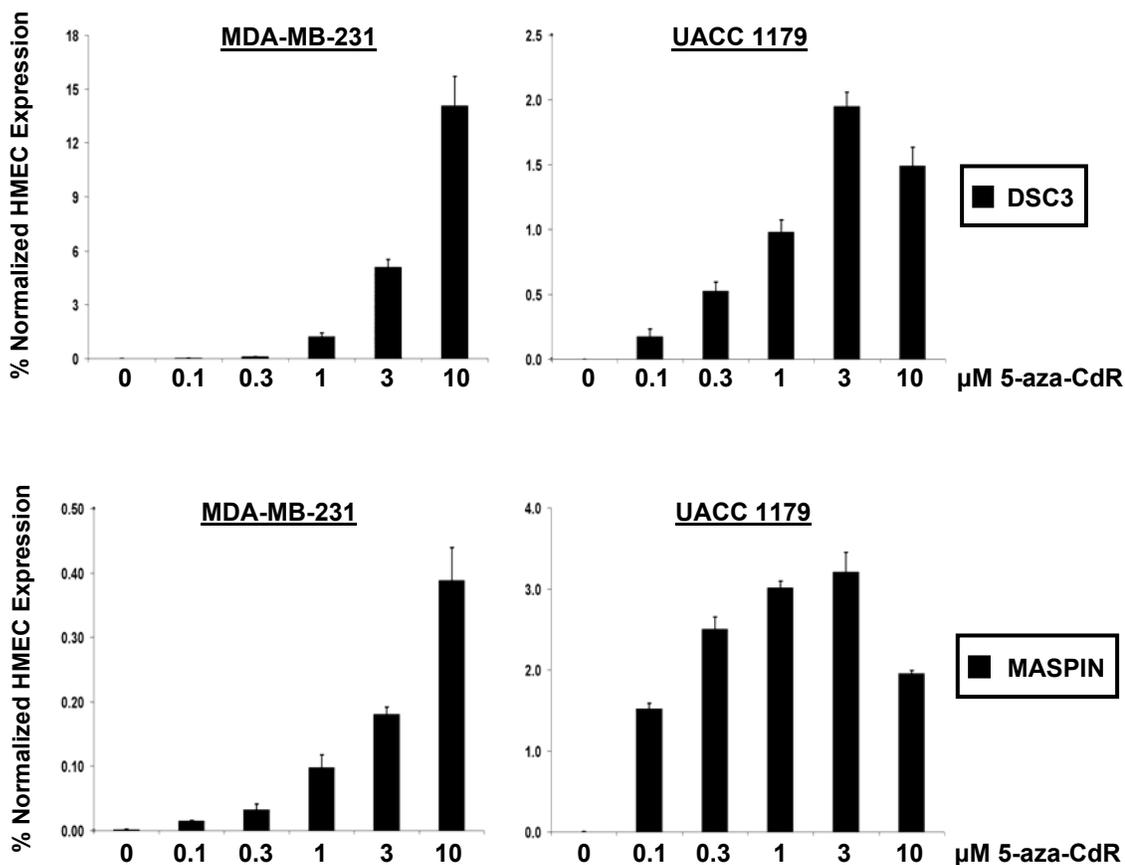


Figure 21. Real time RT-PCR experiments were repeated three times, with expression levels normalized to each sample's respective GAPDH expression and then reported as a percentage of basal HMEC GAPDH-normalized DSC3 and MASPIN expression ($\Delta\Delta C_t$ method). Error bars were calculated as standard error of the mean (\pm s.e.m.). DSC3 and MASPIN expression levels are shown before and after 96 hour treatments with 0.1, 0.3, 1, 3 and 10 μ M 5-aza-CdR in MDA-MB-231 and UACC 1179 breast tumor cells.

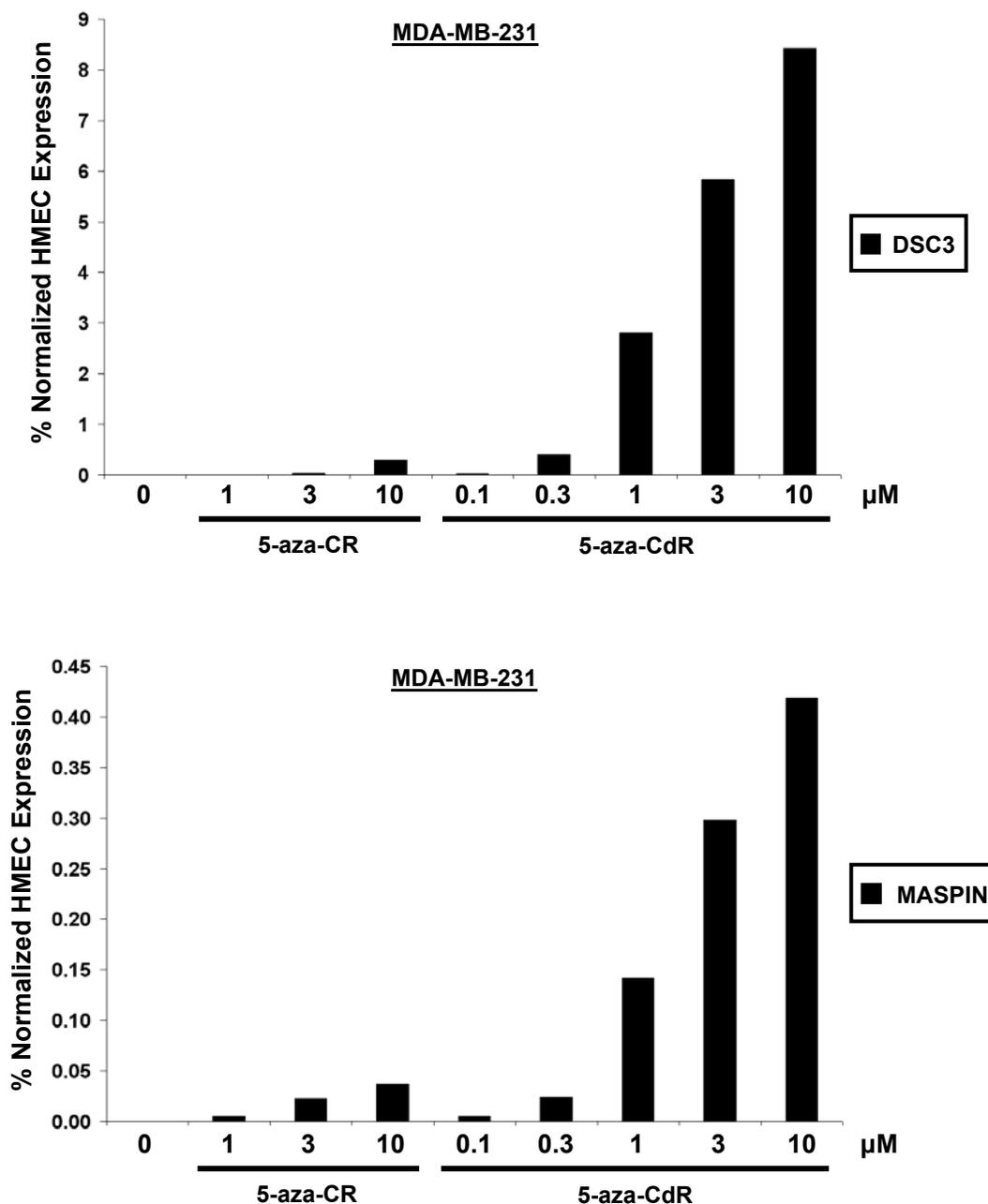


Figure 22. Real time RT-PCR analysis of DSC3 and MASPIN expression in MDA-MB-231 breast tumor cells treated for 96 hrs. with increasing doses of 5-aza-CR and 5-aza-CdR. Expression levels were normalized to each sample's respective GAPDH expression and then reported as a percentage of basal HMEC GAPDH-normalized DSC3 and MASPIN expression ($\Delta\Delta C_t$ method). Error bars were calculated as standard error of the mean (\pm s.e.m.).

5-aza-CdR. For comparison, MDA-MB-231 cells were also treated with increasing doses of either 5-aza-CR or 5-aza-CdR to assess the relative abilities of each drug to induce DSC3 and MASPIN expression (**Fig. 22**). 5-aza-CdR treatment resulted in 10- to 30-fold greater gene induction when compared to the same dose of 5-aza-CR, which is in agreement with the literature (186, 187) and justified the use of 5-aza-CdR for future studies of DSC3 and MASPIN reactivation. In total, this data further supports the idea that the epigenetic silencing of genes such as DSC3 and MASPIN is reversible through the use of pharmacologic inhibitors of enzymes that catalyze repressive epigenetic modifications.

5-aza-CdR does not activate distant, alternative transcription start sites

In an effort to determine whether 5-aza-CdR initiates tumor suppressor gene reactivation from the same CpG island-associated transcription start site as that utilized basally, or whether cryptic upstream sites became active, 5' RACE was performed. Total RNA from MASPIN-positive, normal HMECs, as well as from MDA-MB-231 and UACC 1179 breast tumor cells treated with 5-aza-CdR was converted to cDNA and amplified using primers specific for the 5' end of the predicted MASPIN transcript. For all samples, MASPIN-specific 5' RACE PCR produced amplicons of similar size (**Fig. 23a**), suggesting that all transcripts are initiated from the same region of the MASPIN promoter, whether constitutively expressed in HMECs or 5-aza-CdR-induced in breast cancer cells. Subsequent cloning and sequencing of the MASPIN 5' RACE PCR products confirmed that 5-aza-CdR was not activating a distant, alternative start site, as

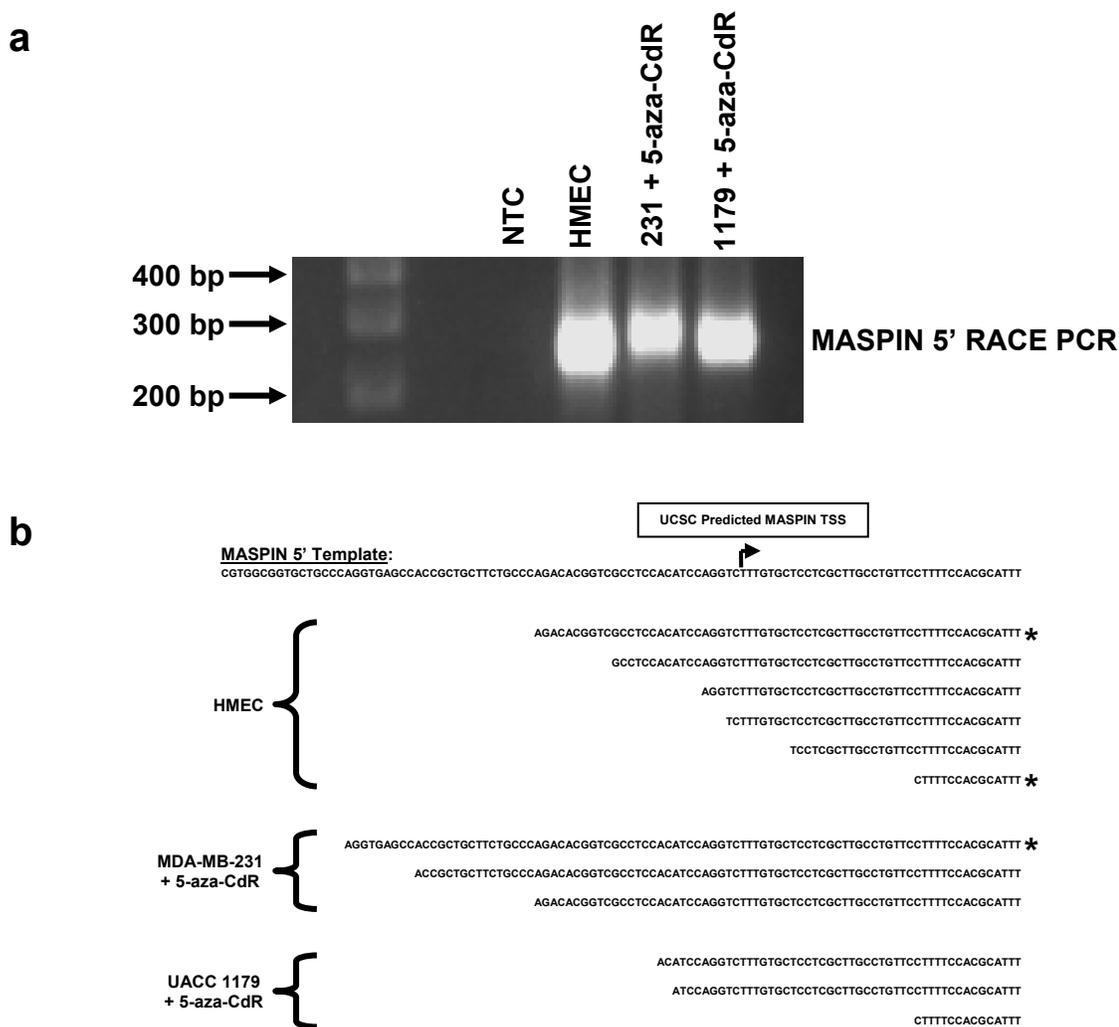


Figure 23. 5' RACE analysis of the MASPIN transcription start site using total RNA from MASPIN-positive, normal HMECs, and from MDA-MB-231 and UACC 1179 breast tumor cells treated with maximal reactivating doses of 5-aza-CdR. **(a)** MASPIN 5' RACE PCR products were run on a 3% agarose gel (shown above) for size determination (predicted size: ~275 bp) prior to PCR product cloning and DNA sequencing. As a negative control, the same 5' RACE protocol was also performed without template RNA (NTC = no template control). **(b)** Summary of MASPIN transcription start sites derived from the sequencing of cloned MASPIN 5' RACE PCR products. Sequences ending with an asterisk (*) denote MASPIN transcripts that had the same start site in multiple clones from the same sample. Shown above is the 5' MASPIN template sequence, with the transcription start site (TSS) predicted in the UCSC human genome database marked by the bent arrow.

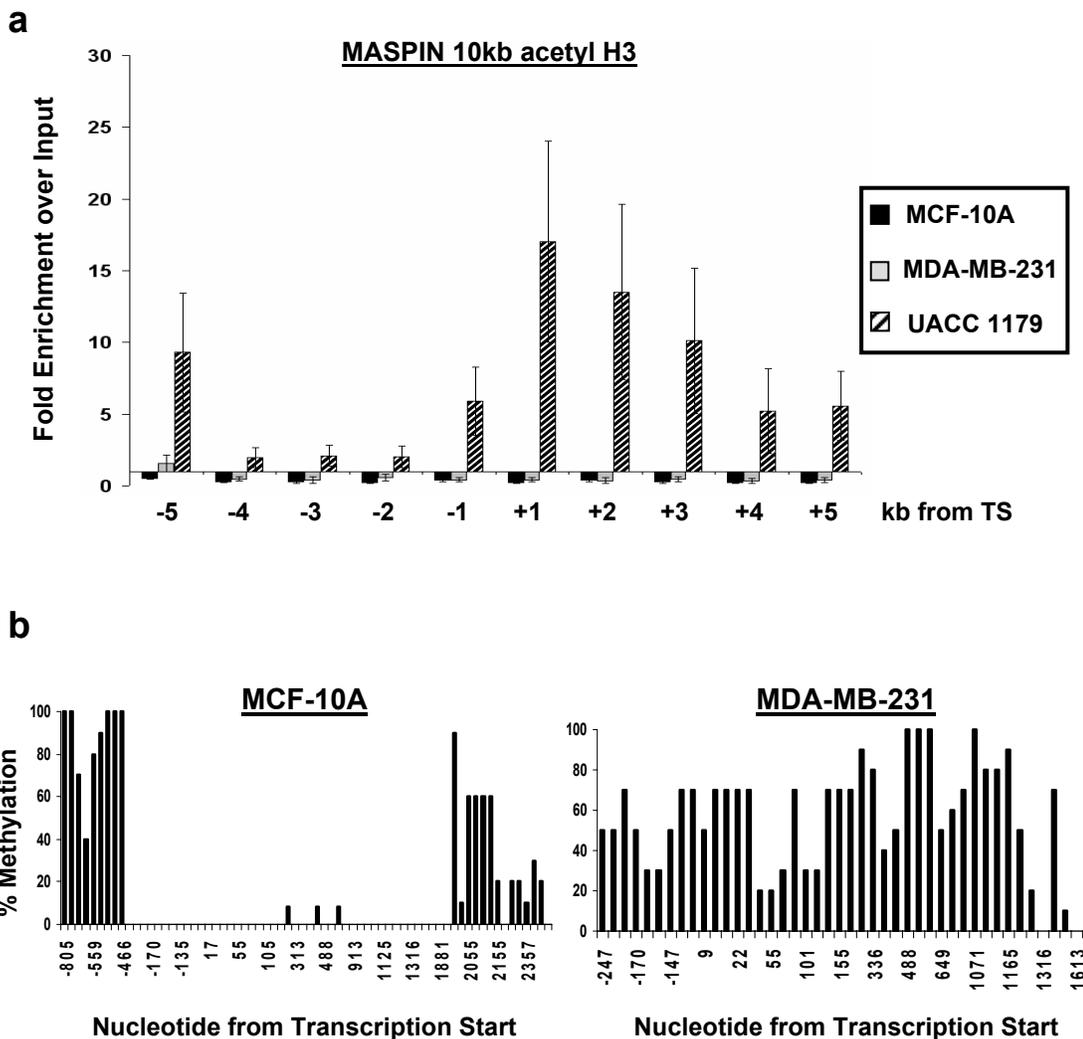


Figure 24. (a) Histone H3 acetylation, and (b) DNA methylation status of normal breast (MCF-10A) and breast tumor (MDA-MB-231 and UACC 1179) cells over an extended region, upstream and downstream of the predicted MASPIN transcription start site, via chromatin immunoprecipitation and sodium bisulfite sequencing, respectively. Acetyl H3 chromatin immunoprecipitation data represents the average of three independent experiments (\pm S.E.M.). For the sodium bisulfite analyses, ten cloned PCR products were sequenced to determine the percent methylation of each of the CpG sites in the region analyzed.

the 5' end of all MASPIN transcripts were mapped to the same localized, CpG island-embedded domain centered around the predicted start site (**Fig. 23b**), a domain that is subject to differential DNA methylation and histone acetylation in MASPIN-positive normal, and MASPIN-negative tumor cells of the breast (**Fig. 24**). Furthermore, it was determined that MASPIN transcription is not initiated from a single start site in either normal HMECs or 5-aza-CdR-treated breast cancer cells, but rather from multiple start sites within this ~75 bp region (**Fig. 23b**). The variation in MASPIN transcription start sites observed both basally in HMECs and after 5-aza-CdR treatment in breast tumor cells, is consistent with the flexible nature of transcriptional initiation seen in TATA-less promoters, such as the MASPIN gene (271, 272).

DSC3 and MASPIN are not reactivated by DNA damage

5-aza-CdR was originally identified as a cytotoxic agent due to its ability to induce DNA damage via the formation of covalent DNA adducts and the obstruction of DNA synthesis (191, 192). Moreover, DNA damage is known to activate p53 to increase its ability to bind to the promoters of target genes (26, 273, 274). To determine whether DNA damage, by itself, was sufficient for DSC3 and MASPIN reactivation in mutant p53 expressing breast tumor cells, MDA-MB-231 cells were treated with increasing doses of the DNA damaging agent doxorubicin. None of the doxorubicin treatments resulted in DSC3 or MASPIN reactivation, even at doses as high as 1000 nM (**Fig. 25**). Thus, these data suggest that the ability of 5-aza-CdR to mediate DSC3 and MASPIN re-expression is a specific effect due to its ability to reverse repressive epigenetic chromatin states in

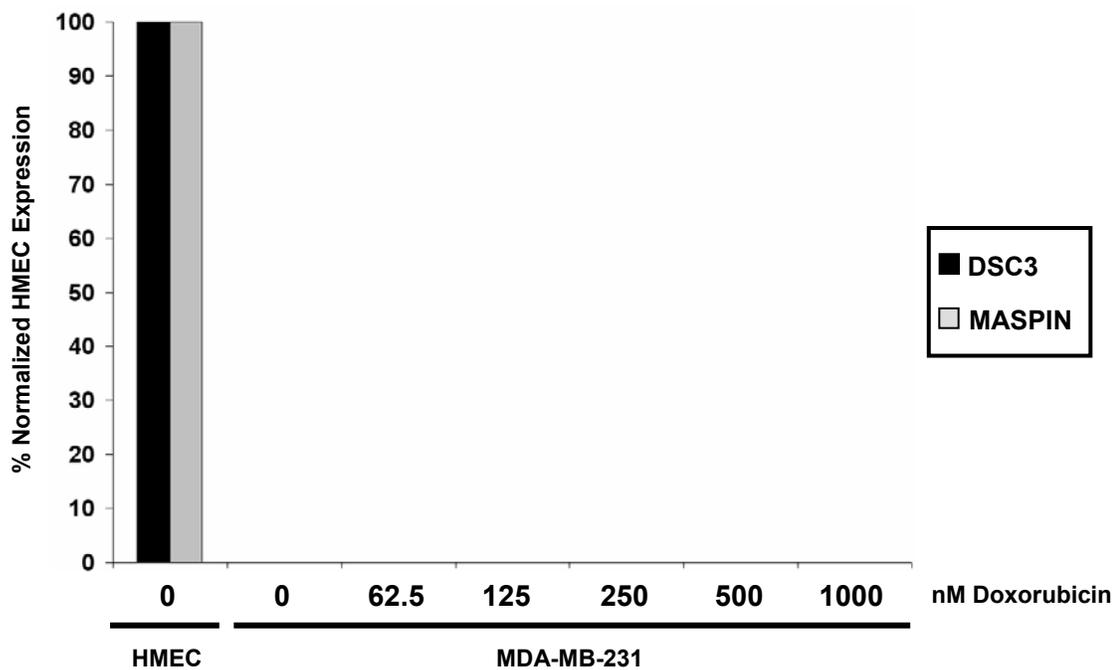


Figure 25. MDA-MB-231 cells were treated with 0, 62.5, 125, 250, 500 and 1000 nM Doxorubicin for 18 hours. Treatments were followed by RT-PCR analysis of DSC3 and MASPIN expression levels, normalized to each sample's respective GAPDH expression and then reported as a percentage of basal HMEC GAPDH-normalized DSC3 and MASPIN expression ($\Delta\Delta C_t$ method).

the promoters of silenced genes, and not a non-specific result of widespread DNA damage.

5-aza-CdR has limited effects on DSC3 and MASPIN promoter DNA methylation

As 5-aza-CdR is capable of inducing re-expression of DSC3 and MASPIN in MDA-MB-231 and UACC 1179 breast tumor cells, we set out to determine its effects on cytosine methylation levels in the promoter regions of both genes. Our breast tumor cell lines were treated once again with a high dose of 5-aza-CdR (10 μ M), followed by extensive sodium bisulfite sequencing of the DSC3 and MASPIN promoters. For this analysis, we compiled sequence data from 94 clones for each gene-associated CpG island in both cell lines, examining the methylation status of 24 CpG sites in the DSC3 promoter and 19 CpG sites in the MASPIN promoter. The percentage of methylation at each CpG site in the promoters of these genes was then calculated, as well as the percentage of methylation across the regions as a whole. Statistical significance of 5-aza-CdR treatment on DNA methylation was assessed using a two-sample test of proportions, comparing the percentage of methylated sites over the entire promoter region analyzed, before and after treatment. The p-value obtained was then tested at the 95% confidence level (p-value ≤ 0.05 were considered statistically significant), to determine whether or not significant changes in regional DNA methylation had occurred following 5-aza-CdR treatment in the DSC3 and MASPIN promoters in both cell lines.

Methylation analysis of the DSC3 promoter in MDA-MB-231 cells revealed a statistically significant reduction in regional promoter methylation after DNA

methyltransferase inhibition, decreasing from 79.6% to 54.9% overall (*p-value < 0.0001), along with the increasing emergence of completely “naked” alleles (**Fig. 26**). Conversely, in UACC 1179 cells, DSC3 promoter methylation was not significantly reduced following 5-aza-CdR treatment (p-value = 0.2911) (**Fig. 26**). It is interesting to note, however, that the DSC3 gene in MDA-MB-231 cells, which experienced the greatest degree of promoter demethylation following 5-aza-CdR treatment, also exhibited the largest increase in gene reactivation (**Fig. 21**). For the MASPIN CpG island promoter, we found that 5-aza-CdR treatment also failed to significantly alter regional methylation of this promoter in either MDA-MB-231 or UACC 1179 cells (p-values = 0.2370 and 0.8593, respectively) (**Fig. 27**). Taken together, these data indicate that while 5-aza-CdR can, in some contexts, reduce cytosine methylation at select loci, the cellular and loci-specific effects of this drug on gene reactivation are likely not limited to DNA demethylation, suggesting that other epigenetic modifications may also be targeted by 5-aza-CdR.

5-aza-CdR induces global DNA demethylation of tumor cells

Although 5-aza-CdR has shown its utility as a DNA hypomethylating drug by both clinicians and researchers seeking to reactivate epigenetically silenced genes in human cancers (194, 195, 212, 214), it appears from our data that the cytosine demethylation effects of this drug may be cell type- and loci-specific. Therefore, since the DSC3 and MASPIN promoters in MDA-MB-231 and UACC 1179 cells did not experience significant cytosine demethylation in all cases after 5-aza-CdR treatment, we

Desmocollin 3

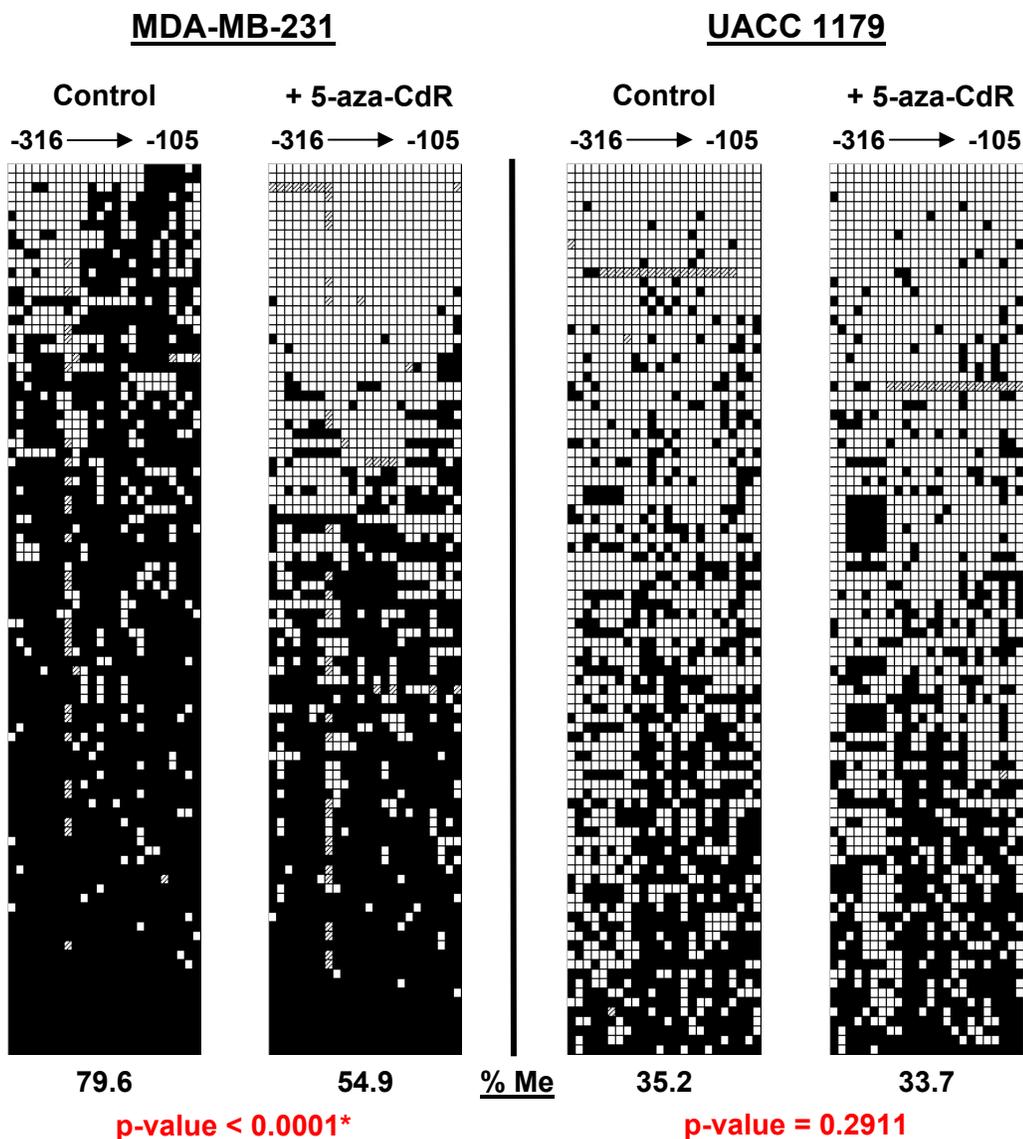


Figure 26. Methylation status of 94 sequenced clones (47 each from two biological replicates), analyzing 24 CpG sites in the DSC3 promoter before and after a 96 hour treatment of MDA-MB-231 and UACC 1179 cells with 10 μ M 5-aza-CdR. In these diagrams, each row represents one cloned PCR product, while the columns contain the data for each of the CpG sites analyzed (■ methylated sites, □ unmethylated sites, ▨ poor sequence data). Clones were sorted from least to most methylated for presentation. The p-values shown below the graphs of treated samples indicate whether or not any differences in regional methylation following treatment were statistically significant (*).

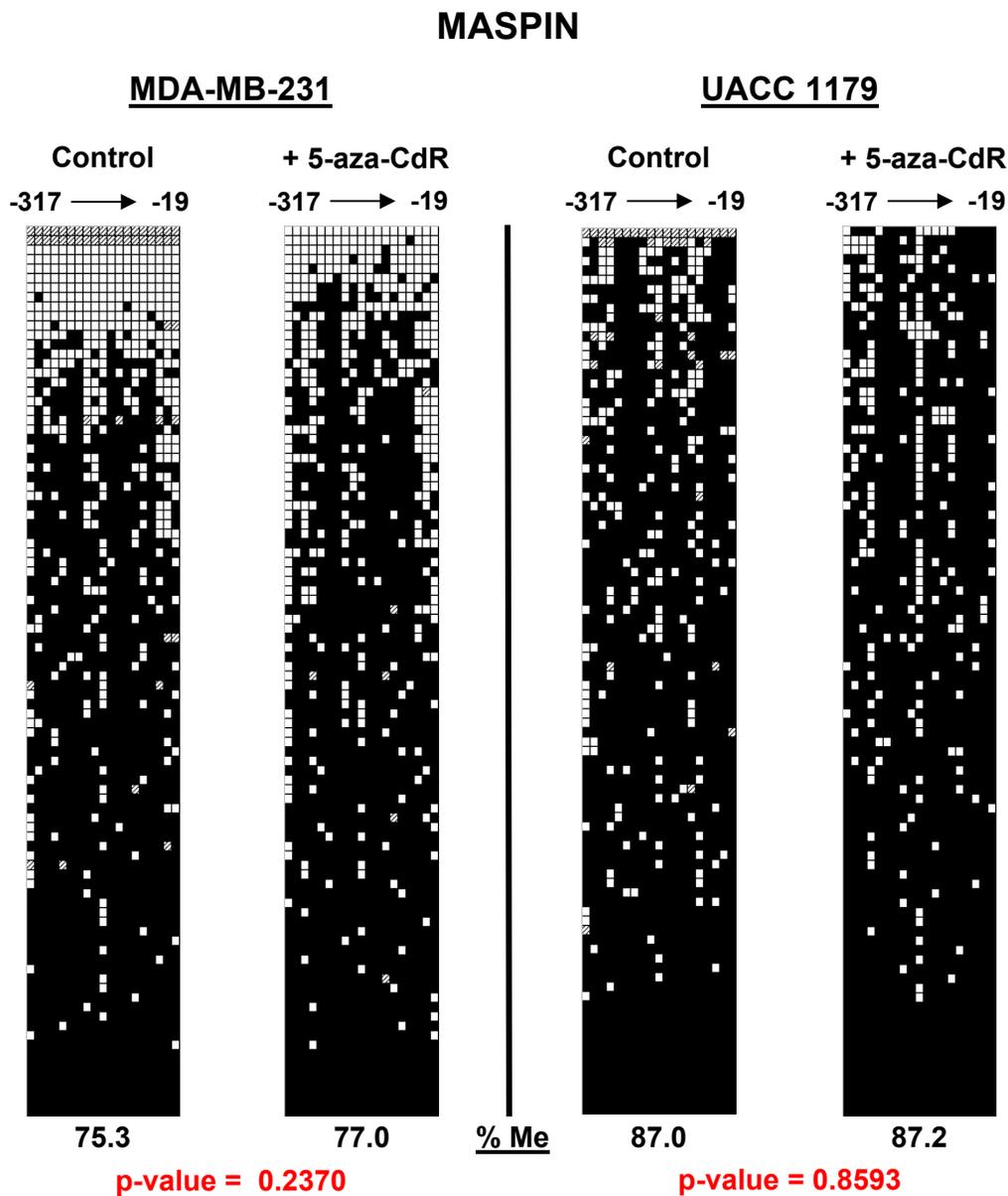


Figure 27. Methylation status of 94 sequenced clones (47 each from two biological replicates), analyzing 19 CpG sites in the MASPIN promoter before and after a 96 hour treatment of MDA-MB-231 and UACC 1179 cells with 10 μ M 5-aza-CdR. In these diagrams, each row represents one cloned PCR product, while the columns contain the data for each of the CpG sites analyzed (■ methylated sites, □ unmethylated sites, ▨ poor sequence data). Clones were sorted from least to most methylated for presentation. The p-values shown below the graphs of treated samples indicate whether or not any differences in regional methylation following treatment were statistically significant (*).

sought to investigate the global effects of this drug in these cell strains. Genomic DNA digested to individual nucleosides was analyzed by LC-MS, to quantitate 5-methylcytosine levels in our breast tumor cells, before and after treatment with 3 and 10 μ M 5-aza-CdR. In MDA-MB-231 cells, total 5-methylcytosine content was reduced by ~65% after treatment with 3 and 10 μ M 5-aza-CdR (**Fig. 28**). Similarly, in UACC 1179 cells, the percentage of cytosines that were methylated decreased by ~50-60% after treatment (**Fig. 28**). Therefore, despite the ability of 5-aza-CdR to induce global CpG demethylation, the 5-methylcytosine levels in the DSC3 and MASPIN promoters in our breast tumor cells were not consistently decreased in all cases, suggesting that the ability of 5-aza-CdR to demethylate specific regulatory regions is context-dependent, and perhaps explains the fact that 35-50% of the original methylcytosine pool in our cells remained methylated after treatment (**Fig. 28**).

5-aza-CdR does not alter the histone acetylation or H3 K27 methylation status of the DSC3 or MASPIN promoters

The epigenetic silencing of genes can result from modifications to both DNA and histones in regions of transcriptional regulation. While DNA hypermethylation is highly associated with transcriptional repression, histone modifications can either repress or promote gene expression (91-94). In light of our data above, showing increased expression of DSC3 and MASPIN in breast tumor cells following 5-aza-CdR treatment without consistent promoter DNA demethylation, we sought to investigate additional mechanisms of action for this drug at the histone level via chromatin

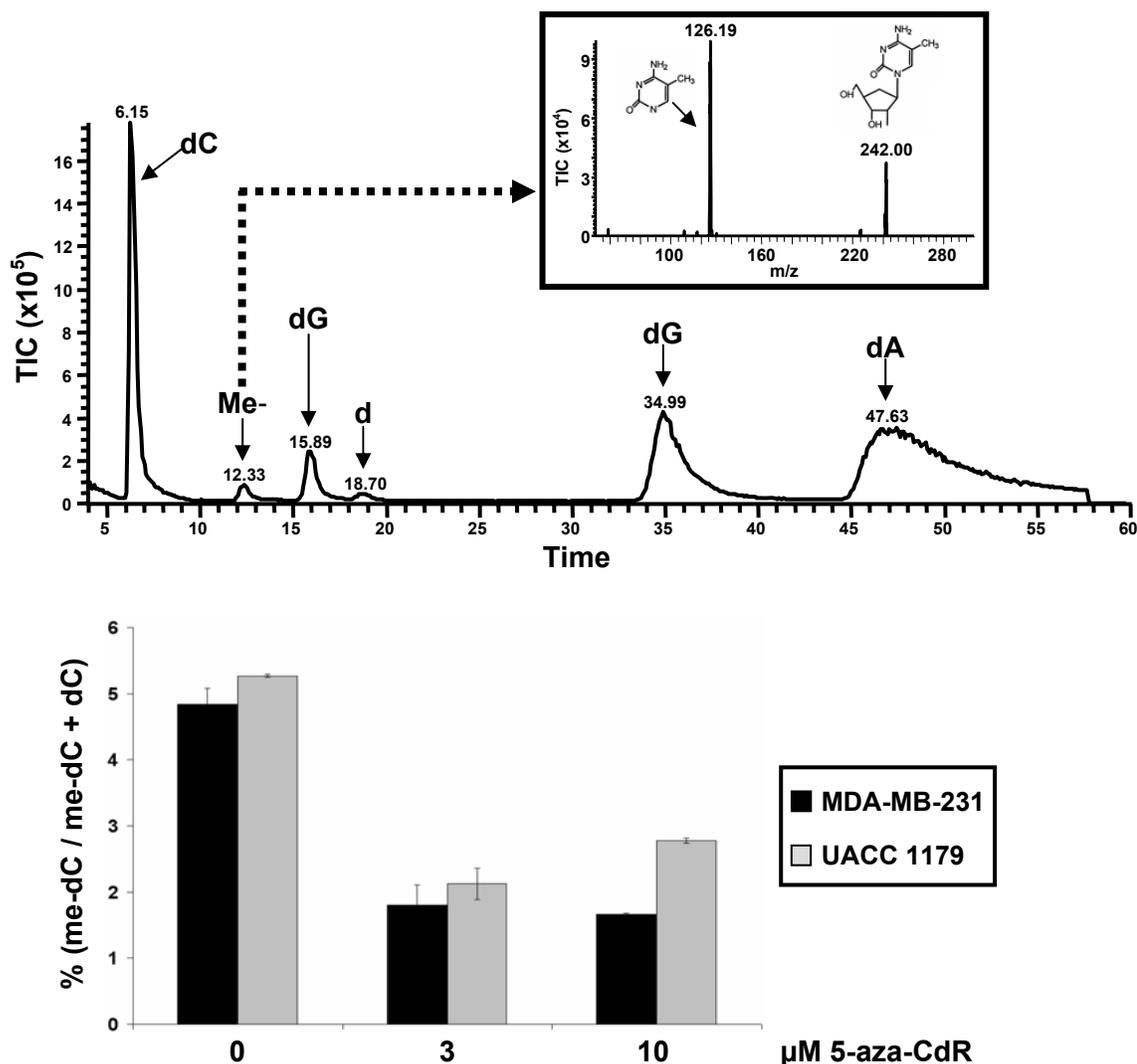


Figure 28. Genomic DNA from MDA-MB-231 and UACC 1179 cells treated with 0, 3 and 10 μM 5-aza-CdR was digested to mononucleosides for LC-MS measurement of 5-methylcytosine levels. Shown above is a representative total ion current (TIC) chromatogram of an untreated UACC 1179 sample. The insert shows the tandem mass spectrum of 5-methyl-dC confirming its structure. MS analysis of biological samples in SIM mode revealed two peaks with the same m/z as that of dG, which were later identified by tandem MS to be the keto-dG (RT: 15.89 min.) and enol-dG (RT: 34.99 min.) tautomers. All treatments and digestions were performed twice and the average percent 5-methylcytosine content is shown graphically for each cell line, before and after treatment. Error bars were calculated as standard error of the mean (\pm s.e.m.).

immunoprecipitation. Among the most widely studied histone modifications include the acetylation of lysine residues on histones H3 and H4 (associated with open chromatin domains), and the methylation of lysines 9 and 27 on histone H3 (associated with closed chromatin domains) (92, 93). As the acetylation of histones H3 and H4 leads to a transcriptionally permissive chromatin state, it is not surprising that both the DSC3 and MASPIN promoters contain underacetylated histones in MDA-MB-231 and UACC 1179 cells (248). However, following 5-aza-CdR treatment, histone acetylation was not markedly increased in either the DSC3 or MASPIN promoters in MDA-MB-231 cells, in comparison to the levels seen in the promoter of the highly expressed house-keeping gene GAPDH (**Fig. 29a**). Additional experiments found that while transcriptionally repressive H3 K27 methylation was enriched in the 5' CpG islands of DSC3 and MASPIN in MDA-MB-231 cells (compare to GAPDH promoter), the levels of this repressive modification were not decreased after 5-aza-CdR treatment (**Fig. 29b**). Thus, histone acetylation and H3 K27 methylation were eliminated as targets of this drug in the reactivation of the epigenetically silenced genes, DSC3 and MASPIN, in breast tumor cells.

H3 K9 di-methylation levels are elevated in DSC3- and MASPIN-negative promoters

Next, since elevated levels of histone H3 K9 di-methylation in the promoter regions of genes is also highly correlated with transcriptional repression (275), we focused further efforts on determining the H3 K9 di-methylation status of the DSC3 and MASPIN promoters in our breast tumor cell lines and to measure the effects of 5-aza-CdR on this repressive epigenetic mark. DSC3 and MASPIN are expressed in a variety

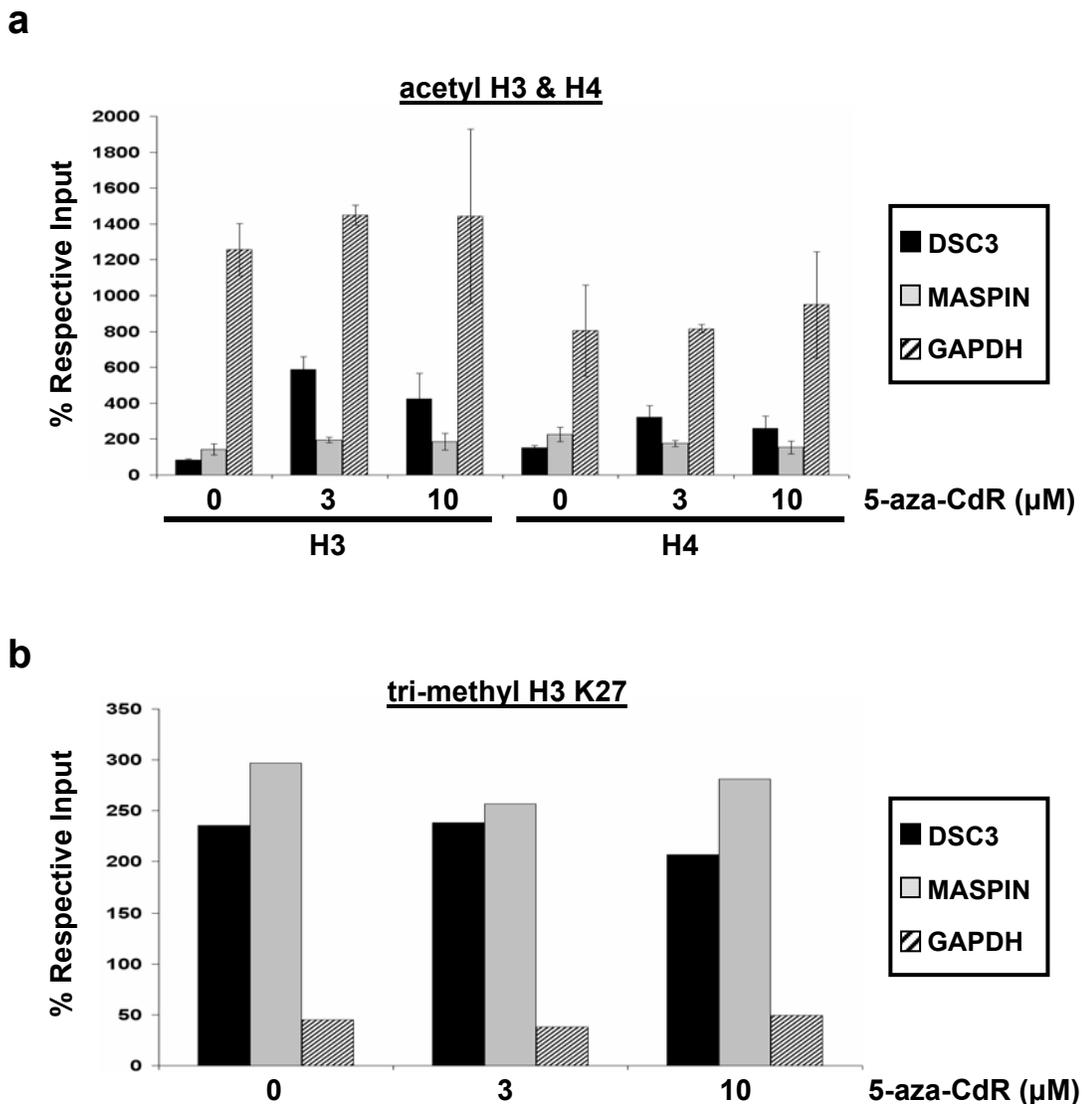


Figure 29. Chromatin immunoprecipitations were performed, using antibodies that recognize acetyl-H3, acetyl H4 and tri-methyl H3 K27. Histone modification levels are graphed as a percentage of their respective input genomic DNA samples. **(a)** Quantitation of *in vivo* H3 and H4 acetylation levels present in the DSC3, MASPIN and GAPDH promoters before and after 96 hour treatments with 3 and 10 μM 5-aza-CdR in MDA-MB-231 breast tumor cells. Average H3 and H4 acetylation levels from three independent experiments are shown along with their standard error bars (\pm s.e.m.). **(b)** Quantitation of *in vivo* H3 K27 tri-methylation levels present in the DSC3, MASPIN and GAPDH promoters before and after 96 hour treatments with 3 and 10 μM 5-aza-CdR in MDA-MB-231 breast tumor cells.

of normal epithelial tissues, but are not expressed in tissues of mesenchymal origin (245, 246). To determine whether inactivation of the DSC3 and MASPIN genes in human epithelial breast cancer strains and normal fibroblastic cells is associated with elevations in repressive H3 K9 methylation, we compared the basal levels of H3 K9 di-methylation in the promoters of these genes in normal and tumor cell lines with known expression status. To this end, we analyzed HMECs, which are DSC3- and MASPIN-positive, along with human foreskin fibroblasts (HFFs) and MDA-MB-231 and UACC 1179 breast tumor cells, which are all DSC3- and MASPIN-negative. Chromatin immunoprecipitations were performed using an antibody against di-methyl H3 K9, followed by real-time PCR amplification with gene-specific primers to quantitate the degree of H3 K9 methylation in the DSC3, MASPIN, and GAPDH promoters (**Fig. 30a**). In HMECs, where DSC3 and MASPIN are highly expressed, H3 K9 di-methylation of their promoter regions was minimal, with both having very low levels of detectable di-methyl H3 K9 (~9% of their respective input amounts). Conversely, in HFFs, the promoters of the DSC3 and MASPIN genes had 7- to 10-fold higher H3 K9 di-methylation levels (66 and 91% of their input DNAs, respectively), consistent with their transcriptionally silent state in these cells. In comparison, in the DSC3- and MASPIN-negative breast cancer cell lines MDA-MB-231 and UACC 1179, H3 K9 di-methylation was significantly elevated in the same regions of both genes, resembling the levels seen in HFFs. As a control, H3 K9 methylation analysis of the GAPDH promoter was also conducted, since this house-keeping gene is ubiquitously expressed in all of the above cell lines and the CpG sites in its promoter are completely unmethylated in all cell types.

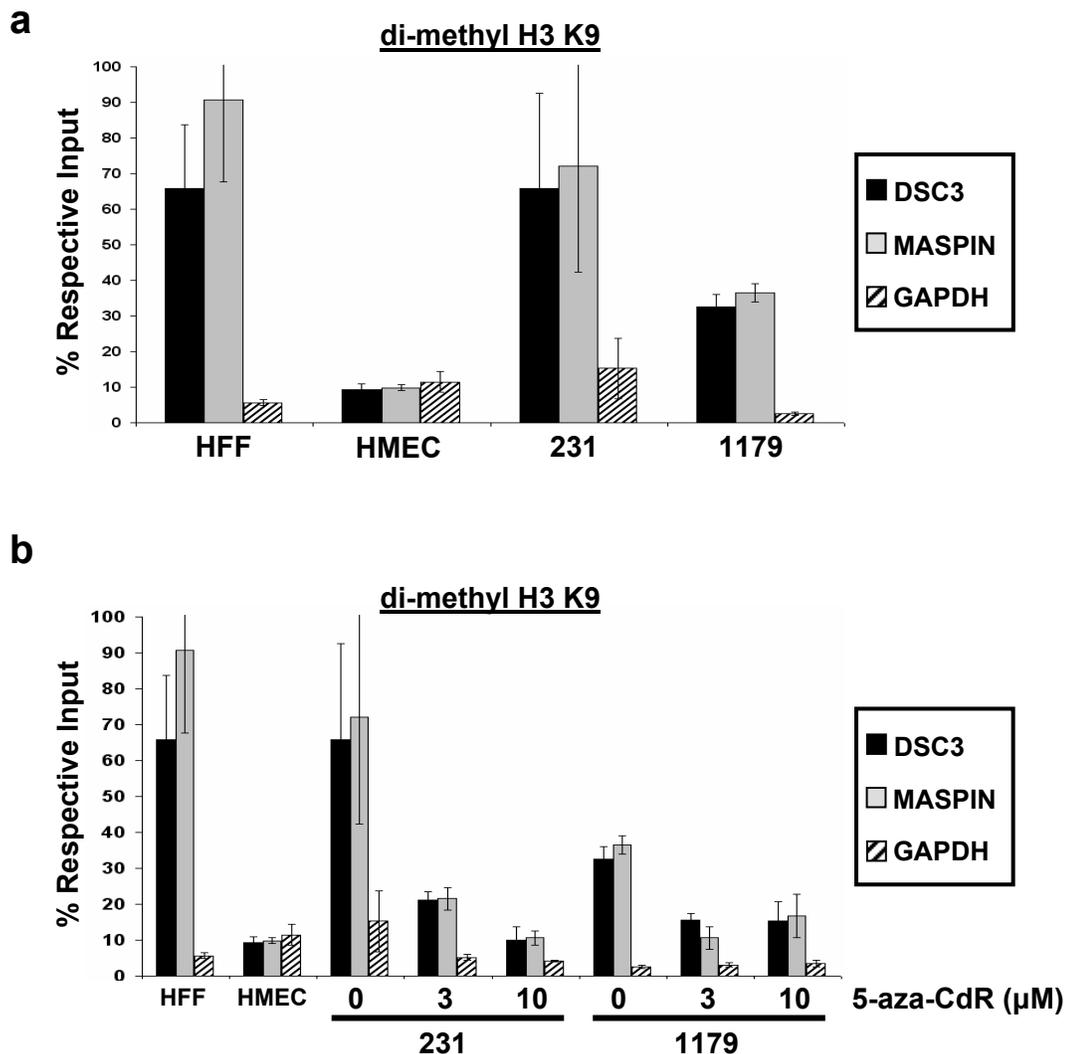


Figure 30. Three independent chromatin immunoprecipitations were performed, using an antibody that recognizes di-methyl H3 K9. H3 K9 di-methylation levels are graphed as a percentage of their respective input genomic DNA samples. Average H3 K9 di-methylation levels are shown along with their standard error bars (\pm s.e.m.). **(a)** Chromatin immunoprecipitation analysis of basal H3 K9 di-methylation levels present in the DSC3, MASPIN and GAPDH promoters of HMEC and HFF primary cultures, along with MDA-MB-231 and UACC 1179 breast tumor cells. **(b)** Quantitation of *in vivo* H3 K9 di-methylation levels present in the DSC3, MASPIN and GAPDH promoters before and after 96 hour treatments with 3 and 10 μ M 5-aza-CdR in MDA-MB-231 and UACC 1179 breast tumor cells. Values for normal DSC3- and MASPIN-positive HMECs, and normal DSC3- and MASPIN-negative HFFs are also shown for comparison.

As expected, minimal levels of this repressive histone modification were detected in the GAPDH promoter in each of our normal and tumor cell lines (from 3-15% of their respective inputs), similar to the levels seen for the DSC3 and MASPIN promoters in HMECs.

5-aza-CdR decreases DSC3 and MASPIN promoter H3 K9 di-methylation

Since the levels of H3 K9 di-methylation in the DSC3 and MASPIN CpG island promoters were found to be elevated in our breast tumor cell lines, in comparison to normal HMECs, untreated tumor cells and those treated with 3 and 10 μ M 5-aza-CdR were analyzed by di-methyl H3 K9 chromatin immunoprecipitation, as above (**Fig. 30b**). The doses of 5-aza-CdR used in these analyses were chosen because they resulted in the greatest levels of DSC3 and MASPIN transcriptional reactivation (**Fig. 21**). In MDA-MB-231 cells, H3 K9 di-methylation levels in the DSC3 promoter decreased significantly in a dose-dependent manner, from 66% of input DNA, to 21 and 10% of input after 3 and 10 μ M 5-aza-CdR treatments, respectively. In the same cell line, we also report similar reductions in H3 K9 di-methylation levels in the MASPIN promoter, from 72% of input before treatment to 22% with 3 μ M and 11% with 10 μ M 5-aza-CdR addition. These experiments were extended to UACC 1179 cells and similar results were seen for both genes, where 2- to 3-fold decreases in H3 K9 di-methylation occurred after 5-aza-CdR treatment. Meanwhile, the ubiquitously expressed GAPDH gene had consistently low levels of this repressive histone methylation mark, with no 5-aza-CdR effect seen in either tumor cell line. In conclusion, while DNA methylation was not consistently

reduced in the silenced DSC3 and GAPDH promoters in our breast tumor cell lines following 5-aza-CdR treatment, histone H3 K9 di-methylation was significantly diminished in all cases and closely linked with gene reactivation. These results suggest an additional mechanism by which 5-aza-CdR acts to reverse the epigenetic inactivation of DSC3 and MASPIN in breast tumor cells, via its ability to reduce repressive histone H3 K9 di-methylation in a manner that does not necessarily require concurrent DNA demethylation.

5-aza-CdR induces global H3 K9 demethylation of tumor cells

As a follow-up to initial studies analyzing the effects of H3 K9 di-methylation at select loci, more recent investigations using global array strategies have found H3 K9 methylation to be highly correlated with heterochromatin formation in a number of gene regulatory regions in both normal and tumor cells, leading to transcriptional silencing (156, 165). These reports illustrate the prevalence of this repressive histone modification as a means of epigenetic gene inactivation and suggest that many repressed genes may be amenable to 5-aza-CdR-mediated H3 K9 demethylation, similar to the results reported here for the DSC3 and MASPIN genes in our breast tumor cell lines. To investigate the extent of 5-aza-CdR-mediated H3 K9 demethylation in the genome as a whole, western blots were performed using protein isolated from untreated MDA-MB-231 and UACC 1179 cells, in addition to those treated with 3 and 10 μ M doses of 5-aza-CdR. Immunoblots probed with di-methyl H3 K9 antibody show the ability of 5-aza-CdR to promote significant reductions in global H3 K9 di-methylation in MDA-MB-231 cells,

with the levels of this histone modification decreasing by 56% following a dose of 3 μM and by 70% following a dose of 10 μM , compared to untreated cells (**Fig. 31**). Similar decreases in genome-wide H3 K9 di-methylation were also witnessed in UACC 1179 cells after treatment with the same doses of 5-aza-CdR (**Fig. 31**). The data from these westerns reveal the ability of 5-aza-CdR to initiate H3 K9 demethylation throughout the genome, not simply at select loci, implying that 5-aza-CdR may have wide-ranging effects on global expression and chromatin architecture through depletion of this repressive histone modification.

Effects of 5-aza-CdR on the Histone Methyltransferase G9A

5-aza-CdR induces global decreases in G9A protein levels

Next, we sought to determine whether 5-aza-CdR treatment had any effect on global levels of G9A, the histone methyltransferase primarily responsible for H3 K9 di-methylation of gene promoter regions. To this end, whole-cell protein lysates were probed for G9A protein levels via western blot, before and after treatment with increasing doses of 5-aza-CdR. The results of this investigation revealed that 5-aza-CdR treatment of MDA-MB-231 and UACC 1179 cells led to dose-dependent reductions in whole-cell G9A protein levels, with the highest doses (3 and 10 μM) resulting in 70-76% decreases overall, in both cell lines (**Fig. 32a**). Over the same dosing range, we did not see reduced levels of G9A transcript levels in either cell line following quantitative RT-PCR analysis of G9A expression (**Fig. 32b**), implying that 5-aza-CdR-mediated decreases in G9A protein levels are likely initiated post-transcriptionally. While the precise mechanism of

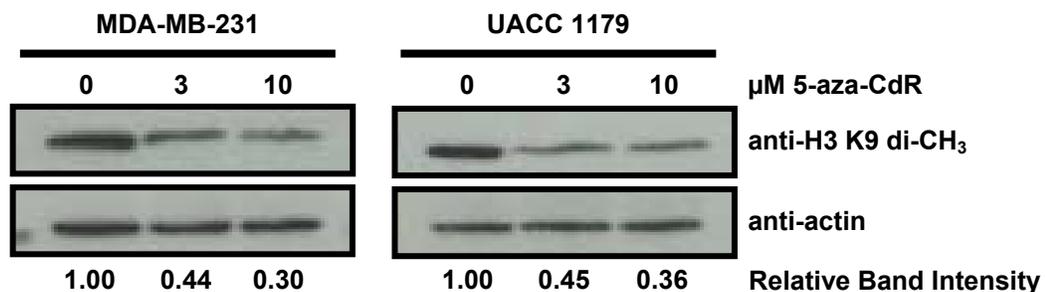


Figure 31. Representative western blots are shown detecting the global levels of H3 K9 di-methylation, before and after treatment of MDA-MB-231 and UACC 1179 cells with 3 and 10 μM 5-aza-CdR for 96 hours, using acid extracted proteins from whole-cell lysates. At least two independent experiments were performed with similar results. Relative protein expression, as determined by integrated densitometry, is shown below each graph, normalized to the actin loading control.

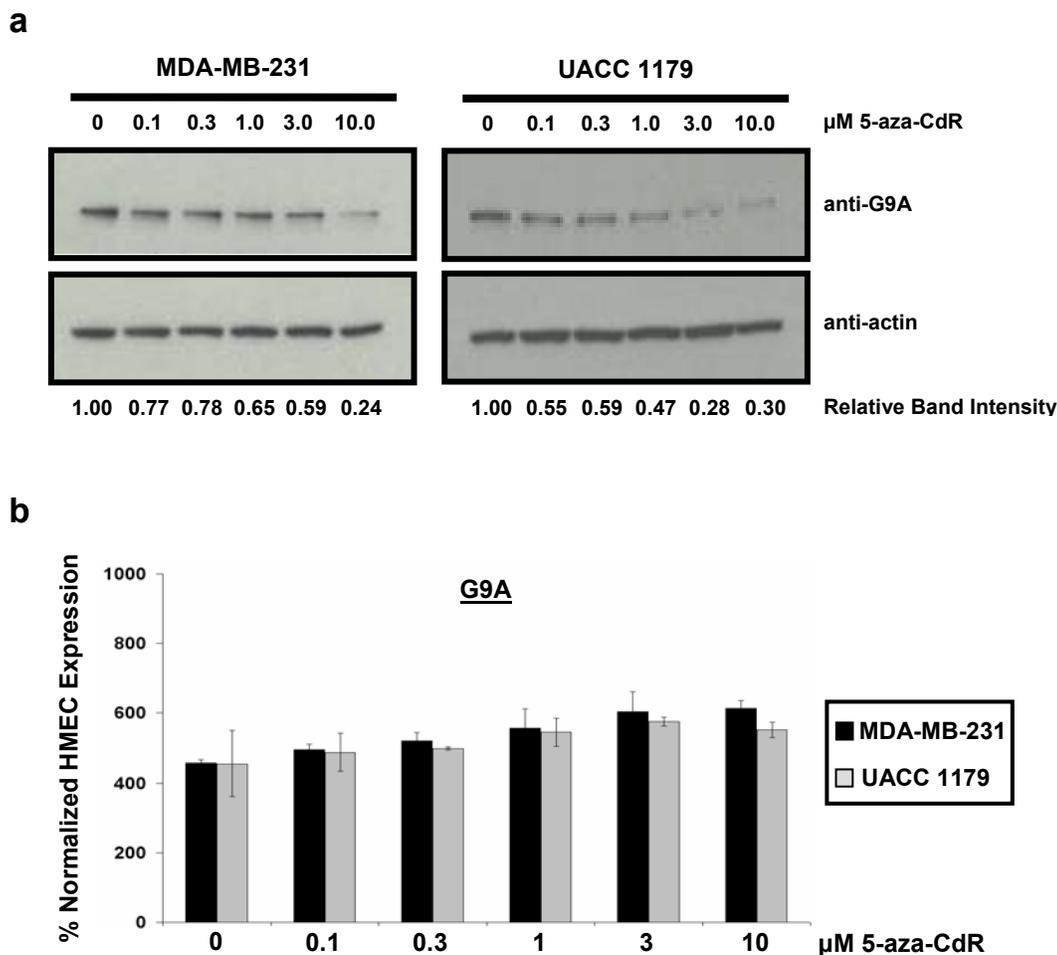


Figure 32. (a) Representative western blots are shown detecting the cellular levels of the histone methyltransferase enzyme, G9A, before and after treatment of MDA-MB-231 and UACC 1179 breast tumor cells with increasing doses of 5-aza-CdR for 96 hours. Three independent experiments were performed with similar results. Relative protein expression, as determined by integrated densitometry, is shown below each graph, normalized to the actin loading control. **(b)** Real time RT-PCR analysis to assess G9A mRNA expression following 5-aza-CdR treatment. Shown in the bar graph are G9A mRNA levels before and after 96 hour treatments with 0.1, 0.3, 1, 3 and 10 μ M 5-aza-CdR in MDA-MB-231 and UACC 1179 breast tumor cells. Experiments were repeated three times, with expression levels normalized to each sample's respective GAPDH expression and then reported as a percentage of basal HMEC GAPDH-normalized G9A expression ($\Delta\Delta$ Ct method). Error bars were calculated as standard error of the mean (\pm s.e.m.).

post-transcriptional G9A degradation following 5-aza-CdR treatment is currently unknown, these decreases in cellular G9A levels post-treatment are consistent with the ability of 5-aza-CdR to reactivate epigenetically silenced genes via demethylation of the K9 residue on histones H3 in the promoters of such genes. Of note, basal G9A transcript levels in these untreated tumor cell lines are approximately 4.5-fold higher than those seen in our reference cell line, normal HMECs (**Fig. 32b**), and may therefore account for the aberrant silencing of tumor suppressor genes in epithelial tumors of the breast via increases in promoter H3 K9 di-methylation. In fact, further analysis of G9A expression in normal breast and breast tumor patient specimens revealed that G9A levels are ~ 3-fold higher on average in breast tumors than in normal breast tissue, in a highly statistically significant manner (*p-value < 0.00001) (**Fig. 33**).

siRNA-mediated knockdown of G9A is sufficient for MASPIN reactivation

Our data here implicate both DNA hypermethylation and elevated histone H3 K9 di-methylation in the silencing of tumor suppressor genes in breast cancer, and demonstrate that 5-aza-CdR can reduce both to varying degrees in association with gene reactivation. In an effort to further examine the significance of 5-aza-CdR-mediated cytosine and histone H3 K9 demethylation in the reversal of gene silencing, a more specific approach was sought. To this end, MDA-MB-231 cells were transfected with G9A- and DNMT1-specific siRNAs, alone and in combination, to see if re-expression of the epigenetically silenced MASPIN gene could be achieved in these cells solely by reducing the levels of key enzymes involved in cytosine methylation and H3 K9 di-

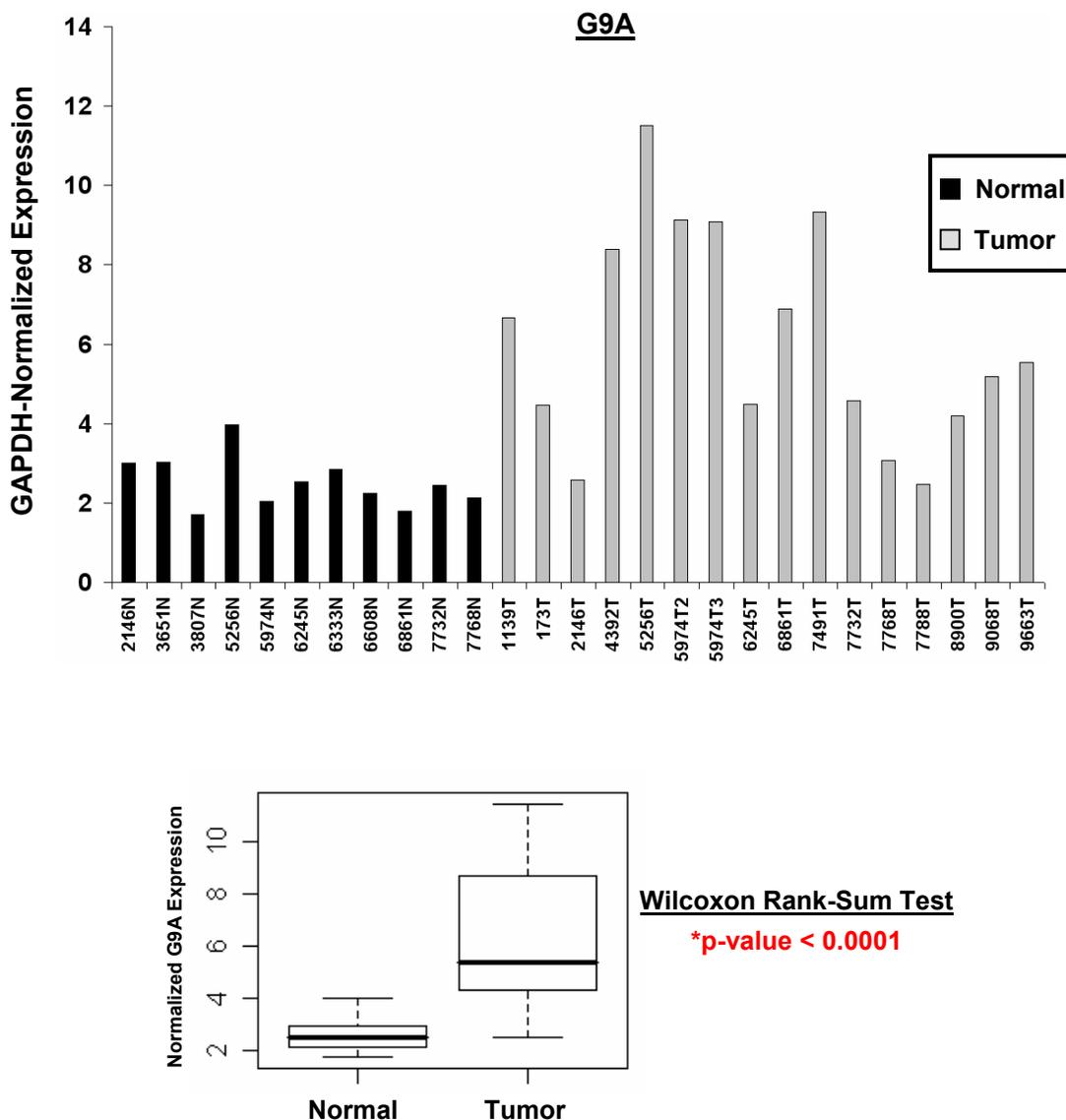


Figure 33. Real-time PCR analysis of GAPDH-normalized G9A expression (Δ Ct method) in normal breast and breast tumor patient tissue specimens. Below is a box plot graphing the same data, with the heavy dark lines representing the median value and the boxes representing the middle 50% of each data set. The entire range of each set of samples is shown by the dashed lines. A Wilcoxon Rank-Sum Test was used to determine whether G9A expression is significantly elevated in breast tumor samples with respect to its expression in normal breast tissue samples at the 95% confidence level by calculating a p-value.

methylation. UACC 1179 cells were not included in this analysis since they are not efficiently transfectable.

By treating MDA-MB-231 breast tumor cells with siRNA duplexes targeting G9A and/or DNMT1 transcripts (either alone or in combination), we were able to reduce their expression levels by ~80-90%, as detected by RT-PCR four days post-transfection (**Fig. 34a**). Following single transfections with either G9A or DNMT1 siRNA alone, RT-PCR analyses after four days revealed the presence of elevated MASPIN transcripts in MDA-MB-231 cells (**Fig. 34b**). When G9a and DNMT1 siRNAs were co-transfected, we saw an even larger increase in MASPIN expression to levels that were more than additive, indicating that both enzymes work cooperatively in the silencing of this gene and are necessary for maximal repression (**Fig. 34b**). In total, these data demonstrate that depletion of the enzymes largely responsible for cytosine methylation and/or histone H3 K9 di-methylation is sufficient for reactivation of these genes and also bolsters the idea that 5-aza-CdR works via inhibition of G9A and/or DNMT1 to induce the reactivation of epigenetically silenced tumor suppressor genes in human cancer.

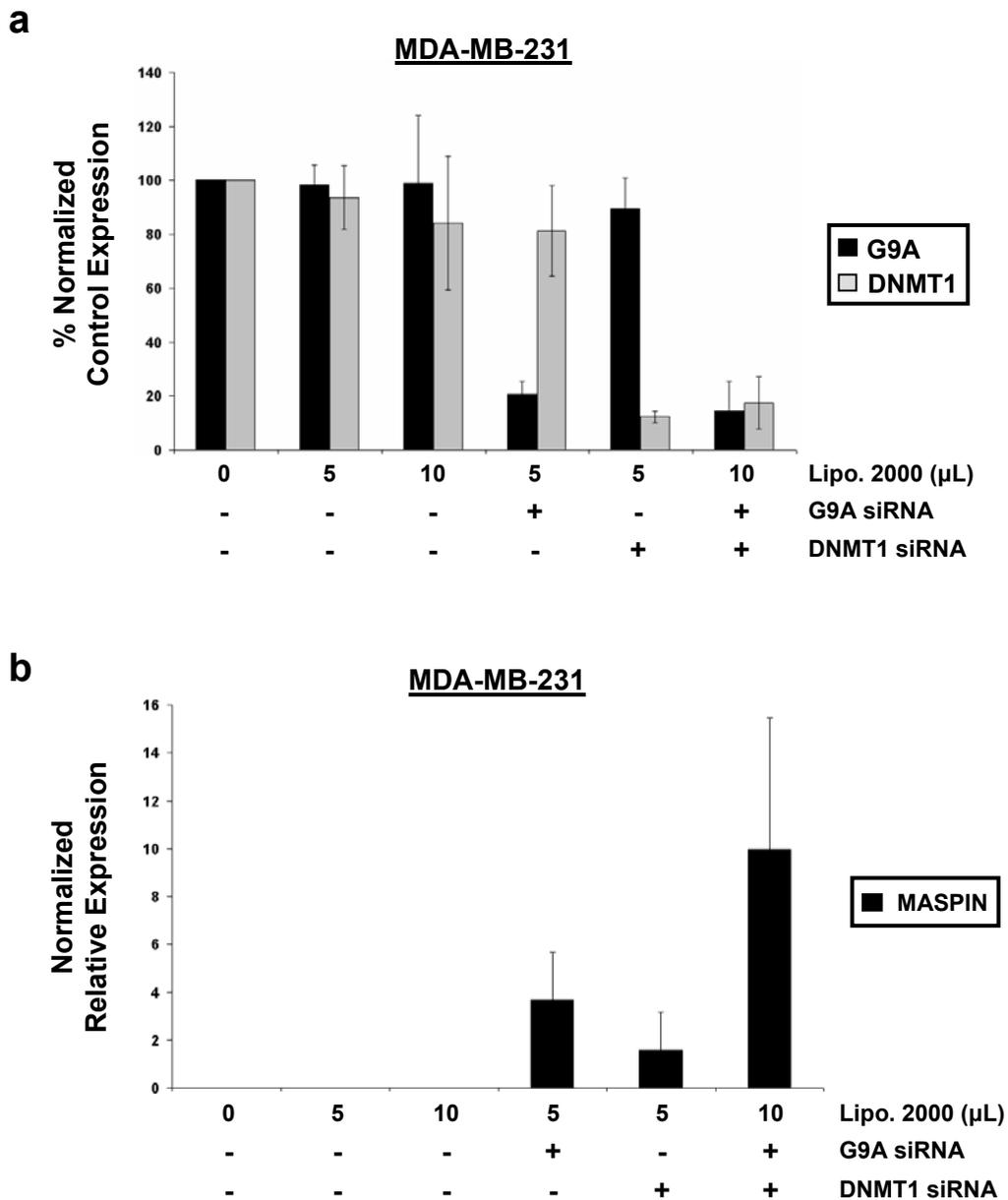


Figure 34. MDA-MB-231 cells were transfected with G9A- and/or DNMT1-specific siRNAs using the indicated amount of Lipofectamine 2000, followed by real time RT-PCR analysis four days post-transfection. These experiments were repeated three times, and error bars were calculated as standard error of the mean (\pm s.e.m.). (a) G9A and DNMT1 expression levels were normalized to each sample's respective GAPDH expression and reported as a percentage of GAPDH-normalized untransfected control G9A and DNMT1 expression ($\Delta\Delta C_t$ method). (b) Relative GAPDH-normalized MASPIN expression levels are shown (ΔC_t method).

DISCUSSION

Overview

The results presented in this dissertation demonstrate the importance of both genetic and epigenetic dysregulation in the silencing of critical tumor suppressor genes during human breast tumorigenesis and the ability of pharmacologic interventions to overcome these deleterious events. Specifically, the anti-metastatic genes MASPIN and DSC3 are often down-regulated in tumors of the breast following loss-of-function mutations in the transcriptional activator p53, along with the aberrant adoption of inaccessible chromatin architectures within their 5' regulatory regions. The identification of these multiple layers of abnormal repression drove the proposal of a targeted dual therapy that combines adenovirally-mediated wt p53 re-introduction with the epigenetic modifier 5-aza-CdR. This combination therapy has been shown here to effectively reverse both facets of MASPIN and DSC3 silencing to induce their expression in cancer cells to the levels seen in normal breast epithelial cells. The precise mechanisms by which MASPIN and DSC3 are synergistically reactivated in response to this dual treatment strategy have also been characterized in the promoters of both genes. The addition of wt p53 led to increases in histone acetylation and chromatin accessibility, while 5-aza-CdR primarily reduced repressive H3 K9 di-methylation levels by mediating dose-dependent, post-transcriptional reductions in cellular G9A histone methyltransferase levels. This mechanism of 5-aza-CdR-mediated gene reactivation is novel in that it can

occur independently of DNA demethylation, which was its originally identified mode of action in the induction of epigenetically silenced genes (186, 190).

Mechanisms of wt p53-mediated Target Gene Reactivation

The purpose of my initial efforts was to identify p53-target genes that were down-regulated in breast cancer cell lines via p53 mutation. Microarray studies analyzing the effect of wt p53 re-introduction into breast cancer cells that express mutant forms of p53 revealed MASPIN and DSC3 as target genes that require wt p53 for their transcriptional activation. Although MASPIN had previously been identified as a p53-target gene in prostate cells (247), no such link had been reported with regard to DSC3. Within the context of cancer, both proteins are involved in suppression of the metastatic phenotype (240, 243, 249-251). The aberrant silencing of these genes in tumor cells, therefore, provides them with the ability to spread beyond the primary site of tumor formation to other distant sites. Once tumors become delocalized, they become inherently more difficult to monitor and treat, making the identification of anti-metastatic genes that are targeted for silencing in tumors of interest to investigators. Moreover, in addition to the established roles that p53 plays in the activation of genes involved in DNA repair, cell cycle arrest and apoptosis (23, 24), the discovery of MASPIN and DSC3 as p53-target genes in breast cancer further extends the array of tumor suppressor functions attributed to p53 to include the inhibition of cell motility and invasion. Thus, it appears that p53

mutation is not only involved in the early stages of carcinogenesis, but also helps facilitate further malignant progression and tumor dissemination.

Although the loss of wt p53 function in many breast tumors is a critical event in the down-regulation of MASPIN and DSC3 expression, it is not the only form of transcriptional repression present in these genes. Previous studies with MASPIN, and later with DSC3, found that the silencing of these genes in breast cancer cells is also tightly associated with the establishment of repressive epigenetic profiles in their promoter regions, highlighted by DNA hypermethylation and histone hypoacetylation (235, 238, 249). An important question that emerges from these studies involves the relationship between p53 and the aberrant epigenetic silencing of p53-target gene promoters. Are mutations in p53 and the aberrant epigenetic silencing of p53-target gene promoters two distinct events with independent probabilities or does mutation of p53 participate in the formation of condensed chromatin domains?

One mechanism by which p53 could facilitate aberrant epigenetic silencing is if p53 functions, in part, to protect target promoters from the inward spread of condensed chromatin through its DNA-binding activity and the actions of associated histone acetyltransferases, such as p300 and CBP (31, 32, 34). Once wt p53 DNA-binding activity is lost, the p53-target regions would become permissive to cellular machinery responsible for *de novo* cytosine methylation, which can target these regions for the recruitment of histone deacetylases and H3 K9 methyltransferases to further aid in their repression (116, 152, 154, 155). Alternatively, several p53 mutants have been shown to possess residual, albeit diminished, DNA binding activity (41, 43, 44). In the case of the

p53-target gene MAD1, binding of mutant forms of p53 results in transcriptional repression through the recruitment of co-repressor proteins such as HDAC1 to the MAD1 promoter (276). Therefore, it is possible that p53 mutation either causes a loss-of-protection through diminished DNA binding and transactivation of the MASPIN and DSC3 promoters, allowing for repressive epigenetic domains to spread inward, or that residual mutant p53 binding attracts repressive complexes that drive epigenetic silencing. Our p53 chromatin immunoprecipitation data revealed that in untreated breast tumor cells, there was residual binding to both the MASPIN and DSC3 promoters in MDA-MB-231 cells, which express a p53 point-mutant, however, in UACC 1179 cells, which express a severely truncated form of p53, there was negligible binding to either promoter. In light of the fact that both genes in both breast tumor cell lines undergo epigenetic silencing of their promoters, yet only one of these cell lines expresses a p53 mutant capable of binding its response elements in these genes, it seems unlikely that the latter hypothesis is true, that residual mutant p53 binding recruits a repression complex to the MASPIN and DSC3 promoters and targets them for secondary epigenetic inactivation. Therefore, the loss-of-protection mediated by wt p53 binding, histone acetyltransferase recruitment and gene transactivation represents a more probable scenario for MASPIN and DSC3 epigenetic silencing in breast tumors. However, while both hypotheses dictate that mutation to p53 precede and initiate epigenetic inactivation, it is also possible that these multiple mechanisms of MASPIN and DSC3 inactivation occur independently of one another. The stable knock-out of p53 in normal breast cells could further address this

possibility, to see if the loss of p53 binding promotes the formation of an epigenetically silenced state in these target promoters.

As described above, I have shown that the adenoviral re-introduction of wt p53 stimulated gene reactivation through increases in histone H3 and H4 acetylation of the MASPIN and DSC3 promoters. This mechanism of p53-mediated gene activation through increases in histone acetylation of the target promoters is in general agreement with other recent reports (29, 32, 268). Concomitant with the increase in histone acetylation was a change in chromatin architecture; the promoter became more accessible to DNA-binding proteins, as measured by MspI accessibility to its cognate site in the MASPIN promoter (248). The question still remains, however, as to how wt p53 is able to bind target gene promoters that are in an epigenetically condensed state to initiate chromatin remodeling. One theory rests in the idea that epigenetic states, while predominantly open or closed, are still under the constraints of equilibrium and subject to some degree of fluctuation. By over-expressing wt p53 in breast tumor cells, it is proposed that this transcription factor is in large enough excess so as to bind its consensus DNA binding sites whenever available, even if this window is brief. Once bound, p53 can initiate re-acetylation of target promoters leading to partial chromatin remodeling and gene reactivation. Additionally, p53 has been shown to associate with the SWI/SNF complex (277), which is an ATP-dependent complex capable of reversing repressive epigenetic domains via an active process, leading to its being dubbed the ‘can opener’ of the genome. It is therefore possible that a physical association between p53 and SWI/SNF in breast tumor cells is capable of enhancing the ability of wt p53 to bind

to target genes during brief periods of access. Co-immunoprecipitation of p53 with various SWI/SNF subunits (BRG1, BRM, hSNF2H, BAF155, BAF170, BAF250, etc. (278)) in both untreated and wt p53-treated breast tumor cells would be a logical starting point to test this theory, followed by chromatin immunoprecipitation experiments to measure the extent of binding by human SWI/SNF complex proteins at the MASPIN and DSC3 promoters in a p53-dependent context. Further studies could also investigate the ability of wt p53 to reactivate epigenetically silenced target genes in BRG1 (the essential SWI/SNF ATPase catalytic subunit) null or inactive cells, or following dominant-negative inhibition using a transfected/infected BRG1 mutant (279).

Although wt p53 was by itself, sufficient for gene reactivation, wt p53 was not sufficient to increase MASPIN and DSC3 gene expression to levels seen in normal mammary epithelial cells. Consistent with this observation are the data which show wt p53 alone cannot stimulate re-acetylation of histones or chromatin opening in breast tumor cells to the levels present in their normal cell counterparts. These results suggest that the aberrant epigenetic silencing that is present in these promoters maintains a repressive effect on gene expression. Indeed, bisulfite sequence analysis revealed that wt p53 did not induce general CpG demethylation of its target promoters. These data indicate that the repressive mechanisms associated with aberrant cytosine methylation, namely the recruitment of histone deacetylase/methyltransferase complexes and the adoption of an inaccessible chromatin state (112-114, 116, 152, 154, 155, 280, 281), can partially resist the positive regulatory effects induced by wt p53. As such, it was hypothesized that inhibition of DNA methylation coupled with the repair of genetic

defects through the adenoviral introduction of wt p53 would enhance MASPIN and DSC3 reactivation.

To test this hypothesis, we treated breast cancer cells with the DNA methyltransferase inhibitor 5-aza-CdR (177, 282) in combination with the re-introduction of wt p53. Results showed that this combination produced a synergistic increase in the expression of both MASPIN and DSC3 in breast cancer cells, to near normal levels. In summary, these results indicate that genetic and epigenetic mechanisms of gene control can cooperate in the long-term silencing of gene expression in human cancer cells. By extension, as silencing of MASPIN and DSC3 are linked to the increased metastatic behavior of tumor cells, the data presented here suggest that cancer therapies that target both genetic and epigenetic facets of gene regulation may provide a new approach to the transcriptional reprogramming of tumors.

Mechanisms of 5-aza-CdR-mediated Reactivation of Epigenetically Silenced Genes

The ability of 5-aza-CdR to reactivate epigenetically silenced genes through the inhibition of DNMTs and DNA methylation is well documented (186, 190, 193-196, 283). Thus, we sought to determine whether 5-aza-CdR induced DNA demethylation as a means of potentiating wt p53-mediated MASPIN and DSC3 re-expression, however, our results suggested that additional mechanisms of 5-aza-CdR-mediated gene reactivation also exist. Extensive sodium bisulfite sequencing of the DSC3 and MASPIN promoters in MDA-MB-231 and UACC 1179 breast tumor cells (94 clones each) was

conducted to definitively quantitate total CpG methylation levels across each promoter before and after treatment with 5-aza-CdR. Statistical comparisons of promoter DNA methylation levels revealed that only the DSC3 promoter in MDA-MB-231 cells experienced significant demethylation ($p\text{-value} \leq 0.05$) following 5-aza-CdR treatment, despite the ability of this drug to induce global DNA hypomethylation in both of our tumor cell lines. Interestingly, DSC3 in these cells also experienced the greatest level of drug-induced re-expression. Although these examples verify the importance of DNA methylation in transcriptional silencing and further support the DNA demethylating activity of 5-aza-CdR, we also saw consistent reactivation of DSC3 and MASPIN in both cell strains even in the absence of significant local DNA demethylation. In addition to our findings, earlier evidence of DNA-methylation-independent mechanisms of action for this drug come from reports of 5-aza-CdR-mediated induction of genes with unmethylated 5' CpG islands (200-202), and gene activation in an organism lacking DNA methylation by its ribose analog, 5-aza-CR (203, 204). It has been postulated that the up-regulation of such genes by 5-aza-CdR may be a result of its ability to induce DNA damage, with subsequent activation of upstream transcriptional initiators, such as p53. While DSC3 and MASPIN are p53-responsive genes, this explanation is unlikely in our case, since both of our breast tumor cell lines express mutant forms of the p53 protein. Furthermore, neither gene was reactivated in MDA-MB-231 cells following treatment with the DNA damaging agent and p53 activator, doxorubicin, over a wide-range of doses. Thus, since CpG island demethylation and DNA damage do not appear to fully explain the ability of 5-aza-CdR to consistently reactivate DSC3 and MASPIN in

our tumor cells, we sought to investigate other potential targets of this drug in the reversal of epigenetic silencing at the histone level.

Histone H3 K27 methylation is an epigenetic mark that has been linked to transcriptional repression (141), while histone H3 and H4 acetylation is associated with transcriptionally active gene promoters (132-134). Although H3 K27 methylation levels were found to be elevated in the silenced MASPIN and DSC3 promoters in breast tumor cells via chromatin immunoprecipitation, 5-aza-CdR treatment did not decrease the levels of this repressive histone modification in the upstream regulatory regions of either gene. In terms of histone acetylation levels, the promoters of both DSC3 and MASPIN are hypoacetylated in MDA-MB-231 and UACC 1179 breast tumor cells, which helps facilitate their transcriptional inactivation. However, following 5-aza-CdR treatment we did not see consistently significant increases in histone acetylation in either promoter in MDA-MB-231 cells, results that are in concordance with a similar lack of re-acetylation seen by Kondo and colleagues when looking at the effects of this drug on a set of silenced tumor suppressor genes in colorectal cancer cells (284). In contrast, other groups have reported that 5-aza-CdR treatment, by itself, is capable of moderately increasing histone acetylation in the promoters of silenced genes in acute myeloid leukemia and bladder cancer (178, 200). Taken together, I conclude that the ability of 5-aza-CdR to induce histone acetylation in certain cases may be due to a secondary effect of this drug. This theory suggests that following the loss of epigenetic repression mediated by 5-aza-CdR, transcription factors such as wt p53 or AP-1/ETS, if present, can

recruit histone acetyltransferases to previously silenced target promoters to induce some degree of re-acetylation (29, 31-34, 285, 286).

Meanwhile, histone H3 K9 di-methylation is another repressive histone modification that has been reported to be elevated in the promoters of silenced genes (140, 142). Further chromatin immunoprecipitation experiments revealed elevated levels of histone H3 K9 di-methylation in the DSC3 and MASPIN promoters of both of the breast tumor cell lines analyzed in this study. Importantly, the ability of 5-aza-CdR to reactivate expression of these genes in tumor cells tracked closely with reductions of this repressive histone modification in their promoter regions, to the levels seen basally in DSC3- and MASPIN-positive normal HMECs. These results are in agreement with previous reports of decreased H3 K9 di-methylation levels in other epigenetically silenced genes following 5-aza-CdR treatment (164, 178, 179). However, our observation that local H3 K9 methylation can be decreased in the absence of significant DNA demethylation is a novel finding, and suggests that these distinct layers of epigenetic repression maintain their independence following 5-aza-CdR treatment, as H3 K9 methylation is not passively lost as a result of DNA demethylation. Additionally, despite the ability of the DNA and H3 K9 methylation machinery to recruit each other to the regulatory regions of repressed genes (116, 152, 155), the loss of H3 K9 methylation does not direct DNA demethylation. This independence may be explained by our observation that the enzymes responsible for DNA and H3 K9 di-methylation are inhibited by 5-aza-CdR in distinct ways, through irreversible binding/sequestration of DNMT(s) (186) and drug-induced decreases in G9A protein levels, respectively. These

findings are significant in that they demonstrate the ability of epigenetically silenced genes to be reactivated by 5-aza-CdR in spite of continued DNA hypermethylation, by an alternative, independent mechanism of action that mediates reductions in transcriptionally repressive histone H3 K9 di-methylation.

In addition to the ability of 5-aza-CdR to reactivate DSC3 and MASPIN expression in association with local H3 K9 demethylation, we also report significant global decreases in H3 K9 di-methylation following treatment. This finding is intriguing, and suggests that loss of H3 K9 di-methylation may be an important event in 5-aza-CdR-induced transcriptional reactivation of many epigenetically silenced loci. In fact, a number of genes have recently been identified as targets of methyl H3 K9-mediated transcriptional silencing in tumor cells (165). With this in mind, the global decreases in H3 K9 di-methylation we have witnessed following 5-aza-CdR treatment suggest that these identified genes may also be targets of reactivation by 5-aza-CdR through H3 K9 demethylation. Future microarray studies analyzing both gene expression and H3 K9 di-methylation levels in gene regulatory regions will help to define the full extent of H3 K9 demethylation induced by 5-aza-CdR in various cell types.

Although H3 K9 demethylation of silenced genes following 5-aza-CdR treatment has been previously described (164, 178, 179), an identification of the mechanism by which this phenomenon occurs has not yet been investigated. Thus, our finding that cellular G9A levels decline in a dose-dependent manner after addition of 5-aza-CdR is exciting in that it further implicates this drug as an inhibitor of H3 K9 di-methylation. One potential explanation for 5-aza-CdR-mediated reductions in G9A levels may reside

in a recent study describing the formation of a heterodimeric complex consisting of G9A and GLP, another mammalian H3 K9 methyltransferase that was found to be necessary for both the efficient deposition of H3 K9 di-methylation, as well as G9A stabilization (146). It is therefore conceivable that 5-aza-CdR could disrupt this interaction, which occurs via their SET domains, leading to enhanced degradation of G9A. If this is true, siRNA-mediated knock-down of GLP should result in concurrent decreases in G9A levels, and the ability of 5-aza-CdR to induce G9A degradation should be diminished in the presence of proteasome inhibitors. Mechanistically, it would be important to conduct *in vitro* co-immunoprecipitation experiments to determine whether radio-labeled 5-aza-CdR interacts with G9A and/or GLP. If 5-aza-CdR is found to associate with G9A and/or GLP, the use of various deletion constructs in the same co-immunoprecipitation experiments described above would allow for the precise mapping of the binding site(s).

Finally, our findings suggest that 5-aza-CdR acts differentially on multiple targets of epigenetic control to reactivate DSC3 and MASPIN in breast tumor cells, by initiating decreases in DNA and/or histone H3 K9 methylation. Therefore, we sought to more precisely investigate the roles of two of the main enzymes responsible for depositing these repressive epigenetic marks, DNMT1 and G9A. To this end, we utilized siRNA targeted against both DNMT1 and G9A, and found that dual knock-down of these enzymes led to greater reactivation of MASPIN than simply decreasing the levels of either methyltransferase alone. Similarly, the greatest levels of re-expression following 5-aza-CdR treatment occurred when DNA and H3 K9 methylation were concurrently decreased (DSC3 in MDA-MB-231 cells), furthering the idea that multiple layers of

epigenetic repression participate in gene silencing. However, while 5-aza-CdR reactivated DSC3 in conjunction with DNA and H3 K9 demethylation of its promoter in MDA-MB-231 cells, its expression in the same cells was not induced by targeted G9A/DNMT1 degradation. This disparity suggests that perhaps 5-aza-CdR has additional effects on other epigenetic modifications crucial to DSC3 silencing or that 5-aza-CdR is capable of reactivating one or more important transcriptional initiators of DSC3 that is also epigenetically down-regulated in breast tumors. Another question that has emerged from these studies relates to the fact that H3 K9 di-methylation was consistently reduced in the DSC3 and MASPIN promoters following 5-aza-CdR treatment, while DNA methylation was not. Our results suggest that the ability of 5-aza-CdR to induce CpG demethylation in these genes is context-specific, and likely due to differences in the components (e.g., DNMTs) of the repression complexes present at each gene regulatory region in a given cell type, as well as the specificity of 5-aza-CdR for inhibiting each of the DNMT isoforms. Ultimately, greater clarification of the cell type- and loci-specific effects of 5-aza-CdR will require the complimentary use of more targeted genetic approaches, such as siRNA or stable knock-out of different combinations of epigenetic modifying enzymes to elucidate/mirror its precise functions in the reactivation of particular genes in different cell types.

In summary, the work presented here demonstrates that the anti-metastatic, tumor suppressor genes MASPIN and DSC3 require wt p53 and accessible promoter chromatin domains for normal expression levels in breast epithelial cells. The mutation of p53 along with the aberrant adoption of repressive chromatin architectures, highlighted by the

hypoacetylation of histones and the hypermethylation of DNA and histone H3 K9 residues, serves to effectively silence these genes in many breast tumors via multiple layers of repression (**Fig. 35**). The addition of wt p53 to mutant p53-expressing breast tumor cells can partially reverse these repressive mechanisms by inducing some degree of histone re-acetylation, however, DNA and H3 K9 methylation remain to keep these genes moderately repressed. 5-aza-CdR pre-treatment further relaxes the chromatin via DNA and/or H3 K9 demethylation to potentiate the transactivation capabilities of wt p53 by allowing increased access to wt p53 and its associated co-activator proteins at the MASPIN and DSC3 promoters. The ability of 5-aza-CdR to inhibit and sequester DNMTs has already been well documented, yet it is now evident by the research presented here that this drug can also mediate reductions in cellular G9A levels leading to local and global decreases in repressive H3 K9 methylation. In fact, it appears from these studies that a significant mode of 5-aza-CdR-induced MASPIN and DSC3 reactivation in breast cancer cells is via reductions in G9A and H3 K9 di-methylation levels, and that concurrent DNA demethylation is not mandatory in all cases. However, we also demonstrate that concomitant DNA and H3 K9 demethylation results in greater gene reactivation than H3 K9 demethylation alone. This observation indicates that DNA and H3 K9 methylation represent multiple layers of repression and that pharmacologic strategies targeting an assortment of modifications involved in epigenetic silencing may prove more efficacious toward a complete reversal of repressive chromatin architectures. Thus, the development of novel inhibitors of G9A and/or H3 K9 methylation, along with

Normal Breast

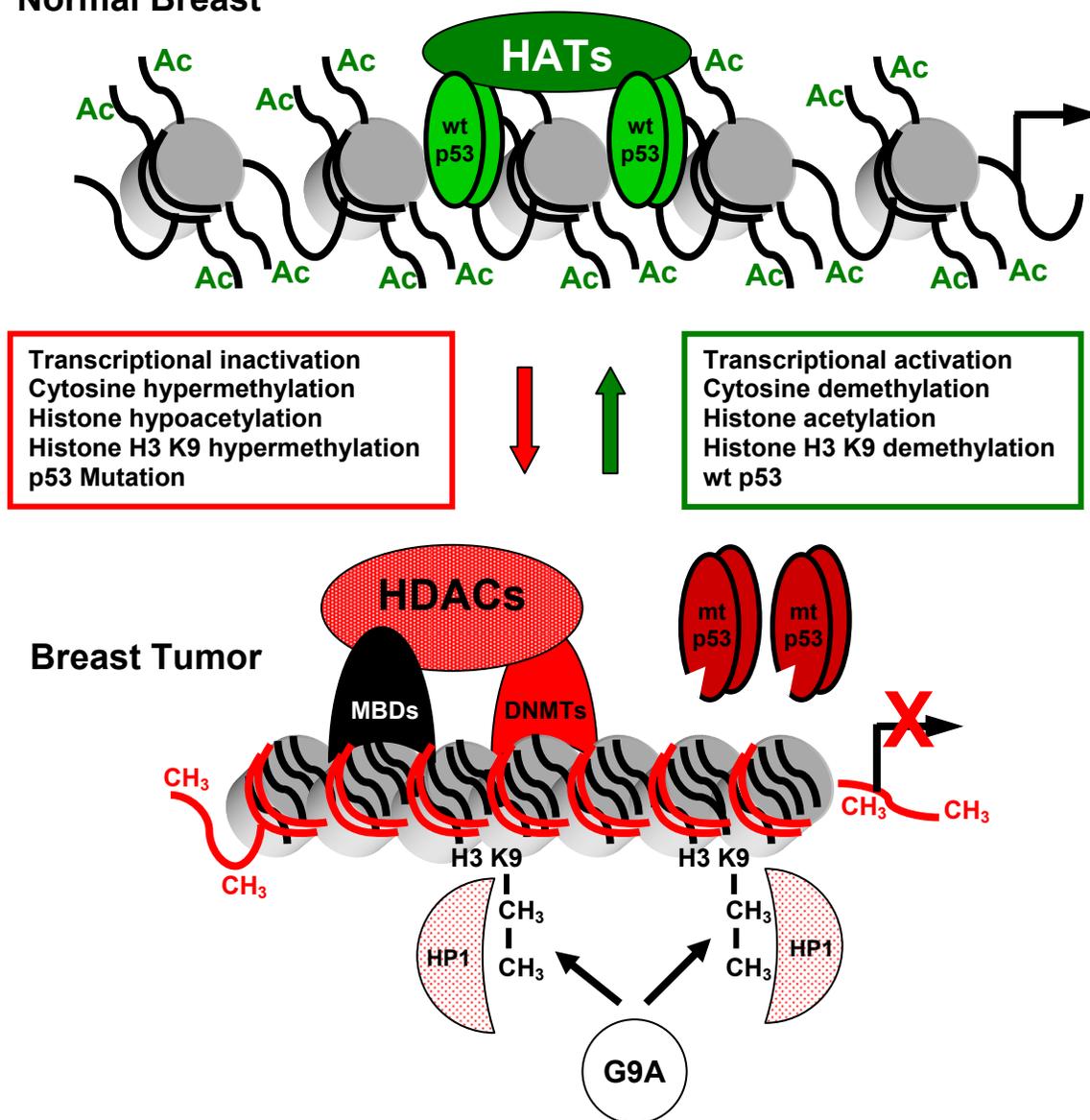


Figure 35. Model of known genetic and epigenetic components of MASPIN and DSC3 gene activation and repression in normal and tumor cells of the breast, respectively. In normal breast cells, both promoters are associated with highly acetylated histones and an open chromatin structure that is accessible to critical transcription factors such as p53, which can recruit HATs to help maintain acetylation in these regions. In breast tumor cells, the p53 gene is often mutated and the MASPIN and DSC3 promoters are in a condensed chromatin state due, in part, to the aberrant methylation of DNA and histone H3 K9 residues. The inhibition of DNMTs and the reduction of G9A levels mediated by 5-aza-CdR treatment relaxes the chromatin in these promoters by demethylating DNA and/or histone H3 K9 residues, allowing re-introduced wt p53 to access its response elements in the MASPIN and DSC3 promoters to induce their re-activation to near normal levels.

rational approaches toward their use in combination therapies with other epigenetic modifying drugs and gene therapy approaches, represent intriguing possibilities for the effective transcriptional reprogramming of tumor cells in a more targeted manner.

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