Epigenetic Silencing of the \( p16^{\text{INK4A}} \) Tumor Suppressor Gene in Human Cancer Cells:

Modulation by DNA Demethylating Agents and Poly(ADP ribosylation)

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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2013
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2013
DEDICATION

I dedicate this thesis to my mom and my sister for their love and support.
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ACKNOWLEDGEMENTS

First and foremost I would like to express my deepest appreciation to my thesis advisor, Dr. Beverly Emerson for allowing me to join her lab and to work on this exciting project.

I would also like to thank my thesis committee members, Drs. Tracy Johnson and Amy Paquinelli. It was an honor and a pleasure to interact with Dr. Johnson and Dr. Pasquinelli regarding my project and I thank them for their invaluable feedback.

Lastly, I would like to thank my mom and my friends for their constant support. They always encouraged me and their faith in me was fundamental. They supported me in challenging times and I could not have succeeded without their love.
ABSTRACT OF THE THESIS

Epigenetic Silencing of the \( p16 \) Tumor Suppressor Gene in Human Cancer Cells: Modulation by DNA Demethylating Agents and Poly(ADP ribosyl)ation

by

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Master of Science in Biology

University of California, San Diego, 2013

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We previously described the existence of a chromatin boundary upstream of the \( p16 \) locus that requires poly-ADP-ribosyl (PARylation)-dependent binding of the zinc finger protein CTCF for proper function. Post-translational modification of CTCF by PARylation is important in mediating CTCF regulation of boundaries and insulators. When \( p16 \) is
epigenetically silenced during early stages of tumorigenesis, CTCF becomes dePARylated and dissociates from the boundary resulting in encroachment of hyper DNA methylation and repressive histone marks from an upstream heterochromatic domain. Using chemical inhibitors of protein PARylation, this study analyzes whether a reduction in new synthesis of PAR residues impacted epigenetic programming and transcription of the active $p16$ locus in human breast cancer cells. We show that upon inhibition of new PAR synthesis, $p16$ expression remains stable and H2A.Z incorporation is increased at the active $p16$ promoter.

The ability of DNA demethylating agents like the FDA-approved drug 5-Aza-2′-deoxycytidine (5-Aza-dc) to reverse gene silencing is well established. However, only low level and transient gene re-expression is achieved with transcriptional silencing inevitably recurring. This study examined if 5-Aza-dc on reversal of $p16$ silencing could be enhanced or stabilized during combinatorial 5-Aza-dc regimes with the PKC activator PMA and the PI3K inhibitor Wortmannin. We show that activation of the PKC pathway, while stimulating PARP-1 activity and protein PARylation, neither augments nor stabilizes the kinetics of 5-Aza-dc induced $p16$ transcriptional activation in $p16$ silenced cells. However, upon inhibition of the PI3K-Akt pathway, 5-Aza-dc induced $p16$ re-expression is transiently increased.
Introduction
Molecular Aspects of the Epigenetic Landscape

During development the same genetic material in cells is altered epigenetically to produce tissue specific phenotypes. Epigenetics refers to changes in gene expression through chemical modifications of DNA and histones rather than changes in the nucleotide sequence \(^1,2\). In fact, DNA can be modified via three mechanisms: DNA methylation, covalent histone modifications, and non-covalent histone modifications \(^1\). An example of DNA methylation occurs during DNA replication where maintenance DNA methylation ensures that methylation patterns are passed on to daughter DNA molecules \(^2\). DNA methylation occurs when a cytosine is methylated in a CpG dinucleotide, where a cytosine is linked to a guanine by a phosphate. Specifically, the cytosine in a CpG dinucleotide is methylated at carbon 5 of the pyrimidine ring \(^3\). This enzymatic process is carried out by DNA methyltransferase enzymes which add a methyl group to cytosine forming 5-methylcytosine \(^1\). Unmethylated CpGs often cluster in CpG rich areas of DNA called “CpG islands” \(^1,4\). The most abundant of the three catalytic DNA maintenance methyltransferases in mammalian cells is DNA methyltransferase 1(Dnmt1) which recognizes CpG cytosines at hemimethylated sites \(^2,5\). Most CpG islands are unmethylated, as is the case near the promoters of housekeeping genes where CpG islands remain unmethylated whether the gene is active or not \(^6\). Also, genes undergoing tissue specific expression, such as human alpha-globin and collagen, contain CpG islands which remain unmethylated even when the associated gene is silenced \(^7\).

A smaller portion of CpG islands become methylated during development via de novo methylation which occurs after DNA replication \(^1,7\). This is true for parental imprinted genes and inactive X-chromosome genes, where CpG islands upstream promoters become de novo
methylated during development leading to transcriptional silencing. One way methylated CpG islands alter transcription is by recruiting methyl-CpG-binding domain proteins. In mammalian cells the presence of 5-methylcytosine in promoter regions inhibits transcription initiation by compromising the binding affinity of sequence-specific transcription factors.

Moreover, methylated CpG islands can mediate transcriptional repression by recruiting methyl-CpG-binding domain proteins which modulate chromatin structure and histone acetylation levels. One such protein is MeCP2, a sequence-independent, 5-methylcytosine dependent transcriptional repressor which recruits a transcriptional co-repressor and a histone deacetylase to silence the target gene.

The second mechanism for DNA modification is covalent histone modification to produce transcriptionally active or repressed chromatin domains. Chromatin is DNA wrapped around histone proteins to form nucleosomes. A nucleosome consists of 146 base pairs of DNA wrapped around a core histone octamer composed of an H3-H4 tetramer and two H2A-H2B dimers. Histones have a globular domain and a flexible charged N-terminus “tail” protruding from the nucleosome. These tails undergo covalent modifications such as acetylation, phosphorylation and methylation. In addition to CpG methylation, X-chromosome inactivation is also characterized by histone modifications such as hypoacetylation of H3 and H4. In fact, acetylation of a lysine on a histone tail neutralizes its positive charge weakening association with nearby nucleosomes. This results in the formation of actively transcribed chromatin (euchromatin) because transcription factors have access to their DNA sequences. On the other hand, a compact aggregation of nucleosomes, occurring for instance when chromatin is hypoacetylated, is referred to as heterochromatin and is associated with transcriptional silencing.
The third mechanism for DNA modification is noncovalent modification of chromatin. One example is the replacement of conventional histones with variant histones which carry their own modification patterns. Variant histones differ from core histones in their primary sequence. The H2A histone has a variant H2A.Z which usually flanks transcription start sites. Studies in yeast have shown that H2A.Z antagonizes heterochromatin spread whereby H2A.Z deposition occurs at several gene promoters in euchromatin. Yeast genes containing H2A.Z in their promoters are not always expressed but often cluster near heterochromatic regions suggesting a role for H2A.Z in delimiting heterochromatin from euchromatin. In fact, yeast cells lacking H2A.Z undergo a spread of silencing complexes resulting in heterochromatization. In the chicken B-globin gene H2A.Z is highly enriched in the region flanking an upstream heterochromatic region. In mammalian cells H2A.Z is depleted from heterochromatinized genes on the inactive X-chromosome.

**Epigenetic Silencing in Cancer Cells**

Clonal tumor expansion relies on the heritability of newly acquired traits such as aberrant changes in gene expression. Such modifications in gene expression in cancer often occur through epigenetic silencing at particular loci. Epigenetic silencing refers to the variety of biological mechanisms which suppress gene expression through changes in locus specific chromatin modification. Post-translation modifications of histones, such as deacetylation, and de novo methylation of CpG islands are examples of mechanisms which suppress gene activity without changing the DNA sequence. Cancer cells utilize such mechanisms to acquire growth advantages over neighboring normal cells. In order to proliferate, cancer cells acquire
aberrant DNA methylation and heterochromatic histone modifications to proliferate in a deregulated manner \(^2,^{14}\).

With the exception of imprinted genes and genes on the inactivated X chromosome, de novo methylation of 5’ CpG islands is uncommon in normal somatic tissues \(^4,^{7}\). On the contrary, one mechanism through which a cancer cell overcomes cell-cycle regulation and increases proliferation is through CpG hypermethylation and silencing of growth regulatory genes \(^2,^{14}\). For example, hypermethylation of the RB1 gene occurs in retinoblastoma, of the estrogen receptor gene in breast cancer, and of the \(p15\) gene in leukemias \(^{13}\). In fact, tumor-suppressor genes containing CpG islands in their promoters, such as \(p15\) and \(p16\), are often de novo methylated and silenced in tumorigenesis \(^5,^{15}\).

**Pharmacological Reversal of DNA Methylation by 5-Aza-2’deoxycytidine**

Abnormal hypermethylation of genes which regulate cellular proliferation is an attractive target for medical intervention. Historically, two chemotherapeutic agents have been used as inhibitors of DNA methyl transferase enzymes: 5-Azacytidine and its deoxy analog 5-Aza-2’deoxycytidine \(^3\). They are pyrimidine ring analogs of cytidine where nitrogen replaces Carbon 5 of regular cytidine.

5-Azacytidine can be incorporated into both DNA and RNA whereas 5-Aza-2’deoxycytidine is only incorporated into newly synthesized DNA \(^3\). Because 5-Azacytidine can also incorporate into RNA, it interferes with tRNA methylation and processing leading to defective tRNA functioning. Protein synthesis is also compromised with 5-Azacytidine.
incorporation into ribosomal RNA where methylation required for ribosomal RNA processing is affected.

Drawbacks of using these agents are toxicity, targeting of normal cells and transient demethylation of promoters. Dnmt-cytosine analog adducts are toxic and mutagenic if not repaired. Promoters which undergo demethylation after treatment with these agents eventually become de novo methylated.

Importance of CTCF in Demarcating Methylated and Repressed Chromatin Domains

In addition to using agents like 5-Aza-2’ deoxycytidine to reexpress epigenetically silenced genes, other elements contribute to appropriate gene expression such as chromatin insulators. Chromatin insulators are DNA elements that inhibit certain interactions with neighboring regions in the genome. Insulators have two functional properties: enhancer blocking and chromatin domain barrier between repressive and active chromatin domains. Enhancer blocking insulators prevent improper interaction of regulatory elements, such as enhancers, with unrelated genes. Chromatin domain insulators buffer genes and their regulatory regions from inappropriate repression by nearby repressive chromatin domains. In vertebrates the major insulator binding protein is CTCF (CCCTC-binding factor).

CTCF is a functionally versatile protein which regulates processes such as transcriptional activation/repression, genomic imprinting, and chromatin insulation. It has an eleven zinc finger central DNA binding domain which is homologous in mouse, chicken and humans. Through diverse combinatorial use of its zinc fingers, CTCF binds to a wide range of sequences. Enhancer-blocking assays identified enhancer blocking insulators such as the
Imprinted control Region (ICR) of the *H19*/*lgf2* genes in mammals. The ICR is situated between *lgf2* and enhancers located 3’ to the *H19* gene. These enhancers are shared between *H19* and *lgf2*. The ICR contains CTCF binding sequences and in the paternal chromosome, these ICR sequences are methylated which inhibits CTCF binding. This is because CTCF’s binding is methylation sensitive. Then, the paternal *lgf2* gene is activated by the enhancers downstream of the *H19* gene. On the same paternal chromosome, the *H19* gene is methylated and silenced. On the contrary, in the maternal chromosome the ICR is not methylated and CTCF binding to the ICR impedes the enhancers from activating *lgf2* leading to *H19* activation. This is an example of CTCF mediated enhancer blocking insulation (Figure 1).

**Figure 1:** Schematic Diagram of CTCF Mediated Enhancer Blocking Insulation. In the maternal ICR (1a), CTCF blocks communication between downstream enhancers and the *lgf2* promoter leading to repression of the maternal *lgf2*. On the paternal *lgf2* (1b), the ICR is methylated and CTCF is unable to bind leading to *lgf2* activation.
Like other proteins, CTCF undergoes post-translational modifications such as Poly-ADP-Ribosylation (PARylation)\textsuperscript{16}. This protein modification is carried out by Poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme that mediates a variety of nuclear processes such as DNA repair, transcription and chromatin structure regulation, induction of cell death in stress conditions, and Poly(ADP-ribose)lation of target proteins\textsuperscript{22}. PARP-1 belongs to a family of PARP genes, but in mammalian cells a significant portion of PARylation is catalyzed by PARP-1\textsuperscript{22}. PARP-1 polymerizes linear and branched chains of ADP-ribose units from donor nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) onto target proteins\textsuperscript{23}. Poly-ADP-Ribosylation is important for cellular signaling and survival as well as chromatin remodeling for transcription\textsuperscript{23}. The negative charge of these PAR chains affects target protein structure and function leading to downstream regulation of processes mediated by substrate proteins which are PARylated\textsuperscript{24}.

**The Tumor Suppressor Gene \textit{p16\textsuperscript{INK4a}} in Human Cancers**

\textit{p16\textsuperscript{INK4a}} is an example of a tumor suppressor gene often altered as an early destabilizing event in tumorigenesis\textsuperscript{15}. In particular, \textit{p16} genetic deletion or epigenetic alteration by hypermethylation is a frequent event in human cancers such as melanoma, pancreatic adenocarcinoma and bladder carcinoma\textsuperscript{15}. It is part of the \textit{INK4A/ARF/INK4B} locus which encodes the three homologous tumor suppressor proteins \textit{p14\textsuperscript{ARF}}, \textit{p15\textsuperscript{INK4b}}, and \textit{p16\textsuperscript{INK4a}}\textsuperscript{15}. Cell cycle progression is promoted by the action of cyclin dependent kinases and \textit{p16} allosteric inhibition of cyclin dependent kinases CDK 4/6 leads to hypophosphorylation of retinoblastoma (Rb) thereby leading to G1 cell cycle arrest\textsuperscript{15}. Furthermore, expression of \textit{p14\textsuperscript{ARF}} inactivates MDM2 which is a negative regulator of tumor suppressor \textit{p53} (Figure 2).
The promoter and first two exons of \textit{p16} contain CpG islands and in various types of cancers it is among the first targets of epigenetic silencing through CpG hypermethylation\textsuperscript{25}. Interestingly, \textit{p16} promoter methylation has been identified in mammary tissues of normal cancer free women which may predispose them to cancer\textsuperscript{26}. Aberrant transcriptional silencing through hypermethylation is also coupled with alterations in the histone code of \textit{p16}. Histone code refers to the collection of histone tail modifications at a particular genomic region which dictate dynamic transitions between heterochromatin and euchromatin\textsuperscript{9}. For example, mono-, di-, and trimethylated histone H3 lysine 9 (H3K9) and lysine 27 (H3K27) characterize stably heterochromatinized regions of the mammalian genome\textsuperscript{27}. Loss of activating mark dimethylated histone H3 lysine 4 (H3K4me2) is another characteristic of heterochromatin\textsuperscript{27}. The histone code of a transcriptionally active \textit{p16} promoter comprises activating histone modifications such as H2A.Z and H3K4me3\textsuperscript{28}. A hypermethylated domain

![Figure 2: The INK4a/ARF/INK4b Locus. This locus encodes p15\textsubscript{INK4b}, p14\textsubscript{ARF}, and p16\textsubscript{INK4a}. Proteins p15\textsubscript{INK4b} and p16\textsubscript{INK4a} bind to and inactivate CDK4/6. Protein p14\textsubscript{ARF} inactivates MDM2.](image)
stops near -1kb of the expressed p16 gene and this domain features repressive histone marks such as H4K20me1, H3K79me1, H3K27me2 and H3K9me3\(^{28}\). On the contrary, a transcriptionally silenced p16 loses activating marks H2A.Z and H3K4me3 near the promoter. At the same time, the hypermethylated domain extends beyond the -1kb region and repressive marks H4K20me1, H3K79me1 and H3K9me3 localize at the promoter of p16\(^{28}\).

![Diagram](image)

Figure 3: Model of a transcriptionally active and silenced p16 locus and the -1kb demarcation between heterochromatin and euchromatin.

Curiously, H2A.Z localizes at the active p16 promoter and is lost when the p16 gene is silenced. Furthermore, in T47D breast cancer cells where p16 is aberrantly silenced, demethylation by 5-Aza-2’deoxycytidine leads to a partial restoration of an active histone code. After demethylation, repressive histone mark H4K20me1 is lost while activating mark H3K4me3 is incorporated again at the promoter. However, H2A.Z is not re-incorporated at the
promoter region, showing that only a partial reversal of the histone code occurs after demethylation of a silenced p16 gene. A similar event occurs with tumor suppressor gene hMLH1 in colorectal cancer cells where this gene is also aberrantly hypermethylated and silenced. The normally expressed hMLH1 features activating marks H3K4me2 and acetylated H3K9. When aberrantly silenced, repressive histone marks di- and trimethylated H3K9 and H3K27 surround the promoter. After 5-Aza-2’deoxycytidine treatment activating marks H3K4me2 and acetylated H3K9 are incorporated while repressive mark H3K9me2 is lost. However, trimethylation of H3K9 and H3K27 is not reversed by 5-Aza-2’deoxycytidine induced demethylation and hMLH1 eventually re-silences. As is the case with p16, demethylation by 5-Aza-2’deoxycytidine leads to a partial restoration of an active histone code and this may be a reason why p16 becomes hypermethylated and silenced again after 5-Aza-2’deoxycytidine treatment. Furthermore, 5-Aza-dc mediated activation of p16 does not reach p16 mRNA induction levels seen in normal human fibroblasts. This suggests that elements other than demethylation are required to maintain a transcriptionally active p16. One such element could be a CTCF-dependent chromatin barrier.

We have previously shown that upstream of the transcriptionally active p16 gene in human fibroblasts and p16-expressing breast cancer MDA-MB-435 cells, a CTCF mediated chromatin barrier segregates the p16 promoter from the adjacent heterochromatin domain at approximately -1 kb. When CTCF is ablated in p16 expressing cells, activating histone H2A.Z levels are reduced at the p16 promoter concomitant with an increase in repressive histone H4K20 me1. However, in p16 non-expressing T47D breast cancer cells, CTCF binding is lost from the upstream boundary and the gene becomes methylated, heterochromatinized and transcriptionally silenced. Although CTCF binds in a methylation sensitive fashion, treatment
of \textit{p16} silenced T47D cells with 5-Aza-2’-deoxycytidine does not restore CTCF binding to the boundary \cite{28}. This suggests that other properties of CTCF may be involved in imparting proper insulator function.

We have shown that not only is CTCF binding required for proper barrier insulation upstream of \textit{p16}, but the post-translational modification of CTCF by Poly-ADP-ribosylation (PARylation) is required for binding \cite{28}. In \textit{p16} expressing cells, CTCF is PARylated and maintains the active boundary, whereas in \textit{p16} silenced cells CTCF becomes dePARylated and dissociates from the boundary \cite{28}. In the latter case, CTCF is bound to PARP-1 and is unable to undergo an efficient PARylation reaction and PARP-1 remains associated with a non-PARylated CTCF. Levels of total cellular proteins which are PARylated are similar between 435 and T47D extracts except for proteins in the CTCF size range whose PARylation is defective in T47D cells \cite{28}. Therefore, the general PARylation mechanism in T47D cells is normal, except for the PARylation of specific target proteins such as CTCF. The particular defect in the CTCF PARylation pathway could be due to specific signaling defects in T47D cells which undermine PARP-1’s modification of certain target proteins. Exploring possible signaling defects affecting the PARylation of CTCF in \textit{p16} silenced T47D cells will potentially restore CTCF’s PARylation and barrier function while also restoring \textit{p16} re-expression. In this study, signaling pathways were targeted as they may not only affect PARP-1’s interaction with specific substrates, but also the expression of \textit{p16} as signaling pathways in general can affect gene expression.


**Signaling Pathway Candidates: PKC and PI3K**

Two protein kinases were selected which have been previously reported to affect \( p16 \) expression. In vitro studies have shown that PARP-1 activity is in part regulated via phosphorylation by regulatory kinases, such as PKC\(^{30}\). PKC is a family of kinases involved in proliferation, apoptosis, differentiation, and migration\(^{31}\). In vitro studies report that phosphorylation of PARP-1 by protein kinase C (PKC) inhibits its DNA binding and catalytic activity\(^{30}\). However, in mouse embryonic fibroblasts, knockout of the PKC scaffolding protein Akap-12 leads to a hyperactivated PKC and increased \( p16 \) expression\(^{32}\). This is because PKC increases MEK signaling which in turn downregulates negative Ras-MEK modulator helix-loop-helix protein Id1, increasing \( p16 \) induction\(^{32}\). This suggests a role for PKC activity in enhancing \( p16 \) expression. The specific effect of PKC activity on \( p16 \) expression in human cancer remains unclear.

The Phosphoinositide 3-Kinase (PI3K)-Akt signaling pathway may also affect \( p16 \) expression in T47D cells. The PI3K-Akt signaling pathway normally regulates cellular processes like proliferation, survival and growth\(^{33}\). This pathway is an important negative modulator of proapoptotic proteins like the FOXO proteins and Bad\(^{33}\). Furthermore, activating mutations of the p110alpha catalytic subunit of PI3K are commonly found in colorectal, breast, and brain tumors\(^{34}\). In fact, in T47D breast cancer cells the PI3K subunit p110a has an activating mutation\(^{35}\). Inhibitors of PI3 kinase such as Wortmannin compete with ATP for binding to the p110alpha catalytic domain by forming an irreversible covalent interaction\(^{34}\). This results in PI3K not being able to phosphorylate and activate its downstream target Akt.
An example of Akt activity affecting \textit{p16} expression has been reported in stromal fibroblasts. Stromal fibroblasts, present in invasive human breast carcinomas, promote tumor growth through stimulation of tumor angiogenesis. Angiogenesis is the growth of new blood vessels which contributes to tumor growth and expansion. Vascular endothelial growth factor A (VEGF-A) is a key factor in mediating this process. A study shows that \textit{p16} downregulation increases stromal fibroblast migration and VEGF-A mediated angiogenesis through activation of Akt \textsuperscript{36}. More work needs to be done to further understand the effects of PI3K-Akt activity on PARP-1 and \textit{p16} expression in human breast cancer.

In this study, I selected the PKC and PI3K-Akt signaling pathways for analysis in a combinatorial treatment with 5-Aza-dc to investigate if they can augment or prolong 5-Aza-dc induced \textit{p16} re-expression in T47D cells.

**Modulation of \textit{p16} Boundary and Expression through Demethylation and Poly-ADP-ribosylation**

This project began in the context of hypermethylated and silenced \textit{p16} in T47D human breast cancer cells where PARP-1 forms an inactive complex with an unPARylated CTCF which no longer binds to the \textit{p16} boundary resulting in \textit{p16} epigenetic silencing. In this study, other factors which may be required for stable \textit{p16} boundary and expression were investigated. To stabilize the transient 5-Aza-2’deoxycytidine induced re-expression of \textit{p16} in T47D cells, combinatorial regimes were established with the PKC activator PMA and the PI3K inhibitor Wortmannin.
PKC can be activated by Phorbol 12-myristate 13-acetate (PMA) a non-hydrolysable, non-physiological analogue of PKC activator DAG (diacylglycerol) \(^{37}\). In this study we found that PMA treatment stimulates PARP-1 autoPARylation and overall PARylation in T47D cells (see Results). Since PARP-1 PARylation of CTCF is defective in T47D cells, a co-treatment with 5-Aza-dc and PMA was conducted to assess if PMA induced stimulation of PARP-1 activity had any effect on the dynamics of \(p16\) re-expression after 5-Aza-dc treatment. Furthermore, the previously described connection between PKC activation in fibroblasts and \(p16\) induction was an additional motivation to assess the effects of PMA treatment on the dynamics of \(p16\) mRNA expression in T47D cells after treatment with 5-Aza-2’deoxycytidine.

Similarly, 5-Aza-2’deoxycytidine was combined with PI3K-Akt inhibitor Wortmannin and changes in \(p16\) induction were measured. Given the previously reported aberrant activation of PI3K in T47D cells as well as the connection between Akt activity and \(p16\) downregulation in tumor stromal fibroblasts, the PI3K-Akt pathway was inhibited in an attempt to stabilize \(p16\) mRNA expression after 5-Aza-2’deoxycytidine.

We have previously shown that expression of \(p16\) is affected by inhibition of PARP-1 activity in 435 cells where treatment with the competitive PARP-1 inhibitor 3-Aminobenzamide (ABA) results in decreased \(p16\) expression \(^{28}\). In 435 cells treatment with 3-ABA inhibits existing and new protein PARylation as stable CTCF PARylation is lost after 3-ABA treatment \(^{28}\). In this study, we inhibited active synthesis of PAR with PJ -34, another competitive inhibitor of PARP-1 catalytic activity \(^{22}\) to assess whether dynamic synthesis of PAR is required to maintain \(p16\) transcriptional activity in \(p16\) expressing cells. In order to assess if dynamic PARylation is required to maintain H2A.Z incorporation at the active \(p16\) gene, H2A.Z incorporation at the
p16 promoter was measured via Chromatin Immunoprecipitation (ChIP) after perturbation of new PAR synthesis by treatment with PJ-34.
Results

Effect of Initial Cell Density on the Efficiency of 5-Aza-dc Incorporation and p16 Re-expression

The first objective was to optimize 5-Aza-dc treatment to re-express p16 in T47D cells. To accomplish this objective, three parameters had to be defined. The first parameter was assessing how the initial cell density of cultured cells affects the efficiency of 5-aza-dc incorporation into cells and p16 re-expression. This is because 5-Aza-dc incorporates into newly synthesized DNA as cells divide. Therefore, it was important to optimize the initial number of cells plated with respect to the available room for cells to divide over time. To this end, T47D cell numbers ranging from 75,000 to 750,000 were treated with a fixed 10µM 5-Aza-dc concentration. Varying cell density does not lead to significant differences in p16 mRNA induction after 5-Aza-dc treatment (Figure 4a).

Optimization of 5-Aza-dc Concentration for p16 Induction

The second parameter was optimizing the concentration of 5-Aza-dc to induce the highest possible p16 re-expression while also taking into account that as the concentration of 5-Aza-dc increases so does cytotoxicity and growth inhibition. Thus, a fixed number of T47D cells (500,000) were treated with 1µM, 5 µM, and 10 µM 5-Aza-dc. Results show that 5 µM is as efficient as 10 µM at re-expressing p16 and that both are more effective than 1µM (Figure 4b). Therefore, concentrations of 5µM and 10µM 5-Aza-dc were selected for further analyses.
To investigate the dynamics of 5-Aza-dc induced \( p16 \) re-expression, a 6 day time-course was conducted with 150,000 cells as initial cell density treated with 5 \( \mu \)M and 10\( \mu \)M 5-Aza-dc. Expression of \( p16 \) was measured every 24 hours after initial 5-Aza-dc treatment on day 0. When \( p16 \) reached maximum induction on day 6, there was no difference in \( p16 \) re-expression between cells treated with 5 \( \mu \)M or 10 \( \mu \)M 5-Aza-dc (Figure 4c). To minimize cytotoxicity and growth inhibition, 5\( \mu \)M 5-Aza-dc concentration was selected for subsequent experiments. An initial plating cell density of 150,000 cells was selected to ensure cells would not overgrow throughout the duration of subsequent experiments.

**Kinetics of \( p16 \) Re-expression After Treatment with 5-Aza-dc**

The third and final parameter was determining when peak \( p16 \) re-expression occurred after 5-Aza-dc treatment and when it would begin to re-silence. A 9 day time-course for induction of \( p16 \) mRNA transcription was conducted in order to assess the dynamics of \( p16 \) induction after a single treatment of T47D cells with 5-Aza-dc. A 5-Aza-dc concentration of 5\( \mu \)M was selected by dose response analysis (Figure 4) and 150,000 cells were used as an initial plating density. Induction of \( p16 \) was measured every 24 hours after 5-Aza-dc treatment (day 0) and results show that maximal \( p16 \) mRNA expression occurs between days 5-6 (Figure 4d). The induction of \( p16 \) significantly decreased on day 7 shortly after peak \( p16 \) re-expression. In addition, \( p16 \) re-expression progressively declined after day 7 but was sustained at low levels to the end of the time-course.
Chapter 2: Stabilization of 5-Aza-dc Induced p16 Expression in T47D Cells with Combinatorial Targeting of Signaling Pathways

Kinetics of p16 Induction with Combinatorial 5-Aza-dc and Wortmannin Treatment

The next objective was to stabilize 5-Aza-dc induced peak p16 re-expression in T47D cells. To assess if the dynamics of peak p16 re-expression could be modulated by the PI3K signaling pathway, the latter was inhibited with Wortmannin. The time-course for this analysis was extended to 15 days in an attempt to capture complete p16 re-silencing after 5-Aza-dc treatment. As a control, 150,000 cells were treated with 5µM 5-Aza-dc (day 0) and p16 mRNA expression was measured every 24 hours after treatment for 15 days (Figure 5a). Similarly, another set of 150,000 cells was treated with 5µM 5-Aza-dc (day 0) and Wortmannin was subsequently added during peak p16 re-expression (day 6). Inhibiting the PI3K pathway with Wortmannin does not significantly change the dynamics of peak p16 mRNA induction by 5-Aza-dc. Also, after day 6 the 5-Aza-dc control and 5-Aza-dc/Wortmannin treatments display similar kinetics of p16 induction suggesting that Wortmannin does not change the dynamics of p16 induction after 5-Aza-dc induced peak re-expression.

PI3K Inhibition by Wortmannin was Effective

As an inhibitor of PI3K, Wortmannin inhibits the catalytic activity of PI3K ultimately resulting in loss of phosphorylation of Akt. To verify that PI3K was effectively inhibited by Wortmannin treatment, a Western blot was performed for phospho-Akt (p-Akt), total-Akt and actin for days 5-15 after combinatorial 5-Aza-dc and Wortmannin treatment. Total-Akt and Actin served as loading controls. Ablation of p-Akt occurred after 1 hour treatment with Wortmannin on day 6 confirming that PI3K was effectively inhibited upon Wortmannin
treatment (Figure 5b). Interestingly, the inhibiting effect of Wortmannin dissipates the next day (day 7) as shown by the induction of Akt phosphorylation on day 7. Therefore, in a similar co-treatment with 5-Aza-dc and Wortmannin the expression of \( p16 \) was measured after 1 hour treatment with Wortmannin.

**Kinetics of \( p16 \) Induction with 5-Aza-dc and Short Co-treatment with PI3K Inhibitor**

**Wortmannin**

Even though Wortmannin had no significant effect on the kinetics of \( p16 \) induction over a long time course of 5-Aza-dc treatment, it was important to assess if during active inhibition of PI3K the expression of \( p16 \) was also affected. A 1 hour treatment with Wortmannin was conducted during peak \( p16 \) re-expression (day 6) after 5-Aza-dc treatment and \( p16 \) mRNA induction was measured immediately after 1 hour of Wortmannin treatment. As a control, 5-Aza-dc induced \( p16 \) mRNA expression was also measured on day 6 without the addition of Wortmannin. Results show that Wortmannin increases \( p16 \) expression immediately after 1 hour compared to the 5-Aza-dc only control (Figure 5c). This increase is transient because it is not sustained with longer co-treatment with 5-Aza-dc and Wortmannin.

**PKC Mediated Modulation of PARP-1 Activity After 1 hour Treatment with PMA**

In T47D cells CTCF forms an inactive complex with PARP-1 wherein CTCF remains unPARylated \(^{28}\). In this complex, PARP-1 does not carry out an efficient PARylation enzymatic reaction to modify CTCF \(^{28}\). Lack of CTCF PARylation results in loss of CTCF binding to the \( p16 \) boundary contributing to \( p16 \) silencing \(^{28}\). To assess if PKC activation can modulate PARP-1 activity in T47D cells, an in vitro \( \beta \)-NAD+ response assay was conducted (Figure 5e). \( \beta \)-NAD+
is the obligate substrate for PARP-1 catalyzed Poly-ADP-ribosylation. T47D cells were treated with PMA for 1 hour in cell culture and untreated T47D cells served as the control. Subsequently, β-NAD+ was added in vitro to nuclear lysates untreated and treated with PMA. β-NAD+ stimulates synthesis of new PAR in untreated T47D cells compared to untreated T47D cells without β-NAD+. Interestingly, treatment with PMA in the presence of β-NAD+ further stimulates new PAR synthesis as well as PARP-1 autoPARylation (Figure 5d). This suggests that PMA mediated activation of PKC stimulates PARP-1 enzymatic activity as also reflected by the increase in PARP-1 auto modification by PARylation. Therefore, PKC activation positively modulates PARP-1’s activity. To assess the full extent of PKC modulation of PARP-1’s activity in T47D cells, p16 mRNA expression was measured after 5-Aza-dc co-treatment with the PKC activator PMA.

**Kinetics of p16 Induction with Combinatorial 5-Aza-dc and PMA Treatment**

To determine if the dynamics of peak p16 re-expression in T47D cells could be modulated by PKC activation, 150,000 T47D cells were treated with 5-Aza-dc (day 0) and PKC activator PMA was added on day 6. Activating PKC with PMA does not significantly change the dynamics of peak p16 mRNA induction. Similarly, after peak p16 re-expression the 5-Aza-dc control and 5-Aza-dc/PMA treatments display similar kinetics of p16 re-silencing (Figure 5e).

**Kinetics of p16 Induction with 5-Aza-dc and Short Co-treatment with PKC Activator PMA**

Treatment of T47D cells for 1 hour with PKC activator PMA increases PARP-1 activity and PAR synthesis. To assess if this PMA-induced PARP-1 activity may also have transiently affected the dynamics of 5-Aza-dc-induced p16 re-expression, p16 expression was measured
before and after 1 hour treatment with PMA. As an additional control a similar experiment was performed in the absence of 5-Aza-dc where cells were treated with PMA only on day 6.

During peak p16 re-expression, 1 hour treatment with PMA on day 6 does not increase the 5-Aza-dc induced p16 re-expression (Figure 5f). No p16 induction occurred with PMA only treatment (data not shown). Although PMA was able to elicit an increase in PARP-1 PARylation activity, this may not necessarily correct the defect in CTCF PARylation by PARP-1. Both short and long-term exposures of 5-Aza-dc treated T47D cells with PMA did not affect the dynamics of p16 mRNA induction.

Re-expression of p16 Correlates with 5-Aza-dc Incorporation into Newly Synthesized DNA

Cells were counted every day for each treatment condition in order to determine if p16 induction was due to cells which had incorporated 5-Aza-dc, re-expressed p16 and stopped dividing or due to dividing p16 re-expressing cells. Cells incorporating 5-Aza-dc would experience the total effect of this agent, namely demethylation of silenced loci as well as growth inhibition caused by the toxicity of this agent. Cells continue to grow, although modestly, throughout the time course indicating that a demethylated p16 is being passed on to newly dividing cells (Figure 5g). At the same time, although a demethylated p16 was passed on to newly dividing cells, the re-expressed loci was temporally diluted out by de novo methylated and silenced p16, as shown by the decreasing p16 induction after peak re-expression (Figure 5a).
Chapter 3: Effects of Dynamic PARylation on p16 Transcriptional Activity and Epigenetic Programming in p16 Expressing MDA-MB-435 Cells

Effects of Dynamic PARylation on p16 Transcriptional Activity

We have previously shown that expression of CTCF-regulated tumor suppressor genes p16 and RASSF1A is affected by inhibition of the PARylation pathway. Treatment of p16 expressing 435 cells with PARP-1 inhibitor 3-ABA results in loss of CTCF PARylation correlating with decreased p16 and RASSF1A expression. Similarly, shRNA-mediated knockdown of PARP-1 leads to decreased p16 expression.

Since inhibition of the PARylation enzymatic pathway decreases p16 expression, dynamic PARylation was perturbed to assess if it would also be required for maintaining p16 expression. Synthesis of new PAR was inhibited by treatment with PJ-34, a potent competitive inhibitor of PARP-1. Here we show that, unlike 3-ABA, PJ-34 preferentially inhibits new PAR synthesis as shown by the Western blot for PAR from p16 expressing 435 cells untreated and treated with PJ-34 (Figure 6a). Lanes 1 and 2 show PARylated proteins in untreated and PJ-34 treated 435 cells and the profile of cellular PARylation was similar between the two conditions (Figure 6a). This result shows that PJ-34 treatment does not affect existing protein PARylation. Upon addition of the obligate PARylation substrate β-NAD+, treatment with PJ-34 inhibits synthesis of new PAR (lane 4) while existing protein PARylation was not affected. In contrast, in the DMSO control (lane 3) synthesis of new PAR occurs as shown by the PAR smear (lane 3).

Upon inhibition of active PAR synthesis by PJ-34, p16 expression was measured (Figure 6b). Given that RASSF1A and C-MYC are CTCF targets like p16, their expression was also measured upon inhibition of new PAR synthesis by PJ-34 treatment (Figure 6b).
Inhibition of new PAR synthesis does not significantly change \( p16 \) mRNA induction. This suggests that \( p16 \) transcriptional activity remains stable under perturbations in dynamic PAR synthesis. Similarly, RASSF1A and \( C-MYC \) are unaffected by perturbations in new PAR synthesis, suggesting the stability in transcriptional activity of these CTCF-regulated genes.

**Effects of Dynamic PARylation on \( p16 \) Epigenetic Programming**

We have previously shown that in \( p16 \) expressing 435 breast cancer cells, shRNA knockdown of CTCF correlates with loss of H2A.Z incorporation at the active \( p16 \) promoter, suggesting that PARylated CTCF in \( p16 \) expressing cells modulates the deposition of this variant histone \(^{28} \). In this study, we assessed whether the PARylation pathway would have to be actively sustained to maintain H2A.Z incorporation at the active \( p16 \) gene. To this end, dynamic synthesis of PAR was inhibited with PJ-34 and H2A.Z enrichment was measured upstream of the active \( p16 \) by Chromatin Immunoprecipitation (ChIP). Interestingly, inhibition of new PAR synthesis leads to an increase in H2A.Z incorporation at the active \( p16 \) promoter (Figure 6c). The gene \( C-MYC \) was selected as a control as it should not be affected by CTCF PARylation. Enrichment of total histone H3 was measured as a control to confirm that incorporation of core histones was not affected by the treatment. Interestingly, incorporation of H2A.Z also increased at the \( C-MYC \) promoter after PJ-34 treatment (Figure 6c). Even though H2A.Z incorporation increased upon PJ-treatment, \( p16 \) mRNA expression did not significantly change compared to the DMSO control, suggesting that the epigenetic programming of the active \( p16 \) gene can sustain changes in variant histone incorporation.
Figure 4: Optimization of Initial Cell Density and 5-Aza-2’deoxycytidine Concentration for p16 Re-expression in T47D Cells

A. T47D cells were plated at numbers ranging from 150,000 to 750,000 and treated with a constant 10µM 5-aza-dc for 48 hours and total RNA was collected after 5 days and p16 mRNA levels relative to GAPDH mRNA levels were measured using RT-qPCR.

B. 500,000 T47D cells were plated and treated with 1, 5, 10 µM 5-aza-dc for 48 hours. After 5 days, p16 mRNA expression was measured relative to GAPDH mRNA levels by RT-qPCR.

C. 150,000 T47D cells were plated and treated with 5µM and 10µM 5-aza-dC on day 0 and p16 expression was measured relative to GAPDH by RT-qPCR every 24 hours for 6 days after treatment.

D. 150,000 T47D cells were treated with 5µM 5-Aza-dc (AZA) on day 0 and p16 expression was measured relative to GAPDH by RT-qPCR every 24 hours for 9 days after 5-Aza-dc treatment.
A. Re-expression of p16 with 10μM 5-Aza-dc

B. p16 Re-expression with Varying 5-Aza-dc Concentration
C. 

**Kinetics of \( p16 \) Re-expression with 5 \( \mu \)M and 10\( \mu \)M 5-Aza-dc**

![Graph showing kinetics of p16 re-expression with 5 \( \mu \)M and 10\( \mu \)M 5-Aza-dc treatment](image)

D. 

**Kinetics of \( p16 \) Re-expression After 5\( \mu \)M 5-Aza-dc Treatment**

![Bar graph showing kinetics of p16 re-expression after 5\( \mu \)M 5-Aza-dc treatment](image)

Figure 4: Continued
Figure 5: Kinetics of 5-Aza-dc Induced p16 Expression in T47D Cells does not Change with Combinatorial Targeting of PI3K and PKC Signaling Pathways

A. RT-qPCR for p16 mRNA expression relative to GAPDH mRNA expression measured every 24 hours for 15 days after treatment with 5µM 5-Aza-dc (AZA) on day 0 or a combination of 5-Aza-dc (AZA) and 1µM Wortmannin added on day 6.

B. Western blot for p-Akt, total Akt and Actin expression for 5-Aza-dc and Wortmannin co-treatment (top panel) and with 5-Aza-dc only (bottom panel). Lack of phosphorylation of Akt on day 6 after 1 hour treatment with Wortmannin confirms effectiveness of this inhibitor.

C. RT-qPCR for p16 mRNA expression relative to GAPDH mRNA expression from a similar experiment as in A. p16 mRNA levels are shown for day 6 after 5-Aza-dc treatment on day 0, with and without a 1 hour treatment with 1µM Wortmannin on day 6.

D. Western blot showing results of in vitro β-NAD+ response assay for nuclear lysates obtained from T47D cells untreated and treated with PMA (100nM) for 1 hour in cell culture. Western blot shows PARP-1, PAR, and TFIIB as a loading control.

E. RT-qPCR for p16 mRNA expression relative to GAPDH mRNA expression measured every day for 15 days after treatment with 5µM 5-Aza-dc on day 0 and co-treatment with 5-Aza-dc and 100nM PMA added on day 6.

F. Similar experiment as in E. RT-qPCR for p16 mRNA expression relative to GAPDH mRNA expression measured on day 6 after 5µM 5-Aza-dc treatment (day 0) with and without 1 hour 100nM PMA treatment on day 6.

G. Cell numbers counted for each day of the 15 days per condition: 5-Aza-dc, 5-Aza-dc plus Wortmannin, and 5-Aza-dc plus PMA.
A. \textbf{Kinetics of p16 Re-expression After 5-Aza-dc and Wortmannin Treatment}

![Graph showing the kinetics of p16 re-expression after 5-Aza-dc and Wortmannin treatment.](image)

- AZA
- AZA + WRT

B. Western blot analysis showing the expression levels of p-Akt, Total Akt, and Actin over different days of treatment.

| Day  | DMSO | DAY 5 | DAY 6 | DAY 6 1 HR | DAY 7 | DAY 8 | DAY 9 | DAY 10 | DAY 11 | DAY 12 | DAY 13 | DAY 14 | DAY 15 |
|------|------|-------|-------|------------|-------|-------|-------|--------|--------|--------|--------|--------|--------|-------|
| p-Akt | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
| Total Akt | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
| Actin | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
C. 

**p16 Re-expression After 5-Aza-dc and Wortmannin Treatment**

![Graph showing relative p16 expression/GAPDH over time](image)

- **DAY 6**
- **Number of days after 5-Aza-dc Treatment**

- **Legend**:
  - AZA
  - AZA + 1hr Wortmannin
  - DMSO

D. 

![Image of gel electrophoresis](image)

- **Untreated**
- **Untreated + PMA**
- **Untreated + β-NAD+**

**Markers**:
- **PAR**
- **PARP-1**
- **TFIIB**

75 Kd, 50 Kd

Figure 5: Continued
E. Kinetics of p16 Re-expression After 5-Aza-dc and PMA Treatment

F. p16 Re-expression After 5-Aza-dc and PMA Treatment

Figure 5: Continued
G.

**Cell Numbers for Each Treatment Set**

![Graph showing cell numbers for each treatment set](image)

Figure 5: Continued
Figure 6: Inhibition of Dynamic PARylation does not Affect p16 Transcriptional Activity and Increases H2AZ Incorporation at the Active p16 Promoter in MDA-MB-435 Cells

A. Western blot showing PARylation levels, as measured with an anti-PAR antibody, after an in vitro β-NAD+ (1mM) response assay of nuclear lysates from 435 cells treated with PJ-34 (10µM) for 24 hours in cell culture and with DMSO as a control. Lanes 3 and 4 show response to β-NAD+ where synthesis of new PAR occurs in the DMSO control (lane 3) but is inhibited by PJ-34 treatment (lane 4).

B. RT-qPCR for p16, c-MYC and RASSF1A mRNA expression relative to GAPDH mRNA expression from DMSO control and PJ-34 treated 435 cells.

C. ChIP analysis to measure enrichment of H2AZ and total Histone H3 near promoter regions of p16 and c-MYC from 435 cells treated with PJ-34 and DMSO as a control.
A.

Different conditions: DMSO, PJ-34 (24 hr), DMSO + β-NAD+, PJ-34 (24 hr) + β-NAD+

Proteins of interest: PAR, PARP-1, TFIIB

Molecular weight markers: 100Kd, 75Kd
Figure 6: Continued

**$p16$ Expression in 435 Cells**

![Bar chart showing relative $p16$ expression in 435 cells after treatment with DMSO and PJ-34 (24 hr).]

- DMSO: Relative expression/GAPDH = 2.0 ± 0.5
- PJ-34 (24 hr): Relative expression/GAPDH = 3.5 ± 0.7

**$C$-MYC and $RASSF1A$ Expression in 435 Cells**

![Bar chart showing relative expression of $C$-MYC and $RASSF1A$ in 435 cells after treatment with DMSO and PJ-34 (24 hr).]

- $C$-MYC: Relative expression/GAPDH
  - DMSO: 80 ± 10
  - PJ-34 (24 hr): 120 ± 20
- $RASSF1A$: Relative expression/GAPDH
  - DMSO: 40 ± 5
  - PJ-34 (24 hr): 80 ± 15
H2AZ Enrichment on \textit{p16} in Untreated and PJ-34 Treated 435 Cells

Total Histone H3 Enrichment on \textit{p16} in Untreated and PJ-34 Treated 435 Cells

Figure 6: Continued
H2AZ Enrichment on C-MYC in Untreated and PJ-34 Treated 435 Cells

Total Histone H3 Enrichment on C-MYC in Untreated and PJ-34 Treated 435 Cells

Figure 6: Continued
Discussion
This study focused on modulating p16 re-expression by 5-Aza-dc in T47D p16 silenced human breast cancer cells. The kinetics of p16 re-expression and re-silencing after 5-Aza-dc treatment were measured. Peak p16 re-expression occurred 5-6 days after treatment and a significant decrease in p16 induction began shortly after peak re-expression and progressively declined. We have previously shown that 5-Aza-dc induced p16 re-expression in T47D cells is not restored to normal p16 expression levels in human fibroblasts. Previous studies established combinatorial regimes with 5-Aza-dc and histone deacetylase inhibitors Trichostatin A (TSA) and 4-phenylbutyric acid (PBA). The rationale for using histone deacetylase inhibitors is that acetylation of histones contributes to a transcriptionally active euchromatin conformation. It has been shown that in the colorectal carcinoma cell line RKO, p16 is hypermethylated and silenced and that 1µM 5-Aza-dc did not lead to detectable p16 re-expression. Also, treatment with TSA alone did not re-activate p16 transcription. However, once 5-Aza-dc and TSA were combined, a synergistic and detectable re-activation of p16 occurred. In another report, p16 was re-expressed in T24 bladder carcinoma cells also using 1µM 5-Aza-dc. Re-expression of p16 was successfully induced at this concentration and combination with PBA induced higher p16 expression only during the window of maximal p16 re-expression (day 3-6). However, this study also reported that combination of 5-Aza-dc with PBA does not change the dynamics of p16 re-silencing after 5-Aza-dc treatment suggesting that inhibition of histone deacetylation is not sufficient to delay p16 re-silencing after 5-Aza-dc treatment.

In this project we show that 1µM 5-Aza-dc is effective at inducing p16 re-expression in T47D human breast cancer cell but 5µM 5-Aza-dc concentration was selected as the optimal concentration for p16 re-expression. Moreover, in T47D breast cancer cells in
particular, we have previously shown that combination of 5-Aza-dc with TSA had no synergistic effect on the re-activation of p16\textsuperscript{28}. Furthermore, 5-Aza-dc induced re-expression of p16 in T47D cells is not restored to levels comparable to normal p16 expression in human fibroblasts\textsuperscript{28}. Therefore, it was important to target signaling pathways which may affect the dynamics of p16 expression in an attempt to augment or prolong 5-Aza-dc induced p16 re-expression. Combinatorial regimes were established with 5-Aza-dc and targeting of PI3K and PKC signaling pathways. It has been previously reported that in stromal fibroblasts activation of Akt correlates with downregulation of p16\textsuperscript{36}. In this project, 5-Aza-dc induced p16 re-expression in T47D cells was increased after inhibition of PI3K which in turn inhibited activation of Akt. Co-treatment with 5-Aza-dc and the PI3-Kinase inhibitor Wortmannin increased p16 induction after 1 hour treatment with Wortmannin compared to 5-Aza-dc only treatment. However this effect was transient and did not change the dynamics of p16 re-silencing after 5-Aza-dc treatment.

It has been reported that in mouse embryonic fibroblasts, increased PKC activity correlated with higher p16 induction\textsuperscript{32}. However, in T47D cells PMA mediated activation of PKC did not significantly change 5-Aza-dc induced p16 re-expression. Nonetheless, we also report here that PMA mediated activation of PKC stimulated PARP-1 activity. This finding was the main motivation for targeting this pathway in an attempt to stabilize p16 re-expression after 5-Aza-dc treatment. In fact, in T47D cells PARylation of CTCF is specifically deregulated as PARP-1 forms an inactive complex with an unPARylated CTCF which no longer binds to the p16 boundary causing p16 silencing. To assess if stimulation of PARP-1 activity by the PKC signaling pathway may correct the defective enzymatic reaction between PARP-1 and CTCF, p16 expression was measured after co-treatment with 5-Aza-dc and PKC activator
PMA. Our results show that \( p16 \) expression does not change after combinatorial treatment with 5-Aza-dc and PMA. This suggests that PMA induced stimulation of PARP-1 PARylation is not sufficient to correct the specific defect in CTCF PARylation in T47D cells. Furthermore, activation of the PKC signaling pathway does not affect \( p16 \) gene expression after 5-Aza-dc treatment of T47D cells.

In this study we also report the effect of dynamic PARylation on the transcriptional activity of \( p16 \) in \( p16 \) expressing MDA-MB 435 breast cancer cells. We have previously shown that treatment with PARP-1 inhibitor 3-ABA inhibited existing protein PARylation as shown by the loss of CTCF PARylation in 435 cells after 3-ABA treatment \(^ {28} \). Concurrently, loss of CTCF PARylation led to a significant reduction in \( p16 \) expression suggesting that existing CTCF PARylation is required for maintaining \( p16 \) transcriptional activity \(^ {28} \). In this study, activity of \( p16 \) transcription was measured after inhibiting active synthesis of new PAR. Upon inhibition of active PAR synthesis by PJ-34, \( p16 \) expression does not change significantly, demonstrating the stability of \( p16 \) transcriptional activity under perturbations in dynamic synthesis of PAR. Synthesis of PAR occurs rapidly in response to physiological and stress-related stimuli as PARylation of a variety of target proteins affects downstream signaling pathways \(^ {22} \). In this study we show that upon inhibition of new PAR synthesis, \( p16 \) expression remains stable under environmental perturbations in signaling pathways elicited by inhibition of dynamic PAR synthesis.

We have also previously shown that shRNA mediated ablation of CTCF in \( p16 \) expressing 435 cells correlated with loss of H2A.Z incorporation at the active \( p16 \) promoter \(^ {28} \). Since CTCF is PARylated in 435 cells \(^ {28} \), in this study we investigated if active synthesis of
PAR was required for maintaining H2A.Z incorporation at p16. Here we show that perturbation in dynamic synthesis of PAR does not inhibit H2A.Z incorporation, but actually increases its incorporation at p16. However, p16 expression is not significantly affected by changes in H2A.Z incorporation suggesting that the epigenetic programming of the active p16 gene can sustain perturbations (but not loss) in variant H2A.Z incorporation.

Collectively, these results show that when active, p16 transcriptional activity and epigenetic programming can sustain perturbations in dynamic synthesis of PAR. Furthermore, the silenced p16 gene in T47D cells can be reactivated by 5-Aza-dc treatment and this re-expression can be augmented transiently by inhibition of the PI3K signaling pathway.
Materials and Methods
Cell Culture

MDA-MB-435 and T47D cell lines were grown in RPMI-1640 media supplemented with 10% FBS. T47D cells were treated with 5’-deoxyAza-2’-deoxycytidine (5-Aza-dc, Sigma) 24 hours after seeding at a final concentration of 5µM for 48 hours and total RNA was extracted from cells every 24 hours after treatment. Cell culture media was replaced with fresh media every 48 hours after treatment with 5-Aza-dc. T47D cells were also treated with 100nM PMA (Phorbol 12-myristate 13-acetate, Sigma) for 1 hour in cell culture or in combination with 5 µM 5-Aza-dc as described in the figure legends. T47D cells were also treated with 1µM Wortmannin (CALBIOCHEM) in combination with 5µM 5-Aza-dc as described in the figure legends. MDA-MB-435 cells were treated with PJ-34 (Hydrochloride hydrate, Sigma) at a final concentration of 10µM for 24 hours.

Western blots

Cells were washed with PBS and trypsinized with 0.25% Trypsin and lyzed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Deoxycholic acid, 0.1% SDS, 50mM Tris-HCl pH 8.0, 5mM EDTA), supplemented with 1x protease Inhibitor, 0.5mM PMSF, 20mM NaFl, 0.2mM NaOVa, and 5mM Sodium Butyrate for 30 minutes on ice. Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C. A protein assay was carried out using the Pierce BCA Protein Assay Kit and protein concentrations were measured by Spectrophotometry. Proteins were separated with SDS-PAGE and transferred to Hybond- C-extra membranes. Membranes were blocked with 1x TBS containing 0.005% tween and 5% non-fat milk and probed with antibodies towards phospho-Akt (Cell Signaling, Ser 473, Cat: 193H12), total Akt (Cell Signaling, Cat: 9272), Actin (Sigma), TFIIB(Santa Cruz
Biothechnologies), PARP-1 (Santa Cruz Biothechnologies) and Poly-ADP-ribose (Millipore).

The protein-bound antibodies were visualized with horseradish peroxidase labeled secondary antibody using the Super Signal system (Pierce Chemical) as substrate. The chemiluminescence was detected by autoradiography.

RT-qPCR

Total RNA was isolated using Trizol reagent (Life Technologies). Total RNA (500ng) was used for reverse transcription. SuperScript III kit (Invitrogen) and random hexamers were used for first-strand cDNA synthesis. Reactions were carried out in triplicate and the fold change in p16 expression was normalized to GAPDH housekeeping gene using the 2-ΔΔCt method. List of qPRC primers is below:

Table 1: List of qPCR Primers

<table>
<thead>
<tr>
<th>RT-qPCR Primers</th>
<th>FORWARD</th>
<th>REVERSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>CTGCCCAACGCACCGAATA</td>
<td>GCGCTGCCCATCATCATGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGTTGCCCATCAATGACCCCT</td>
<td>GACGGTGCCCATGGAATTGC</td>
</tr>
<tr>
<td>CMYC</td>
<td>CAGCTGCTTAGACGCTGGATT</td>
<td>GTAGAAATACGGCTGCACCGA</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>AATGCCAGATCAACAGCAAC</td>
<td>GCTTCTTGCTGGAGGGGCACAA</td>
</tr>
</tbody>
</table>

Chromatin Immunoprecipitation (ChIP)

435 cells, cultured in 15cm dishes, were cross-linked for 15 minutes by addition of formaldehyde (1% final concentration) in the cell media. Cross-linking was stopped upon addition of Glycine to a final concentration of 125 mM for 5 minutes. After 3 washes with 1X PBS, cells were scraped and lysed in ChIP cell lysis buffer (10mM Hepes pH 7.9, 0.5% NP-40, 1.5mM MgCl2, 10mM KCl, 0.5 mM DTT) containing protease inhibitors on ice for 10 minutes. Lysates were then re-suspended in SDS lysis buffer (50mM Tris-HCl pH8.1, 0.5% SDS, 10mM
EDTA). Lysates were subsequently sonicated and 1.5mg of lysate was pre-cleared for 1 hour using 40 µl of a 50% slurry of protein G-Sepharose beads (GE Healthcare). For immunoprecipitation, the indicated antibodies were added to pre-cleared lysates along with 40 µl of a 50% slurry of G beads and incubated at 4°C overnight. Antibodies used were: α-H2AZ (Active Motif, Cat: 39113), α-Histone H3 (abcam, Cat: ab 1791-100). Immuno-complexes were collected the next day and washed once with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris-HCl pH 8.1), once with high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 500mM NaCl, 20mM Tris-HCl pH 8.1), once with LiCl wash buffer (0.25M LiCl, 1% NP-40, 1% Sodium Deoxycholate, 1mM EDTA, 50mM Tris pH 8.1), and twice with TE. Each wash was done for 5 minutes.

Immuno-complexes were then eluted in 220 µl of buffer containing 50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% SDS after incubation at 65°C for 10 minutes. Crosslinks were reversed from immuno-complexes by addition of proteinase K at 42°C for 2 hours and then at 65°C for overnight. DNA was purified by phenol-chloroform extraction. Input samples were treated similarly and associated DNA was identified by qPCR. PCR primers are listed below:

Table 2: List of ChIP qPCR Primers

<table>
<thead>
<tr>
<th>ChIP qPCR Primers</th>
<th>FORWARD</th>
<th>REVERSE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CMYC</strong> (-8Kb)</td>
<td>CAGTAGCAGCCTTGCTTAGC</td>
<td>GGATTTCGCCTTTTCAC</td>
</tr>
<tr>
<td><strong>CMYC</strong> (-2Kb)</td>
<td>ATCCAGGCGCGATGATCTC</td>
<td>GCTGCAAAGCGCTTTCC</td>
</tr>
<tr>
<td><strong>CMYC</strong> (-400 bp)</td>
<td>CCTACCCCTCTCAACGACAGC</td>
<td>CGTCGAGGAGAGCAGAGAAT</td>
</tr>
<tr>
<td><strong>CMYC</strong> (+50 bp)</td>
<td>AGAAGGCCAGGCTTCTTC</td>
<td>CTGCTCTCGCAGATTTACTAC</td>
</tr>
<tr>
<td><strong>p16</strong> Region E (-205 bp)</td>
<td>AGTTAGGAAGGTTGTATCGCG</td>
<td>GCCTGCCAGCAAAGGCGTT</td>
</tr>
<tr>
<td><strong>p16</strong> Region D (-520 bp)</td>
<td>ACCCTTGCCCCAGACAGCCGT</td>
<td>TTCCTTTTTGAGAGTCTGGACT</td>
</tr>
<tr>
<td><strong>p16</strong> Region B (-3Kb)</td>
<td>CATGTTGGTGTGCTGACCCA</td>
<td>CATTGTGCCTATAGAAATGAA</td>
</tr>
<tr>
<td><strong>p16</strong> Region A (-5Kb)</td>
<td>GGTATCTCTCACCTCAAGCGTA</td>
<td>AGGCAATAGCTCTACACTGAG</td>
</tr>
</tbody>
</table>
Nuclear Extraction

Nuclear lysates were made from untreated MDA-MB-435 and T47D cells, T47D cells treated with 100nM PMA for 1 hour in cell culture and MDA-MB-435 cells treated with PJ-34 for 24 hours in cell culture. Cell pellets were re-suspended in hypertonic membrane lysis buffer (10mM Hepes pH 8.0, 1.5mM MgCl2, and 10mM KCl) for 15 minutes on ice. 0.3% Igepal-CA630 was then added to lyse cell membranes and lysates were centrifuged for 2 minutes at 1000xg and supernatant was removed. The nuclear pellet was re-suspended in one packed pellet volume of Nuclear Extraction Buffer (20mM Hepes pH 8.0, 1.5mM MgCl2, 300mM NaCl, 200uM EDTA and 25% glycerol) and incubated on ice for 30 minutes. Samples were then centrifuged at 4°C for 15 minutes at maximum speed and the nuclear supernatant transferred to a new tube. The nuclear extract NaCl concentration was adjusted to 150mM with Dilution Buffer 1 (1M Hepes pH 8.0, 1M MgCl2, 0.5M EDTA) added in an equal volume as the Nuclear Extraction Buffer.

β-NAD+ Response Assay

Nuclear lysates re-suspended in Nuclear Extraction Buffer were obtained from MDA-MB-435 and T47D as above. A protein assay was carried out using the Pierce BCA Protein Assay Kit and protein concentrations were measured by Spectrophotometry. Subsequently, protein concentrations were equalized among all lysates. The NaCl concentration for each lysate was adjusted from 300mM to 150mM using Dilution Buffer 1 as above. Subsequently, β-NAD+ (Sigma) was added in vitro to a final concentration of 1mM in the lysate and incubated on ice for 1 hour. Proteins of interest were analyzed by SDS PAGE.
References


