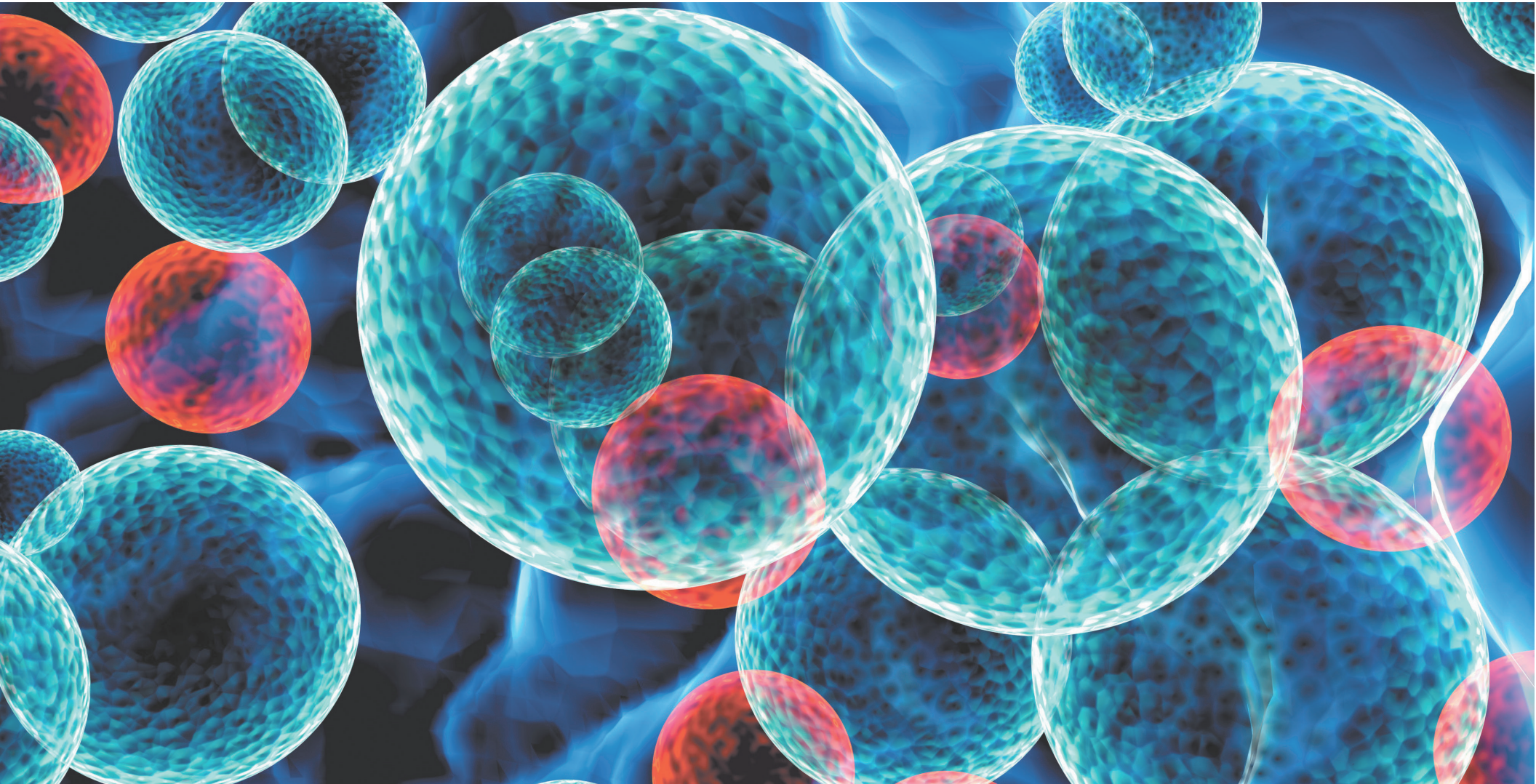


DNA Methylation and **CANCER**



Enabling Epigenetics Research



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Introduction

The emerging role of epigenetics in development and disease

Epigenetics, or the heritable changes in gene function that occur independent of DNA sequence, has claimed a prominent position in research of developmental and disease processes. Countless studies link epigenetic alterations to developmental processes, including X-chromosome inactivation,

genomic imprinting, axis patterning and differentiation, as well as to diseases such as cancer, chromosomal instabilities and mental retardation. Because genetics alone does not provide an adequate explanation for the complexity of these processes, a deeper understanding of the role of epigenetics is critical to unraveling the underlying mechanisms responsible for regulating development and disease.

Epigenetics in Cancer

The epigenetic state of the cell is controlled by the activity of proteins that add and remove small chemical modifications to histones and directly to DNA. Aberrant epigenetic regulation can lead to changes in gene expression and the development of cancer. The link between epigenetics and cancer has been substantiated through the identification of mutations in, or altered expression of, epigenetic regulator proteins in many different types of

cancer. These mutations have been identified in all three major classes of epigenetic proteins: the *Writers* (enzymes that deposit modifications), the *Erasers* (enzymes that remove modifications) and the *Readers* (proteins that recognize and bind epigenetic modifications). Recent drug development strategies that target these enzymes have succeeded in resulting in FDA approved cancer drugs with many more on the horizon.

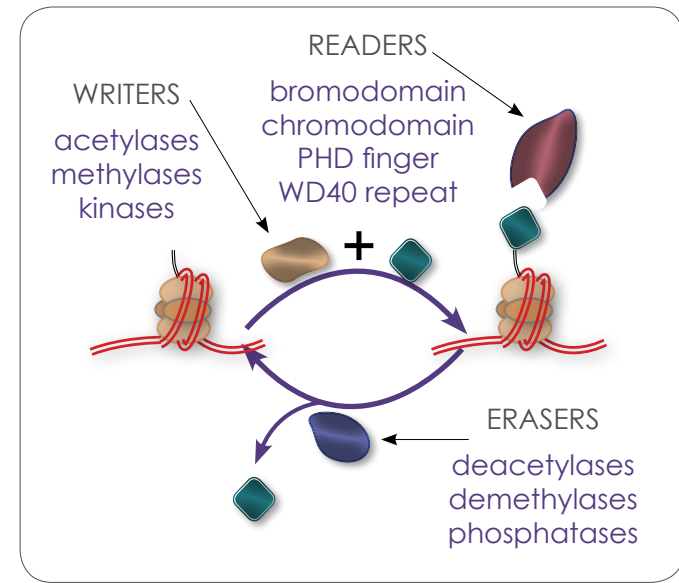
Writers, Erasers & Readers

Writers add modifications

The “Writers” are a group of enzymes that act on histones and covalently add small modifications, such as methyl and acetyl groups. Enzymes that add acetyl groups to histones are called histone acetyltransferases, or HATs, while enzymes that add methyl groups to histones are called histone methyltransferases, or HMTs. Mutations and/or misregulation of these genes are found in many different human tumors. For example, EZH2, an H3K27 methyltransferase, is overexpressed in many tumors and EZH2-inactivating mutations are found in large B cell lymphomas. In another example, the methyltransferase MLL is mutated via rearrangements and translocations in mixed-lineage leukemia, and more than 60 known MLL fusion partners have been described. MLL associates with the H3K79 methyltransferase DOT1L, and this association has led to the discovery that enhanced H3K79 methylation is associated with overexpressed genes in MLL-fusion leukemias.

Erasers remove modifications

The “Erasers” are a group of enzymes that act on

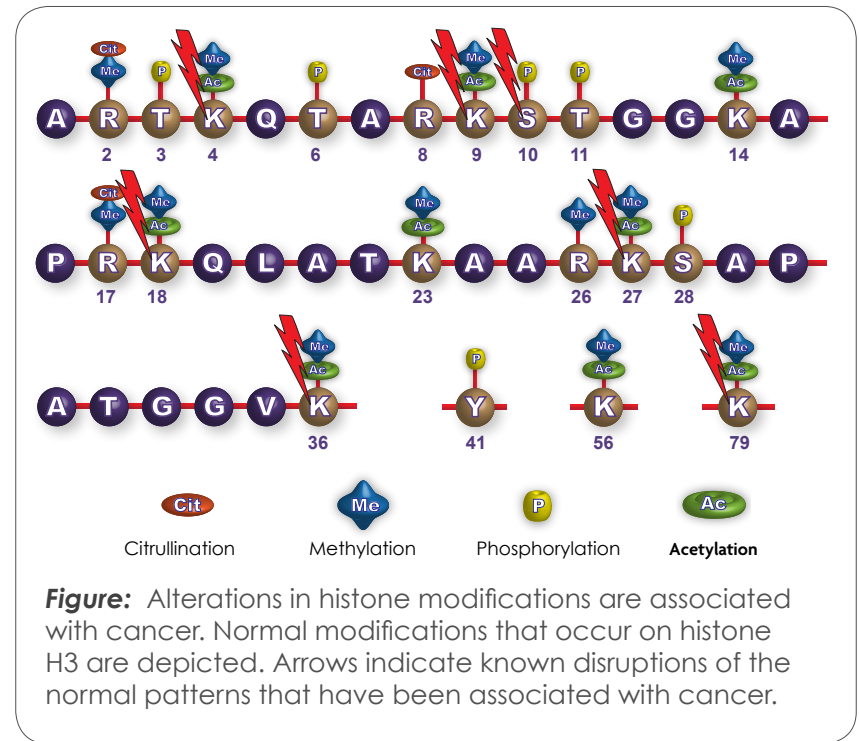


histones to remove small covalent modifications. Enzymes that remove acetyl groups from histones are called histone deacetylases, or HDACs, while those that remove methyl groups from histones are called histone demethylases, or HDMs. Mutation and misregulation of many different HDAC and Sirtuin genes have been associated with cancer. There are currently two FDA approved drugs (and four more that have progressed to

phase II clinical trials) that function by inhibiting HDAC activity. Additionally, the histone demethylase LSD1 has been implicated in cancer. LSD1 is an H3K4/K9 demethylase that is over-expressed in multiple cancer types, and small molecule inhibitors that target LSD1 have been identified that have potential as therapeutics.

Readers help interpret modifications

The “Readers” are a group of proteins that do not have enzymatic activity but that function by recognizing and binding to specific epigenetic modifications. These proteins are thought to influence chromatin structure and function either by recruiting other proteins or by blocking Writers and Erasers from accessing specific modifications. The recent discovery of compounds that specifically inhibit BRD4 binding to acetylated lysines in H3 and H4 has created much excitement around this emergent class of new drug targets.



DNA Methylation & Hydroxymethylation

Changes in DNA methylation have been well characterized in cancer. In general, the cancer epigenome is characterized by global DNA hypomethylation and promoter-specific DNA hypermethylation, which often leads to silencing of tumor suppressor genes. This link to cancer is supported by the finding that mutations in DNMT3A are found in acute

myeloid leukemia, and there are currently two FDA approved cancer drugs that target DNMTs. The recently characterized DNA modification 5-hydroxymethylcytosine (5-hmC) has also been linked to cancer. Both a reduction in 5-hmC and a reduction in expression of the TET enzymes that convert 5-mC to 5-hmC have been reported in breast, liver, lung, prostate and pancreatic cancers.

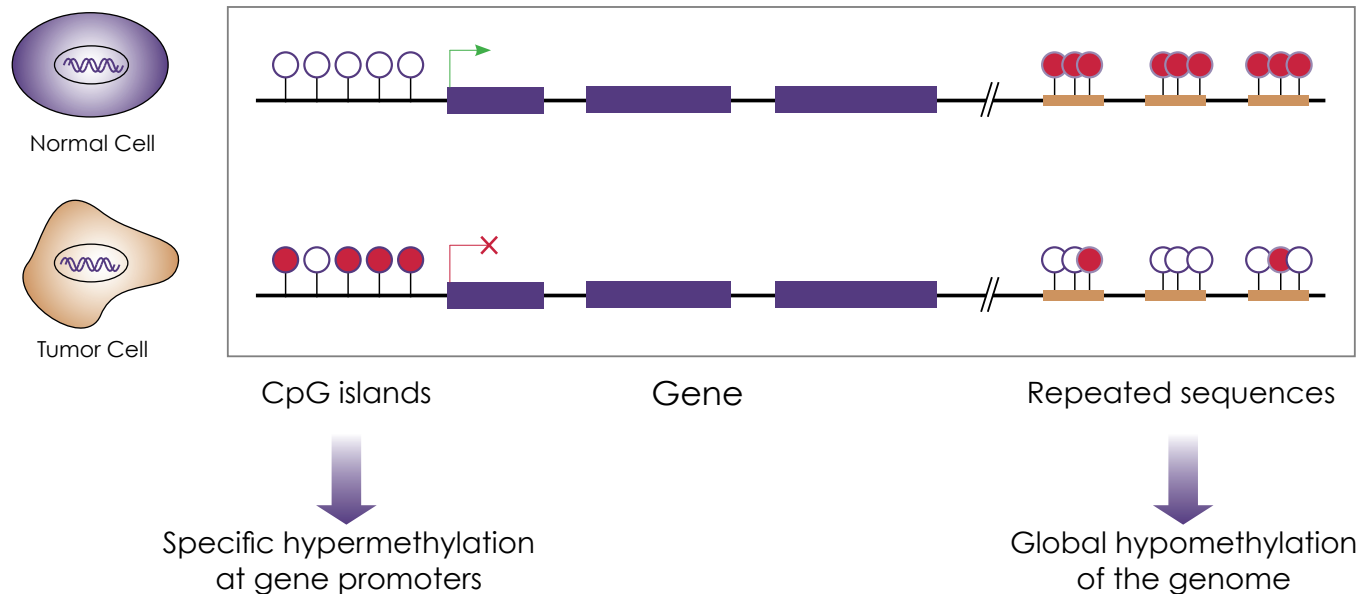


Figure: In normal cells, repetitive elements are generally methylated while CpG islands in promoter sequences are typically unmethylated. In tumor cells, the reverse is observed, with global hypomethylation of the genome occurring due to loss of DNA methylation at repetitive elements, along with locus-specific DNA hypermethylation at promoters of tumor suppressor genes that leads to their transcriptional inactivation. *White lollipop: non-methylated CpG, red lollipop: methylated CpG.*

DNA Methylation and Gene Regulation

DNA methylation is an important regulator of gene expression and genomic organization. It occurs at the cytosine bases of eukaryotic DNA, which are converted to 5-methylcytosine by DNA methyltransferase enzymes. DNA methylation appears almost exclusively in the context of CpG dinucleotides. These dinucleotides are relatively rare in the mammalian genome, and tend to be clustered in what are called CpG islands. Approximately 60% of gene promoters are associated with CpG islands and are normally unmethylated. CpG methylation of gene promoters

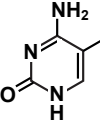
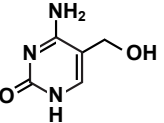
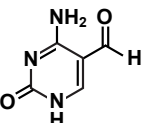
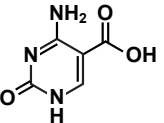
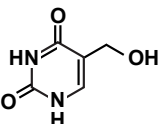
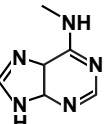
is usually associated with transcriptional silencing which can occur through a number of mechanisms including the recruitment of methyl binding domain (MBD) proteins. DNA methylation is involved in a number of cellular functions, such as embryonic development, genetic imprinting, X chromosome inactivation and control of gene expression. Alterations in normal DNA methylation patterns, either local increases that result in silencing of specific cell cycle regulatory genes or a global reduction in DNA methylation, are involved in many different types of cancer.

DNA Methylation Variants

A role for 5-hydroxymethylation

5-hydroxymethylcytosine (5-hmC) was recently identified as a novel variant of DNA methylation resulting from the enzymatic conversion of 5-methylcytosine (5-mC) into 5-hmC by the TET family of cytosine oxygenases. This form of methylation is prevalent in neurons and embryonic stem cells. In 2011, researchers studying cerebellar and hippocampal cells showed that 5-hmC patterns change significantly during development and aging in mice, raising speculation that 5-hmC may represent another layer of gene regulation (Szulwach, K.E. *et al.*, *Nature Neurosci*, 2011).

Although a role for 5-hmC as a true epigenetic mark remains to be identified, several studies have shown its potential as a demethylation intermediate and suggest that this may be its central role.

	5-mC	5-methylcytosine. Repressive mark at enhancers and promoters, enriched in active gene bodies, recruits specific binders.
	5-hmC	5-hydroxymethylcytosine. Oxidized 5-mC, demethylation intermediate. Active mark at enhancers and gene bodies, recruits specific binders.
	5-fC	5-formylcytosine. Oxidized 5-hmC, demethylation intermediate, recruits DNA repair machinery.
	5-caC	5-carboxycytosine. Oxidized 5-fC, demethylation intermediate, recruits DNA repair machinery.
	5-hmU	5-hydroxymethyluracil. Oxidized form of thymidine, recruits DNA repair machinery.
	6-mA	N6-methyladenine. Active mark, enriched at TSS, influences nucleosome positioning, demethylation by TET homologues, correlation with H3K4 methylation.

5-caC & 5-fC

In addition to 5-hydroxymethylcytosine, other DNA variants have been identified and characterized. Researchers have shown that the TET family of cytosine oxygenase enzymes, which convert 5-methylcytosine into 5-hydroxymethylcytosine (5-hmC), can further oxidize 5-hmC into 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). Both of these DNA modifications have been shown to exist in mouse ES cells, and 5-fC has also been identified in major mouse organs. Both 5-fC and 5-caC exist in the paternal pronucleus, concomitant with the disappearance of 5-methylcytosine, suggesting that these DNA variants may be intermediates in DNA demethylation (Figure 1). One hypothesis for the mechanism of DNA methylation suggests that 5-caC is excised from genomic DNA by thymine DNA glycosylase, thereby returning DNA to an unmethylated state. Other research suggests that replication-dependent dilution accounts for paternal DNA demethylation during preimplantation development. Recent findings indeed show that maternal DNA is methylated and paternal DNA is hydroxymethylated (Figure 2).

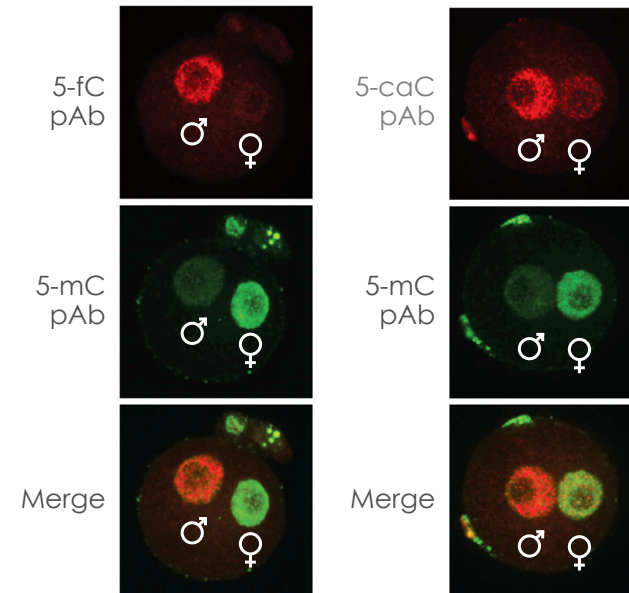


Figure 1: Representative confocal images of fertilized oocytes that were co-stained with [5-Formylcytosine \(5-fC\)](#) and [5-Carboxylcytosine \(5-caC\)](#) antibodies (red), and a [5-methylcytosine \(5-mC\) antibody](#) (green).

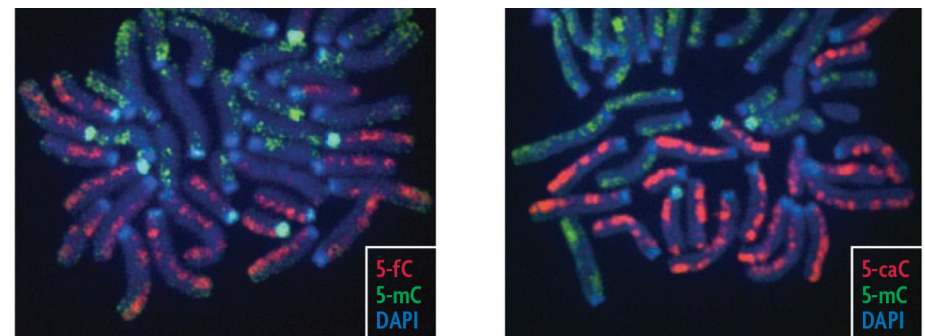


Figure 2: immunofluorescent images of mitotic chromosome spreads that have been co-stained with [5-Formylcytosine](#) (left) or [5-Carboxylcytosine](#) (right) antibodies reveal differential staining of maternally and paternally derived chromosomes. Half the chromosomes are enriched for 5-fC (left) or 5-caC (right), and half for 5-mC (Images provided by Dr. Yi Zhang and described in Inoue *et al.*, *Cell Research*, 2011).

Enzymes Involved in DNA Methylation

CpG methylation involves the transfer of a methyl group to position 5 of cytosine (5-methylcytosine, or 5-mC) by the action of the DNA methyltransferase enzymes (DNMTs). Three families of DNMTs have been identified: DNMT1, DNMT2 and DNMT3. The DNMT3 family contains two active methyltransferases, DNMT3A, DNMT3B and one DNMT3-like protein (DNMT3L) which has no DNA methyltransferase activity but does function as a co-factor of DNMT3A. The defined role of DNMT1 is to ensure the maintenance of cytosine methylation during DNA replication, while DNMT2 does not methylate DNA but has been identified as the first RNA cytosine methyltransferase. The complex series of events leading to a repressive chromatin state involves the coordinated regulation of DNA methyltransferases, Methyl-CpG binding proteins (MBD proteins) and the Kaiso family of proteins. The family of MBD proteins include MeCP2, MBD1, MBD2, MBD3 and MBD4.

DNMT1	The most abundant DNMT in somatic cells. DNMT1 shows a preference for methylating hemi-methylated DNA. It is considered to be a maintenance DNA methyltransferase that is important in the maintenance of specific patterns of methylation throughout cellular divisions.
DNMT2	DNMT2 lacks the large N-terminal regulator domain common to other eukaryotic methyltransferases, but it possesses a catalytic domain. Although referred to as a DNA methyltransferase, DNMT2 does not methylate DNA, but instead is the first RNA cytosine methyltransferase to be identified.
DNMT3A	Required for genome-wide <i>de novo</i> methylation and is essential for the establishment of DNA methylation patterns during development. It modifies DNA in a non-processive manner and also methylates non-CpG sites. It plays a role in paternal and maternal imprinting and is required for methylation of most imprinted loci in germ cells.
DNMT3B	Has been shown to be important in the regulation of specific patterns of DNA methylation, specifically <i>de novo</i> methylation. Mutations in the DNMT3B gene cause the immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome.
DNMT3L	Nuclear protein with similarity to the DNMT family of proteins, but does not function as a DNA methyltransferase. It serves as an essential regulator of DNMT3A and DNMT3B, recruiting them to sites for deposition of <i>de novo</i> DNA methylation. DNMT3L is expressed in the presumptive gametes in mammalian embryos and is required for the establishment of genomic imprinting. It has a PHD-like domain that allows binding to chromatin through interactions with the N-terminal tail of histone H3 that is devoid of lysine 4 methylation.
MBD1	MBD1 is recruited to both methylated and non-methylated CpGs via separate domains: the CXXC domain targets the protein to non-methylated CpGs whereas the MBD domain targets the protein to methylated CpG. It is generally associated with the repressive chromatin state through coordinated regulation of DNA methyltransferases and two other groups of proteins called the Methyl-CpG binding proteins (MBD proteins) and the Kaiso family of proteins.
MBD2	Transcriptional repressor which is associated with histone deacetylases (HDAC) in the Sin3 and the MeCP1 complexes in mammalian cells. The HDAC complexes, also containing MBD3 and MeCP2, are involved in packaging the genomic DNA into inactive chromatin.
MBD3	Regulates transcription by forming a Mi-2/NuRD complex with nucleosome remodeling and histone deacetylase (HDAC) activities in mammalian cells. MBD3 has been shown to interact, not with 5-methylcytosine methylated DNA, but with DNA containing 5-hydroxymethylcytosine (5-hmC) methylation. MBD3 co-localizes with TET1 and is required for normal levels of 5-hmC in embryonic stem cells.

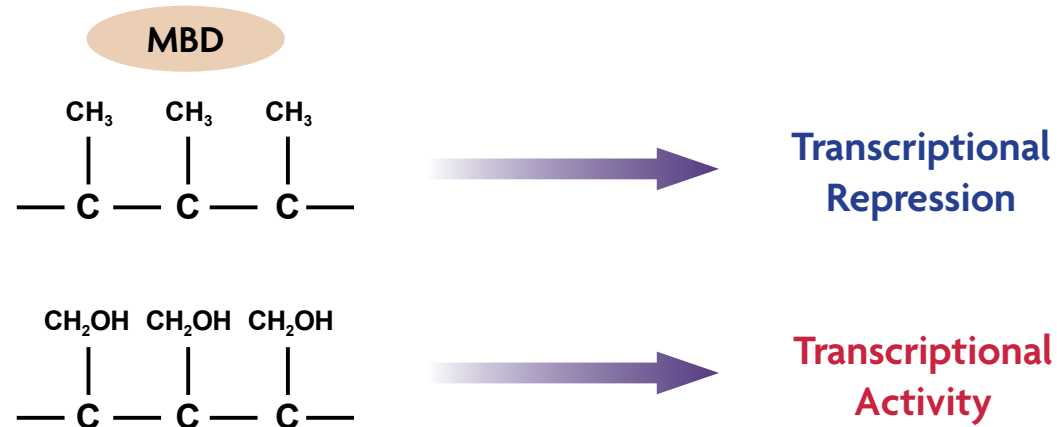
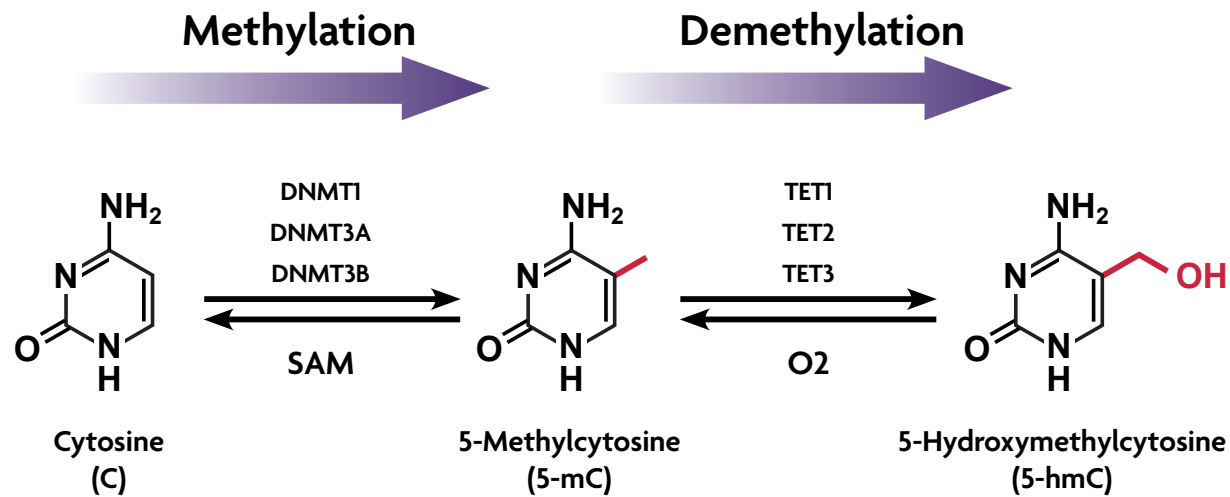
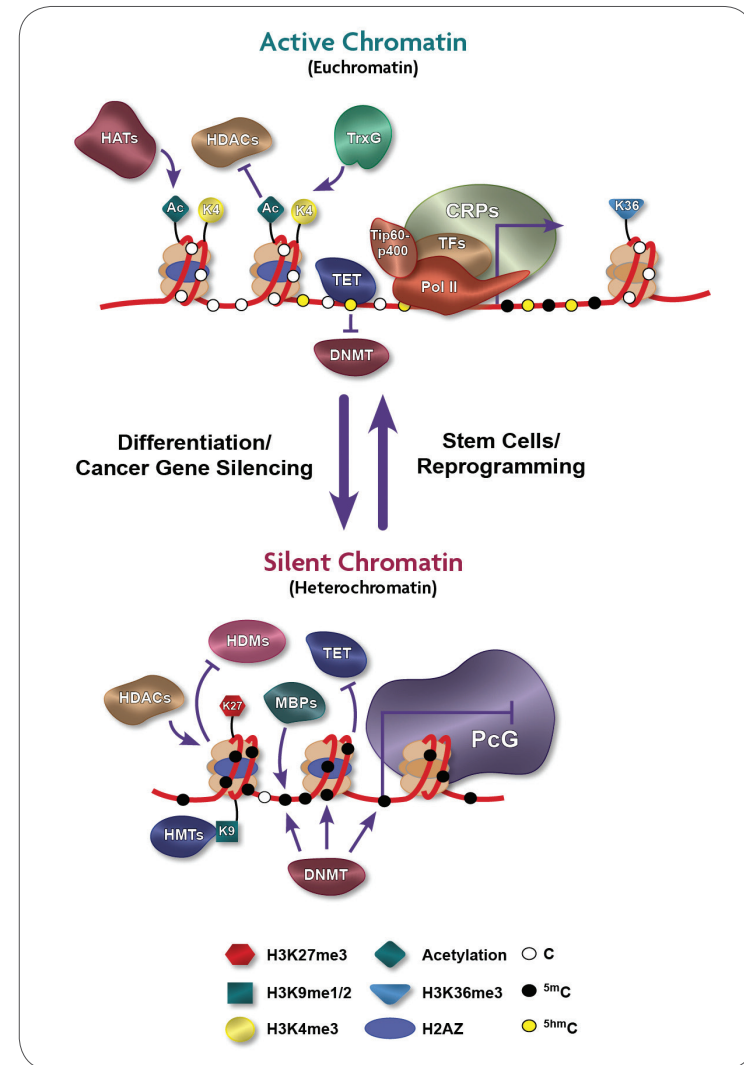


Figure: Depiction of DNA methylation enzymes involved in the methylation/demethylation. The diagram shows the various enzymatic reactions that lead to the DNA methyl variant byproducts 5-methylcytosine (5-mC, CH₃) and 5-hydroxymethylcytosine (5-hmC, CH₂OH). DNA methylation leads to transcriptional repression while DNA hydroxymethylation by Tet enzyme-mediated oxidation of 5-mC is associated with transcriptional activation.

DNA Methylation & Gene Silencing

DNA methylation-mediated gene silencing is a critical regulatory event in both differentiation and tumorigenesis. Active genes have an open chromatin (euchromatin) structure and display DNA hypomethylation at gene promoter regions. They are also characterized by H3K4 & H3K36 methylation and H3 & H4 acetylation. These modifications function to neutralize histone charges and recruit chromatin remodeling proteins (CRPs) that lead to unraveling of the chromatin structure, allowing access to the basal transcriptional machinery. In contrast, gene silencing results in the recruitment of DNA methylation machinery (DNMTs, MBPs), chromatin modifiers (HDACs, HMTs) and repressive complexes, such as Polycomb (PcG) proteins. This leads to chromatin condensation (heterochromatin) and DNA hypermethylation. Condensed chromatin is characterized by H3K27 & H3K9 methylation. Together, the cofactors and regulatory proteins effecting these epigenetic modifications define the chromatin landscape that dictates the expression profile of the cell.



TrxG, Trithorax group; **PcG**, Polycomb group; **HATs**, Histone acetyltransferases; **HDACs**, Histone deacetylases; **TFs**, Transcription factors; **HDMs**, Histone demethylases; **DNMT**, DNA methyltransferase; **TET**, Ten-eleven translocation enzymes; **HMTs**, Histone methyltransferases; **MBPs**, Methyl binding proteins.

Tools to Analyze DNA Methylation

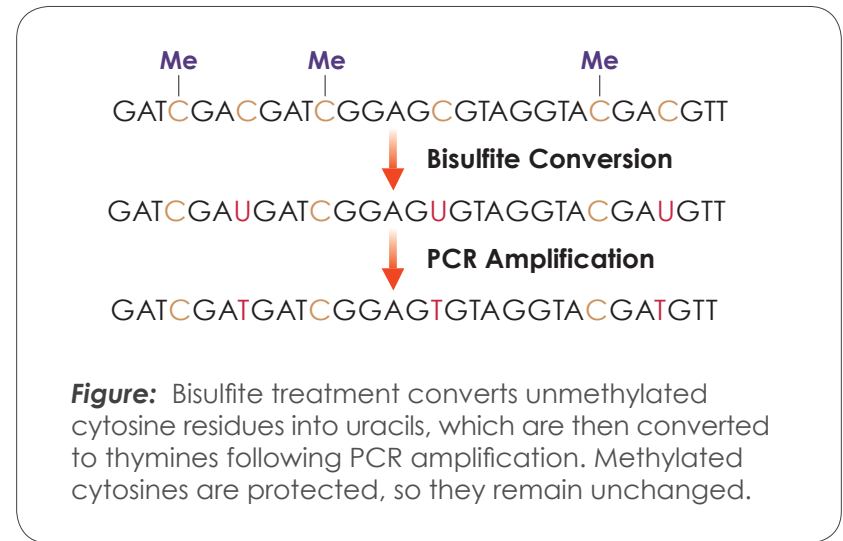
The cancer epigenome is marked by alterations in a myriad of epigenetic features, including DNA methylation, nucleosome positioning, and histone post-translational modifications. These changes influence gene regulation and can serve as signatures of cancer. To better understand how changes in DNA modifications influence normal and diseased states, much research depends on the ability to accurately detect and quantify DNA methylation. There are various approaches that are commonly

used for DNA methylation analysis. Gene-specific methods such as bisulfite conversion and targeted enrichment methods for DNA fragments containing 5-mC and 5-hmC enable the analysis of DNA methylation status at a specific locus. There are also global DNA methylation analysis techniques available to analyze correlations between global cellular changes in genomic 5-mC content and factors such as environmental exposures, lifestyle or clinical outcomes.

Bisulfite Conversion

Bisulfite conversion followed by DNA sequencing is considered the gold standard in gene-specific DNA methylation analysis as it provides single-base-pair resolution of the DNA methylation profile. The conversion reaction occurs as a three-step deamination of cytosine residues into uracil. As only unmethylated cytosine residues are susceptible to bisulfite conversion, the original methylation state of the DNA can be determined.

In the bisulfite conversion reaction, DNA is first treated with sodium bisulfite to convert cytosine residues into a cytosine-bisulfite derivative. The second step is an irreversible hydrolytic deamination of the cytosine-bisulfite derivative that results in a uracil-bisulfite derivative. The final step is desulfonation of the uracil-bisulfite derivative to uracil. Only unmethylated cytosines are susceptible to the bisulfite reaction, therefore 5-mC and 5-hmC remain the unchanged. Following bisulfite conversion, the DNA is often amplified by PCR where the uracils are converted to thymines or followed by sequencing to reveal single-nucleotide resolution information about the methylation status of a specific loci or the whole genome.



Active Motif's **Bisulfite Conversion Kit** simplifies the analysis of DNA methylation and minimizes DNA fragmentation. For more information, visit us at www.activemotif.com/bis-conv.

Active Motif also offers services for bisulfite sequencing. For more information on Active Motif's DNA methylation services, please visit www.activemotif.com/services.

Methylated DNA Enrichment

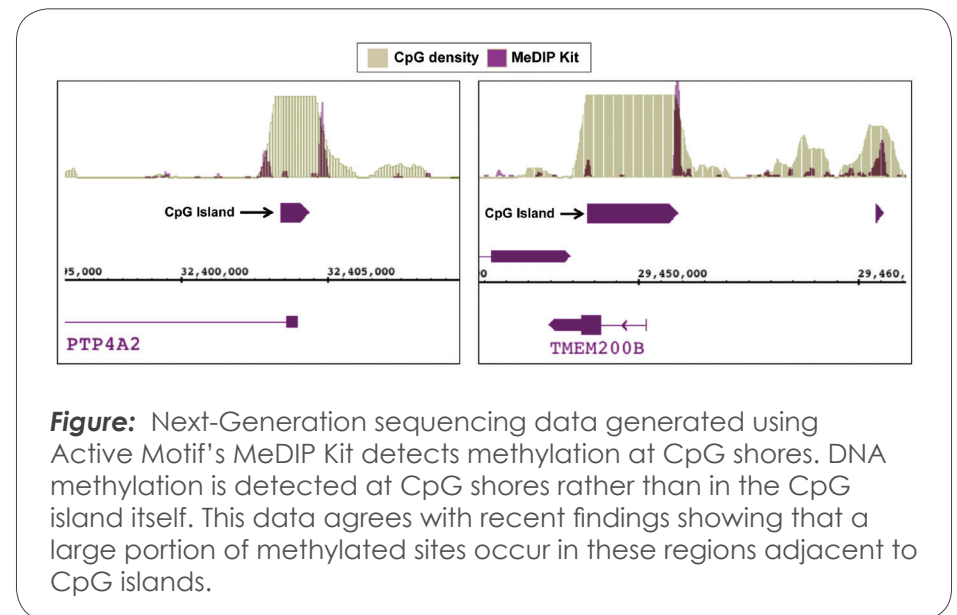
Affinity enrichment is a technique that is often used to isolate methylated DNA from the rest of the DNA population. This is usually accomplished by antibody immunoprecipitation methods or with methyl-CpG binding domain (MBD) proteins. Each method has advantages and disadvantages.

Methylated DNA Immunoprecipitation (MeDIP) is an antibody immunoprecipitation method that utilizes a 5-methylcytosine antibody to specifically recognize methylated cytosines.

The discovery of 5-hydroxymethylcytosine (5-hmC) as a modification within genomic DNA has led to the introduction of **hMeDIP**, immunoprecipitation of 5-hmC containing DNA. The hMeDIP method uses a 5-hydroxymethylcytosine antibody to specifically recognize 5-hmC DNA.

The specificity of the antibodies used enables selective enrichment of either 5-mC (MeDIP) or 5-hmC (hMeDIP) methylated DNA. MeDIP techniques are ideal for the enrichment of DNA fragments containing cytosine methylation regardless of the sequence context. While most DNA methylation in mammalian tissues occurs in

a CpG context, studies have found that 15-20% of total cytosine methylation in embryonic stem cells occurs at sequences other than CpG. For researchers interested in specific enrichment of either 5-mC and 5-hmC methylation, or researchers needing to identify total cytosine methylation, the MeDIP and hMeDIP techniques are ideally suited for these applications.



Another method for the enrichment of methylated DNA fragments uses recombinant methyl-binding protein MBD2b, or the MBD2b/MBD3L1 complex, as in the **MethylCollector™ Ultra Kit** to separate CpG methylation from the rest of the genomic DNA. The MBD enrichment technique uses the specificity of the protein binding site to selectively capture 5-methylcytosine DNA. One advantage of a methyl-CpG binding protein enrichment strategy is the input DNA sample does not need to be denatured, the protein can recognize methylated DNA in its native double-strand form. Another advantage is that the MBD protein binds only to DNA methylated in a CpG context to ensure the enrichment of methylated CpG DNA, making this technique ideal for researchers studying CpG Islands.

Regardless of the affinity enrichment method, enriched DNA can be used in many downstream applications, such as endpoint or real-time PCR analysis of the methylation status of particular loci in normal and diseased samples, bisulfite conversion followed by cloning and sequencing, or amplification and labeling for microarray analysis.

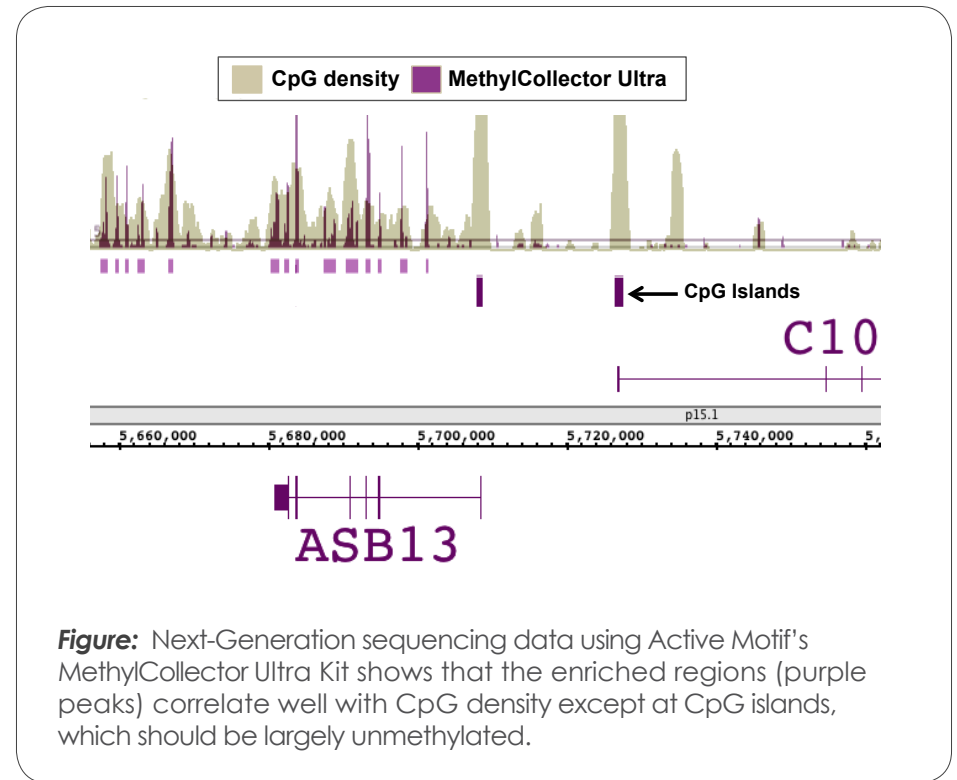


Figure: Next-Generation sequencing data using Active Motif's MethylCollector Ultra Kit shows that the enriched regions (purple peaks) correlate well with CpG density except at CpG islands, which should be largely unmethylated.

Global DNA Methylation Analysis

DNA methylation occurs primarily in the context of CpG dinucleotides. In mammals, 70-80% of CpG dinucleotides are methylated. These CpG sites are mainly found within repetitive regions and are normally methylated to repress transcription. Global DNA hypomethylation is a common feature of various degenerative human states, including aging, developmental and neuropsychiatric disorders, and cancer. Because repetitive elements comprise almost 50% of the human genome and account for more than one-third of genome-wide DNA methylation, the global hypomethylation that is observed in cancers is most likely primarily due to hypomethylation at repetitive elements. DNA hypomethylation leads to activation of dormant repeat elements and the subsequent aberrant expression of associated genes. It also leads to chromosomal instability and increased mutation rates.

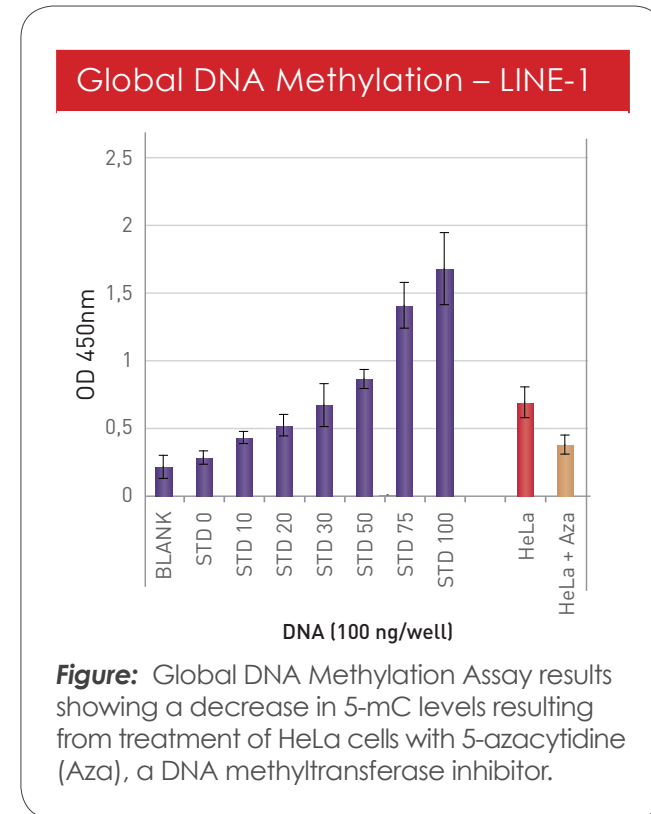
Because global hypomethylation is a hallmark of most cancers, the need to measure global DNA methylation is essential. Gene-specific DNA methylation analysis techniques do not provide information about the global levels of DNA

methylation within a genome. However, several methods are commonly used for detection of 5-mC levels in the genome. The gold standard is high performance liquid chromatography (HPLC). Other variations on this method, such as HPLC tandem mass spectrometry (LC-MS/MS), thin-layer chromatography and liquid chromatography/mass spectrometry are also used. In **chromatographic approaches**, DNA is digested down to single nucleotides and unmethylated and methylated cytosines are quantified. Although these methods are highly quantitative and reproducible, they are labor-intensive, require special equipment and expertise and large amounts of high quality DNA.

One way to get around the sample material requirements of chromatographic approaches is to utilize **PCR-based methods**, such as Alu and LINE-1 assays that measure the methylation status of genomic repeat elements, or methylation sensitive restriction enzyme assays such as the luminometric methylation assay (LUMA). However, these approaches only provide information about the levels of DNA methylation, not about the specific sequences that are being analyzed.

Another approach that is commonly used to measure global DNA methylation levels is an **ELISA-like format**. The advantage of this approach over other methods is that it is high-throughput, requires minimal sample material and is simple to use. For these types of assays, DNA is immobilized onto a well of a 96-well plate either by passive absorption or using an antibody specific for either DNA or 5-mC for capture. A second antibody is used for colorimetric detection of the levels of either 5-mC or DNA within the immobilized DNA. Although these assays are more user-friendly and simpler to use, they often lack sensitivity, and passive adsorption capture methods reduces specificity.

A new ELISA-based approach developed for Active Motif's **Global DNA Methylation – LINE-1 Kit** uses a LINE-1 probe for DNA hybridization rather than non-specific passive adsorption to provide for improved sensitivity and reproducibility. For more information on the Global DNA Methylation – LINE-1 Kit, visit www.activemotif.com/gdm.



Products for DNA Methylation Research

Active Motif offers a comprehensive portfolio of products and services for DNA methylation research including antibodies, recombinant enzymes, kits and services for bisulfite conversion and methylated

DNA enrichment, as well as quantitative assays for high-throughput analysis of global DNA methylation. Simply click on the links below for more detailed information on these products.

Antibodies & Enzymes



A comprehensive portfolio of antibodies and proteins for DNA Methylation analysis.

- [DNA Methylation Antibodies](#)
- [5-mC Antibodies](#)
- [5-hmC Antibodies](#)
- [5-caC Antibodies](#)
- [5-fC Antibodies](#)
- [DNA Methylation Enzymes](#)

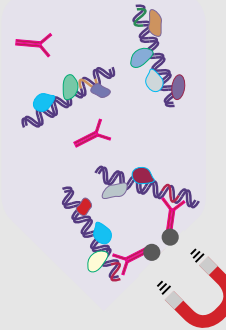
Bisulfite Conversion



Bisulfite Conversion Kits and Sequencing Services to enable analysis of methylation patterns to differentiate between methylated and unmethylated sequences.

- [Bisulfite Conversion Kit](#)
- [Bisulfite Sequencing Services](#)

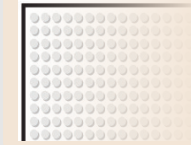
Methylated DNA Enrichment



A broad selection of kits for enrichment of methylated DNA variants (5-methylcytosine, or 5-mC and 5-hydroxymethylcytosine, or 5-hmC).

- [MeDIP](#)
- [hMeDIP](#)
- [MethylCollector™ Ultra](#)
- [Hydroxymethyl Collector™](#)
- [Services](#)

Quantitative Assays



Simple, high-throughput ELISA-based assays for measuring changes in global DNA methylation levels or DNMT activity.

- [Global DNA Methylation Assay](#)

For more information on DNA methylation products, please visit us at www.activemotif.com/dna-methylation.

Enabling Insight into the Cancer Epigenome: A Case Study

Epigenetic changes are hallmarks of human cancers. Changes in DNA methylation as well as histone modifications have been found in every tumor type, both benign and malignant, studied to date and have been associated with the progression of colorectal cancer. Thus, gaining further insight into these complex regulatory mechanisms is crucial to understanding disease susceptibility,

initiation and progression. In a recent study performed by the laboratory of Dr. Manuel Perucho at the IMPPC in Barcelona, numerous assays from the Active Motif product line were utilized in colorectal cancer studies to analyze the role of epigenetic aberrations, in particular global hypomethylation, in the development of cancer.

Case Study:

Because changes in global methylation are a hallmark of many human diseases, including cancer, simpler methods than HPLC or bisulfite sequencing are warranted for correlative studies that analyze variances in genome-wide DNA methylation status. Active Motif's **Global DNA Methylation – LINE-1 Assay** uses a unique hybridization approach that quantitates 5-methylcytosine (5-mC) levels at LINE-1 repeats as a surrogate measure of global methylation. This approach offers better specificity and reproducibility than other available methods that utilize non-specific passive adsorption (Figure 1).

Quantify changes in global DNA methylation levels

The global hypomethylation observed in cancer cells primarily reflects the somatic demethylation of DNA repetitive elements. Hypomethylation predisposes normally compacted DNA to decondensation which can lead to chromosomal instability and tumor development and/or progression.

Through a genome-wide analysis of DNA methylation alterations, a specific family of non-coding

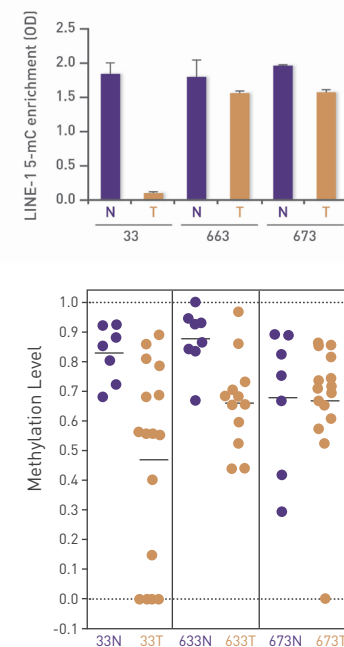


Figure 1: LINE-1 methylation levels of four human colon cancer cases, determined by the Global DNA Methylation – LINE-1 assay (top) and bisulfite sequencing (bottom).

pericentromeric DNA repetitive elements, SST1, was found to exhibit a wide degree of demethylation in colorectal tumors.

As LINE-1 demethylation is a commonly used marker of global hypomethylation, the LINE-1 methylation levels were determined by the Active Motif **Global DNA Methylation – LINE-1 Kit** (Figure 1, top) and bisulfite sequencing (Figure 1, bottom) in a subset of colorectal cancer patients in which SST1 methylation levels had been determined. The data reveal both assays yield similar estimated levels of LINE-1 hypomethylation in tumors compared to corresponding normal tissue.

Further, the analyses reveal that demethylation occurs in both repeats, especially in LINE-1s, in a high proportion of the patients. The results also demonstrate that demethylation of SST1 positively correlates with demethylation of LINE-1 (Figure 2).

Assess chromatin features associated with DNA methylation in normal & diseased cells

Investigation into the chromatin changes that occur during loss of methylation at SST1 repeat elements in colon and ovarian cancer cell lines

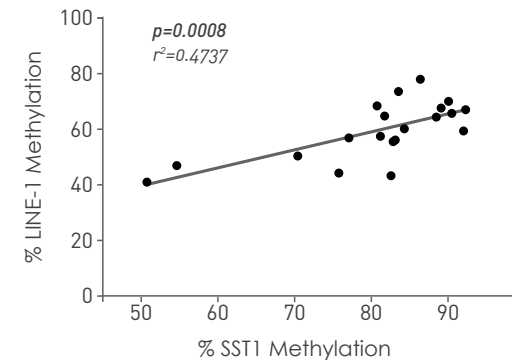


Figure 2: Demethylation of SSTS1 correlates with LINE-1 demethylation ($p=0.0008$), a commonly accepted marker for global DNA methylation.

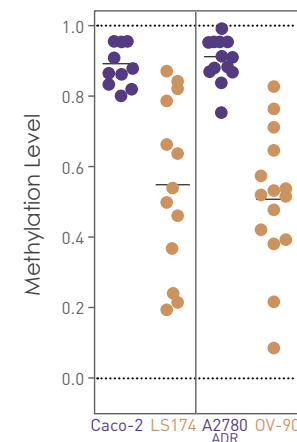


Figure 3: SSTS1 methylation levels of individual clones analyzed by bisulfite sequencing in colon and ovarian cancer cell lines. One clone represents one single SSTS1 element.

revealed that, while the cell lines with retained SST1 methylation (Figure 3) exhibit enrichment of Histone H3 lysine 9 trimethylation (H3K9me3), SST1 demethylation is associated with Histone H3 lysine 27 trimethylation (H3K27me3), an epigenetic repressive mark of facultative heterochromatin (Figure 4).

Heterogeneity of SST1 methylation levels was observed in the LS174 T colon cancer cell line employed to study the putative effects of SST1 demethylation on chromatin structure. Therefore, Active Motif's **ChIP-Bis-Seq Kit**, which offers a method to directly assess DNA methylation patterns associated with chromatin modifications (or chromatin-associated factors), was utilized to test whether SST1 H3K27me3 actually co-occurs in association with demethylated SST1 elements. LS174 and Caco-2 chromatin was immunoprecipitated with an anti-H3K27me3 antibody followed by bisulfite treatment of isolated DNA. The SST1 methylation status of H3K27me3-associated DNA was then determined by bisulfite sequencing (Figure 5). The ChIP-Bis-Seq results confirmed that the H3K27me3-associated SST1 elements are actually demethylated. For more information, please visit www.activemotif.com/chip-bis-seq.

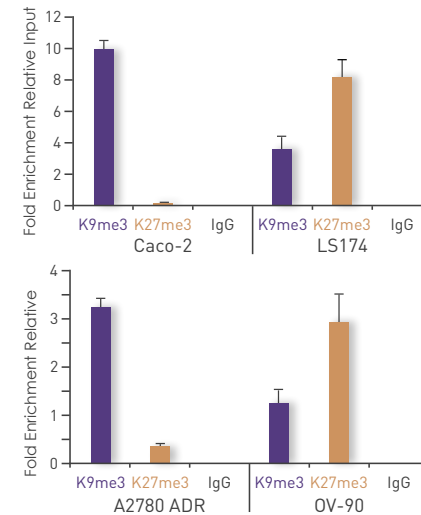


Figure 4: SST1 methylation correlates positively with H3K9me3 levels and negatively with H3K27me3. ChIP analysis corresponding to data sets in Figure 3 show demethylation of SST1 is associated with increased H3K27 trimethylation and a reduction in H3K9me3. Fold change calculated relative to input.

Verify results obtained from cell models in primary tissues

Cell model systems often do not reflect true biology. Thus, it is important to confirm results in primary tissues. The Active Motif **ChIP-IT® FFPE Chromatin Preparation Kit** and **ChIP-IT® FFPE Kit** were used on colon primary FFPE samples and confirmed the shift from high levels of DNA methylation and H3K9me3 in normal tissues to SST1 hypomethylation and increased levels of H3K27me3 in tumor cells (Figure 6). These findings suggest that demethylation of SST1 elements may be a marker of an epigenetic reprogramming event associated with changes in chromatin structure that may ultimately affect chromosomal integrity.

For more complete information on ChIP-IT products for primary cells, visit www.activemotif.com/chip.

Data was provided courtesy of Dr. Johanna Samuelsson and Dr. Manuel Perucho. Samuelsson *et al.*, in preparation.

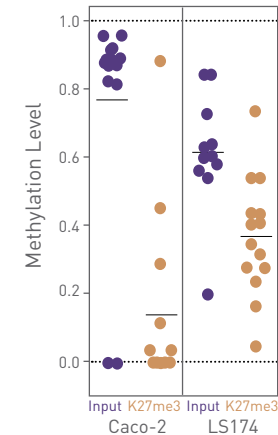


Figure 5: SST1 elements associated to H3K27me3 are highly demethylated.

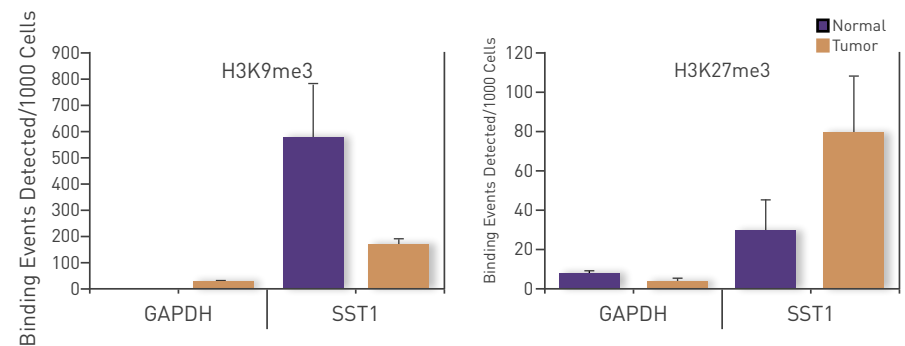


Figure 6: ChIP analysis of histone modifications associated with SST1 repetitive elements in colon cancer FFPE primary tissue samples.

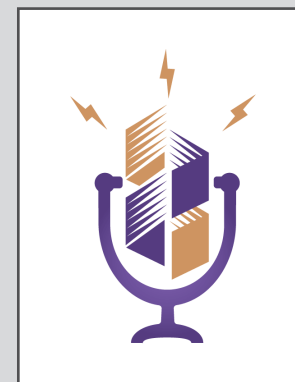
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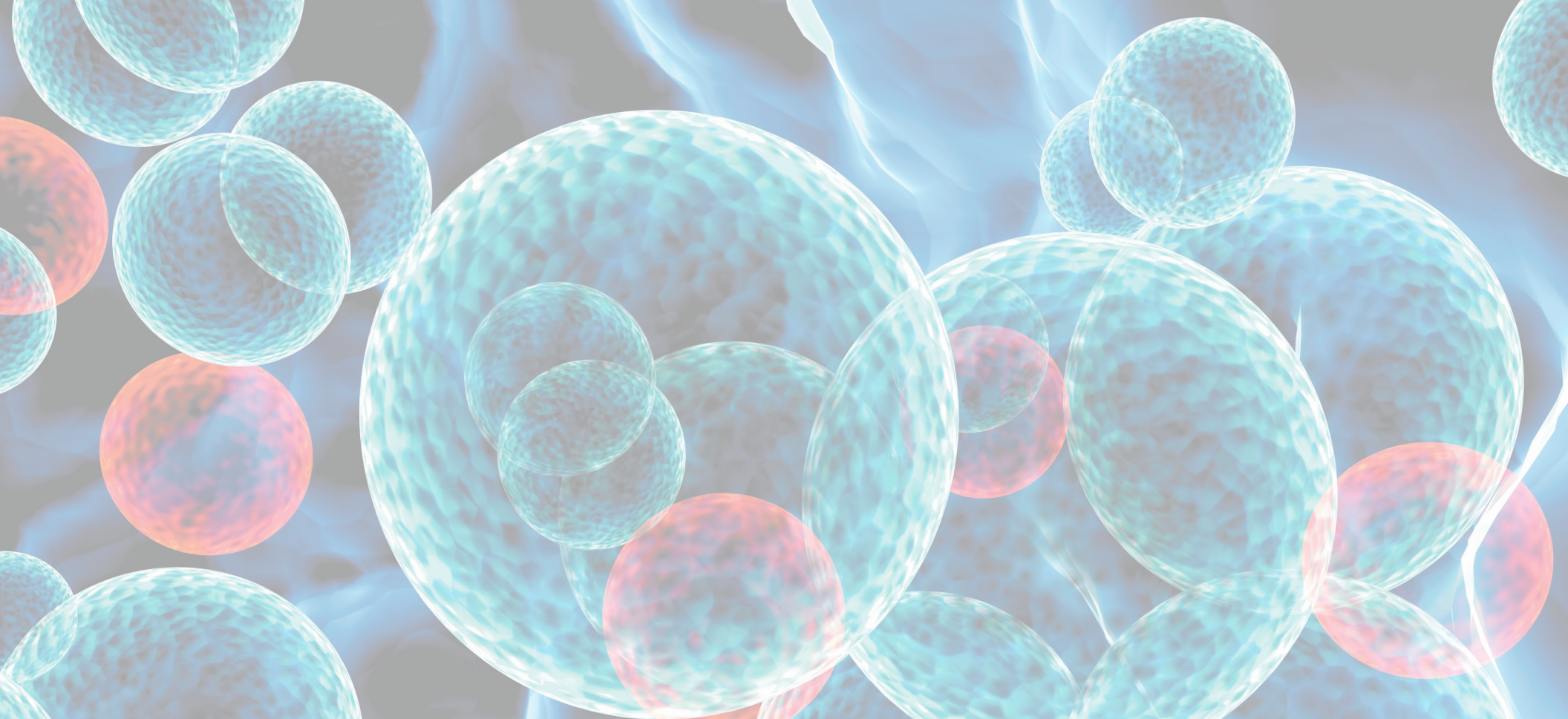
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