EPIGENETIC METHYLATION AND ITS IMPLICATION IN CANCER AND NEURODEGENERATION

by

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ABSTRACT OF THE THESIS

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Epigenetics has become a fast-growing area of study in cellular biology. An epigenetic trait is defined as a stably inherited phenotype resulting from changes in a chromosome without alterations in the DNA sequence (1). These types of modifications are essential for normal cellular function, assisting in the activation or repression of necessary genes in various stages of development. There are instances, though, in which the modifications can be altered to induce irregular gene transcription. In these cases, the results can provoke various forms of disease. In mammals, epigenetic methylation has been found to play an important part in all forms of cancer, with two key areas of alteration. These are the specific methylation of sequences of DNA, as well as modifications on the histones surrounding DNA. Since the discovery of their involvement in the change of gene expression, histone modifications and DNA methylation have been implicated in diseases other than cancer, such as neurological disorders including schizophrenia and Alzheimer's disease. One very important aspect of epigenetic methylation is its reversibility. This key property has created a promising field of epigenetic therapy,

which has led to the development of several FDA approved drugs for cancer treatment. It has also generated several new and exciting ideas for future paths of treatment.

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List of Abbreviations:

AD	Alzheimer's disease
AML	acute myeloid leukemia
AMPKs	AMP-activated protein kinases
APOE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
AXIN	axis inhibitor
BRCA1	Breast Cancer 1, Early Onset
C	cytosine
CBP	CREB-binding protein
CBX	chick homeobox
CDKL5	cyclin-dependent kinase-like 5
CDKN2A/B	cyclin-dependent kinase inhibitor 2A/B
ChIP	chromatin immunoprecipitation
CTCL	cutaneous T-cell lymphoma
CUP	cancer of unknown primary site
DAC	Dacogen; 5-aza-2'-deoxycytidine
DNA	deoxyribonucleic acid
DNMTs	DNA methyltransferases
EED	Embryonic Ectoderm Development
EOAD	early onset Alzheimer's disease
ERβ	estrogen receptor beta
ES	embryonic stem cells
EZ	Enhancer of Zeste
FDA	Federal Drug Administration
GABA	γ-aminobutyric acid
GAD67	glutamic acid decarboxylase 67
GFP	green fluorescence protein
HATs	histone acetyltransferases
HDACs	histone deacetylases
HDMs	histone demethylases
HIF	hypoxia inducible factor
hMLH1	human mutL homologue 1
HMTs	histone methyltransferases
HNPCC	hereditary non-polyposis colorectal cancer
HOX	homeobox
HP1	heterochromatin protein 1
ICF	Immunodeficiency, Centromeric Instability, and Facial Abnormalities syndrome
JMJD6	Jumonji domain-containing 6 protein
K	lysine

LAM	Laser Assisted Microdissection
LKB1	liver kinase B1
LOAD	late onset Alzheimer's disease
MAPT	microtubule-associated protein tau
MARKs	microtubule affinity regulating kinases
MBPs	methyl-CpG-binding domain proteins
MDS	myelodysplastic syndrome
MECP2	methyl-CpG-binding protein 2
MGMT	O^6-methylguanine-DNA methyltransferase
miRNA	micro RNA
MSP	methylation-specific polymerase chain reaction
mRNA	messenger RNA
MTHFR	methylenetetrahydropholate reductase
***N*-MNU**	*N*-methyl-N-nitrosourea
NHL	non-Hodgkin's lymphoma
NSD2	Nuclear Receptor-binding SET Domain 2
PcG	Polycomb group
PFC	prefrontal cortex
PHD	plant homeo domain
PJS	Peutz-Jeghers syndrome
Pol II	polymerase II
PRC2	Polycomb Repressive Complex
PRMTs	protein arginine methyltransferases
PSEN1	presenilin
PTMs	post-translational modifications
R	arginine
RASSF1A	Ras association domain family 1 isoform A
Rb	retinoblastoma
RCC	renal cell carcinoma
(RT)-PCR	reverse transcription polymerase chain reaction
RTT	Rett syndrome
SAM	S-adenosyl-L-methionine
SAM	significance analysis of microarrays
SET	Suppressor of Variegation, Enhancer of Zeste, and Trithorax
STK11	serine-threonine kinase 11
SUZ12	Suppressor of Zeste 12
TSA	trichostatin A
UTX	ubiquitously transcribed tetratricopeptide repeat gene on X chromosome
VHL	von-Hippel Lindau
VPA	Valproic acid

I. Introduction:

There are over 200 different types of cells in the human body. Each type maintains a unique cellular identity represented by the specific sets of genes they transcribe. Because the genetic material in each of these cells is essentially identical, there must be strict regulation of gene expression in each cell within the human body. This transcriptional regulation is carried out by controlling the accessibility to genes. This layer of control is achieved by the packaging of DNA into particular arrangements. DNA is wrapped around proteins called histones, which are structurally organized and condensed into chromatin. These histones and their associated proteins are described in detail below. Histone proteins can be modified, and these modifications serve as important regulators in the transcription of necessary genes throughout the life cycle of an organism. In mammals, the methylation on specific sites of DNA called CpG islands is an additional method of transcriptional control. These cellular processes are collectively called epigenetics, a term defined by the capability of cells to transmit their tissue-and stage-specific gene expression patterns to daughter cells without mutation of the DNA sequence, thereby making these changes reversible. As we will see throughout this paper, these epigenetic mechanisms are often used in combination in order to successfully maintain transcriptional regulation. Although there are numerous epigenetic mechanisms that have the ability to regulate transcription, I will focus here on the methylation of histones and CpG islands.

Defects in epigenetic mechanisms have the potential to induce various diseases, including cancer and neurodegenerative disorders such as schizophrenia or Alzheimer's disease. The reversibility of DNA methylation and histone modifications has created a

new field of therapeutics with great potential in treating such diseases. Before these diseases and the newly created field of therapeutics are discussed, I will explain a few of the definitive models surrounding epigenetic regulation of the genome read-out, as well as describe some of the important contributing factors.

A. Epigenetic inheritance:

It has been reported that distinct cell types have specific patterns of epigenetic aberrations heritably transferred from one generation to another (2). These imprinted modifications on specific sequences of DNA can be altered by environmental influences, and not only does this affect the directly exposed organism, but there have been experiments showing inheritance of these modifications in future generations, called a "transgenerational effect" (3). Although specific mechanisms involved in causing such deleterious states have only begun to be studied, mechanisms have been hypothesized of how DNA methylation can be altered, and subsequently inherited into future organisms (3, 4).

What constitutes a transgenerational effect? To put it simply, a transgenerational phenotype requires a reprogramming of the germ line (3). To test this, one has to investigate at least three generations of a mother that has been exposed to an environmental exposure, therapeutic treatment, or physiological stress (5). Figure 1 describes how to test for a transgenerational effect. Upon gestation, if an F0 mother is exposed to an inducing agent, the developing F1 generation is also exposed, as well as their germline cells, noted in Figure 1 as the F2 generation. Therefore, to observe the inheritance of a newly introduced germline reprogramming, research must be carried out to the F3 generation. If the deleterious effect is carried out to the F3 or beyond, a transgenerational epigenetic effect has occurred.

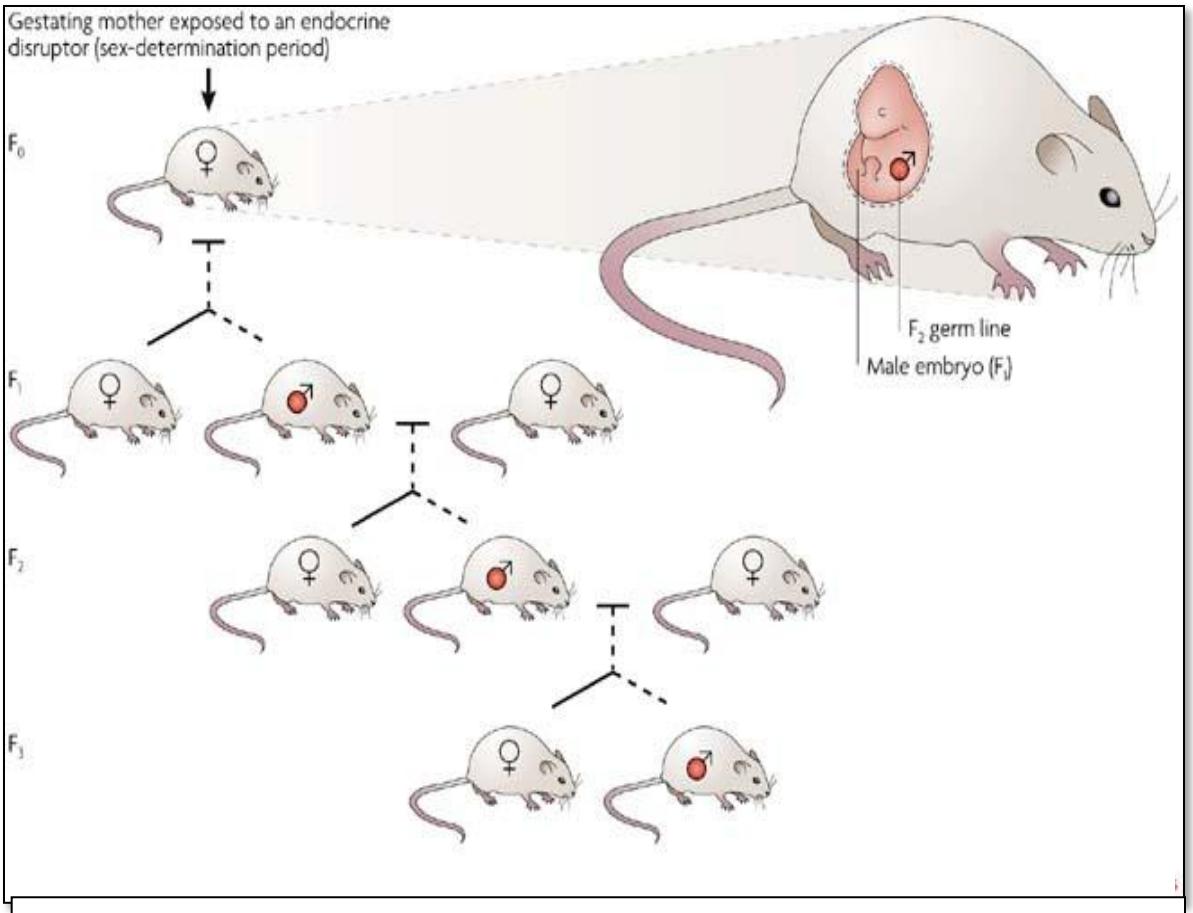


Figure 1: Germline transmission of epigenetically regulated transgenerational phenotypes. In the gestating mother, there is exposure of the F_0 female, the F_1 embryo and the F_2 generation germ line to environmental factors. The transgenerational transmission of disease phenotypes (described in text) through the male germ line is indicated (red). Both male and female offspring develop disease, but the transgenerational phenotype is transmitted only paternally after exposure to vinclozolin. Taken from Jirtle (2007).

Vinclozolin, a commonly used fungicide in vineyards, is also an antiandrogenic endocrine disruptor. It has been used in several experiments on gestating female rats to specifically induce deleterious effects on developing F1 male embryos (2, 3). Vinclozolin binds to the developing steroid receptors within the gonadal cells, inducing an altered pattern of DNA methylation during development. The observed results of this altered methylation state are reduced spermatogenic capacity, increased spermatogenic cell apoptosis, and decreased sperm number and motility in the fully grown F1 male. These traits were successfully transmitted to three subsequent generations of male rats (3). This was the first example of an epigenetically controlled reprogramming of the male germline by

a widely used agricultural chemical.

B. Background:

1. The Nucleosome:

One of the longstanding questions in the field of biology was how the DNA in a single human cell, which measures about 2 meters in length, could be contained within a cell about 5-10 μm in diameter? This amazingly high compaction ratio is accomplished by organizing DNA into chromatin. The fundamental repeating building block of chromatin is the nucleosome core particle, which consists of 147 bp of DNA wrapped around a histone octamer. The octamer consists of a tetramer of two copies of the histones H3 and H4 each, which is bound by two H2A/H2B heterodimers. Nucleosome core particles interact with the linker histone, H1, to form the nucleosome proper, which covers about 200 bp of DNA. Nucleosomes are further organized into densely packed fibers by non-histone proteins, and ultimately compact into chromosomes. The N-terminal domains of each of the eight histones project from the nucleosome core particle, as do many of the C-termini (6). These regions of the histones are mainly subjected to posttranslational modifications, which serve as important signals in the epigenetic regulation of genome accessibility.

2. Histone Modifications:

Once thought of as static protein complexes, nucleosomes have since been discovered to be subjected to multiple post-translational modifications (PTMs) that alter the accessibility of the associated DNA. The amino acid targets for PTMs are predominantly located on the histone tails, which protrude from the nucleosome core into the surrounding milieu. Recent studies have shed light on the important role of nucleosome

modifications in diverse cellular processes such as DNA replication, transcription, and cellular differentiation (7). Histone tails can be modified in a variety of different ways. The best known types of histone modifications are methylation, acetylation, and phosphorylation (8). Considering that about 240 amino acid residues within a nucleosome are available for PTMs, the possibilities of combinatorial modifications within histones are vast. This is illustrated in a snapshot of the amino acids available for chemical modifications in the histones H3 and H4 shown in Figure 2. The identification of enzymes responsible for particular histone modifications has been the subject of intense investigation in the last 10 years (8). Figure 2 shows that several distinct classes of enzymes can modify histones at the same amino acid sites, which often function in a tissue- and

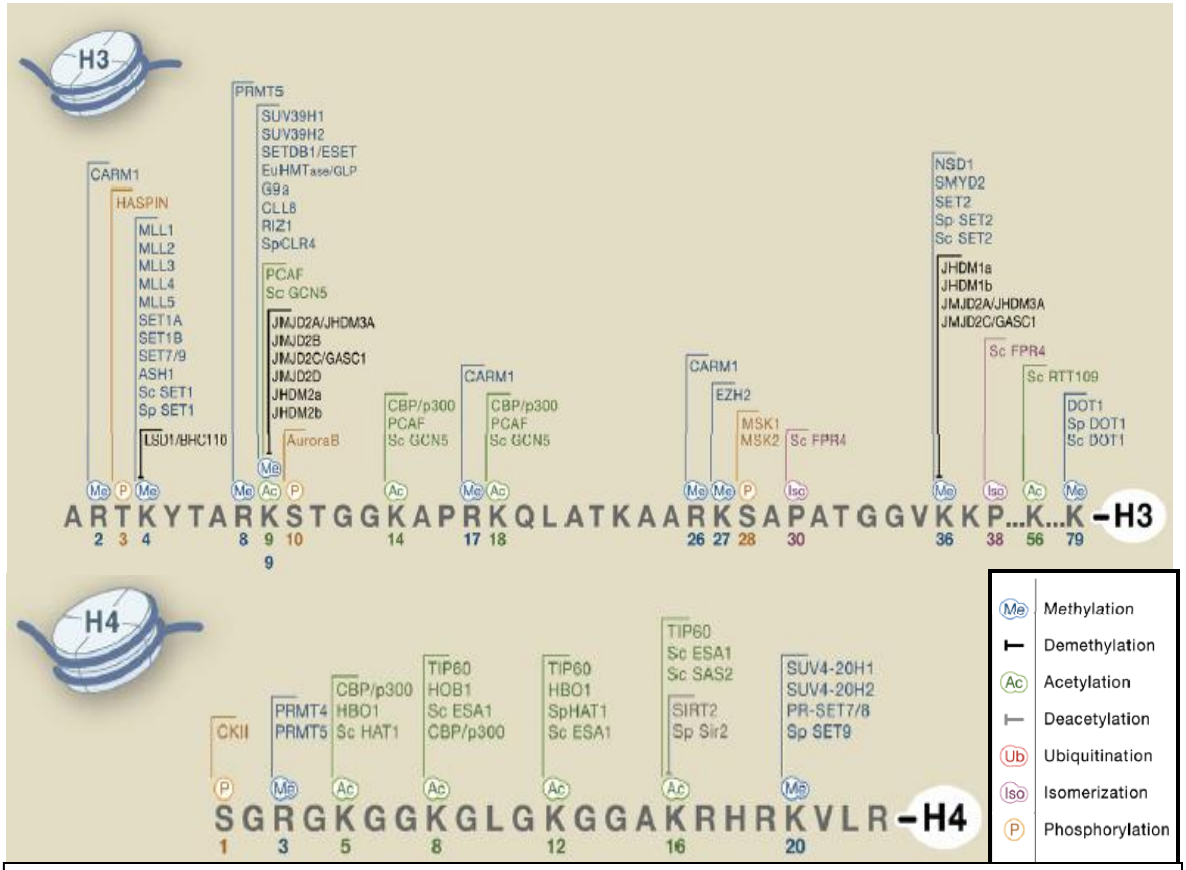


Figure 2: Map of posttranslational modification sites in histone H3 (top), and H4 (bottom). Box in bottom right shows possible modifications. Amino acid positions are given in single letter code. On top of amino acid sequences are listed modifying enzymes (colors correspond to modifications they introduce or remove; see legend). Taken from Kouzarides (2007).

stage-specific manner. This figure also shows enzymes that are responsible for the removal of certain histone PTMs, illustrating that most- but not all- epigenetic modifications are reversible. Some of these important histone modifying enzymes are described in detail below.

Acetylation on histone tails occurs at lysine (K) residues and is one of the more common modifications. This PTM is usually found at sites of active transcription although some acetylations like H4K12 acetylation are found in repressed chromatin. Histone acetylation is introduced by histone acetyltransferases (HATs), and these enzymes are commonly recruited to promoter elements by transcriptional activators (7, 9). In some cases, the activity of HATs can free up DNA from histones and subsequently make activator binding sites accessible, leading to active transcription (9). This sequence of events is an example of an epigenetic signal that frequently occurs in epigenetic mechanisms. In this instance, the PTM directly recruits other chromatin proteins that decondense DNA. It will be shown that some PTMs can alternatively be responsible for the condensation of DNA.

Histone acetylation can be reversed by histone deacetylases (HDACs), which are believed to interact with transcription repressors to silence gene expression (9). An interesting recent finding indicates that HDACs are also recruited by methyl-CpG-binding domain proteins (MBPs) to methylated CpG islands, resulting in the deacetylation of neighboring promoters. This association creates a combinatorial effect of transcriptional repression of certain genes in mammals, which will be discussed in more detail below (10).

Histones can be methylated on K and arginine (R) residues, which can result

either in gene activation or repression depending on their position within the nucleosomes and the degree of methylation (11). At Ks, up to three methyl groups can be added, while Rs can be mono- or dimethylated. Trimethylation of K is usually considered as biologically most significant and appears to have profound impact on the active or repressed state of the associated chromatin region. Three methylation sites in histone H3 are implicated in active transcription: H3K4, H3K36, and H3K79 (8). H3K4me3 marks promoters of transcribed genes in all eukaryotes, while H3K36 methylation is found downstream of these promoters. While the H3K4-specific histone methyltransferases (HMTs) were shown to interact with Polymerase II (Pol II) at promoters, H3K36 methyltransferases only interact with elongating Pol II (8). Both methylations are believed to have positive impact on transcription initiation and elongation. Little is known about the function of H3K79 methylation in transcription.

Three different K methylation sites are linked to transcriptional repression: H3K9, H3K27, and H4K20. H3K9 methylation is implicated in the formation of silent heterochromatin through recruitment of DNA methylating enzymes to the promoters of genes (8). It has been recently challenged that alternatively, H3K9 methylation downstream of promoters of expressed genes assists in transcription elongation (8). This has been explained by the fact that the generation of decondensed chromatin in the body of genes exposes cryptic promoters, from which nonsense RNAs can be expressed. H3K36 and H3K9 methylation recruit “repressive” chromatin modifiers like HDACs to mask these cryptic promoters, thereby ensuring proper transcription (12). These findings demonstrate that chromatin compaction as such is not strictly linked to gene silencing. The methylation on H3K27 sites has been implicated in the silencing of developmental gene

expression including the *Homeobox* (*HOX*) gene clusters, and as we will see, is frequently associated with disease-linked chromatin defects.

Arginine methylation is also involved in either transcriptional activation or repression. Similar to K methylation, the methylation of R sites is an important PTM involved in structural remodeling of chromatin (13). The enzymes responsible for R methylation are protein arginine methyltransferases (PRMTs), and are recruited to promoters by transcription factors (8).

While the loss of methylated histones from genomic DNA has been observed, it was assumed that these are lost due to methyl-histone exchanging factors. The removal of methyl groups from nucleosomes was considered as unlikely since K- or R-demethylation involves the formation of chemically aggressive radicals, which could damage the fragile genomic DNA. However, the recent identification of several K-specific histone demethylases (HDMs) has clarified this issue (12). The Jumonji domain-containing 6 protein (JMJD6) is the only known R demethylase to date (13). A more detailed review of the role of specific HDMs is described below.

3. The Histone Code Hypothesis:

Two specific mechanisms exist for the function of histone PTMs. The first is the disruption of contacts between structured nucleosomes in order to "loosen" chromatin, and the second is the recruitment of non-histone proteins (8). These proteins have specific

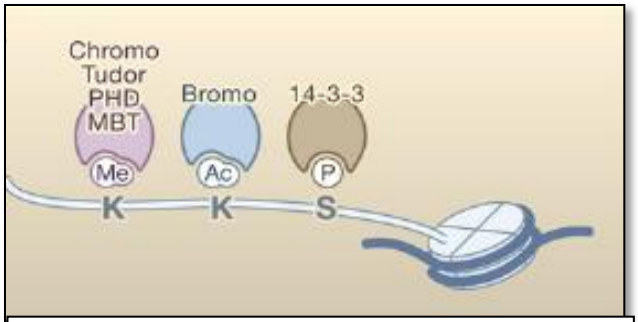


Figure 3: Recruitment of proteins to histones. Protein domains used for the recognition of methylated lysines, acetylated lysines, or phosphorylated serines on histone tails. Taken from Kouzarides (2007).

binding domains that have high affinity to particular histone modifications. Certain protein domains and the PTMs they recognize are shown in Figure 3. Histone methylation is recognized by chromo-like domains and nonrelated plant homeo domains (PHDs), acetylation is recognized by bromodomains, and phosphorylation is recognized by a domain typical for 14-3-3 proteins (8).

The domains in Figure 3 that are attracted to PTMs are subunits of multiprotein complexes that have various, and sometimes competing, control over transcription. There are two mechanisms of how PTMs can determine the outcome of the chromatin region affected. Chromatin structural proteins like the heterochromatin protein 1 (HP1) directly bind to H3K9me3 and further condense the underlying chromatin (8). Other "readers" associate with "writers" to form stable multiprotein complexes. These "writers" then introduce further modifications to already modified nucleosomes (8). The recent evidence that specific proteins are recruited to particular PTMs has led to what is called the "histone code hypothesis." Certain PTMs form a docking platform for the recruitment of subsequently acting histone modifiers that associate with the "readers" of these PTMs. This explains the often observed tight overlap of two or more PTMs within the genome. Therefore, the histone code hypothesis describes the existence of an inherited blueprint of covalent modifications within a cell, and posits that it will determine the structure and activity of various chromatin regions (9). However, the ultimate biological outputs are much more difficult to interpret than what was initially hypothesized. A better understanding of how these modifications interact through combinatorial control and how they affect cellular functions is needed to more accurately predict the outcomes of histone modifications.

4. Histone Methyltransferases:

Histone methylation is carried out by the enzymes histone methyltransferases (HMTs), which have enzymatic activity towards Rs and Ks within histones. The conserved catalytic domain of the enzymes modifying Ks has been derived from the three founding members of this protein family, namely Suppressor of Variegation, Enhancer of Zeste, and Trithorax (SET). SET domain proteins predominantly methylate Ks in the histone tails of H3 and H4, and few residues in the histone cores (14). H3K27me3, introduced above as an important repression-linked modification, is important in the early development of many organisms. This modification is dependent on specific Polycomb group (PcG) proteins that interact with the Enhancer of Zeste-type (EZ) H3K27 methyltransferases. In fact, EZ is the only H3K27 HMT identified so far, with two isoforms existing in humans, namely EZH1 and EZH2. In particular, EZH2 has been implicated in H3K27 methylation at developmentally regulated loci during early embryogenesis. Like most histone modifiers known to date, EZH2 forms a stable complex with regulatory factors assisting in its proper targeting to developmentally regulated genes. These include factors like Embryonic Ectoderm Development (EED), Suppressor of Zeste 12 (SUZ12), and others to form the Polycomb Repressive Complex 2 (PRC2) (15, 16). Different isoforms of some of these subunits can generate a number of

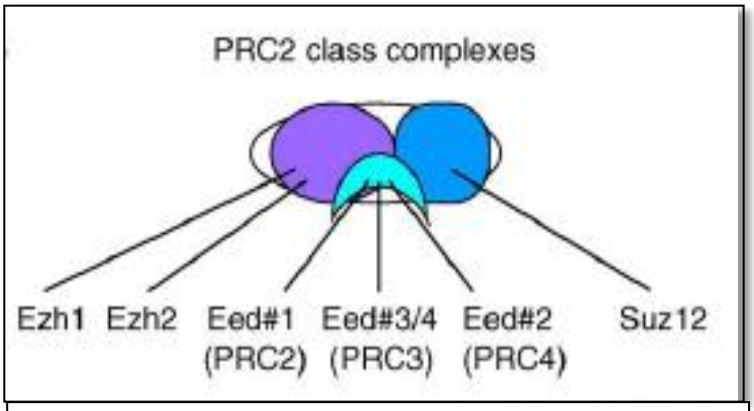


Figure 4: Complexes formed by vertebrate PcG proteins. The subunits of the core PRC2 complexes are indicated. It is likely that many combinations of subunit isoforms associate with each other to form distinct sub-complexes. The unlabelled white oval represents the fact that there are other PcG proteins, which are omitted here. Taken from Kerppola (2009).

PRC2-type subcomplexes. The general structure of PRC2 class core complexes is depicted in Figure 4.

The function of PRC2 proteins has been investigated in mice by examining phenotypes of targeted mutations in genes encoding individual subunits of the complex (17). Mutations in each of the PRC2 subunits eliminate H3K27 methylation necessary for development, causing early embryonic lethality soon after gastrulation (17). This suggests that the PRC2 complex is necessary and sufficient for the control of this developmental PTM. The H3K27me3 modifications also promote association of PRC1, another PcG

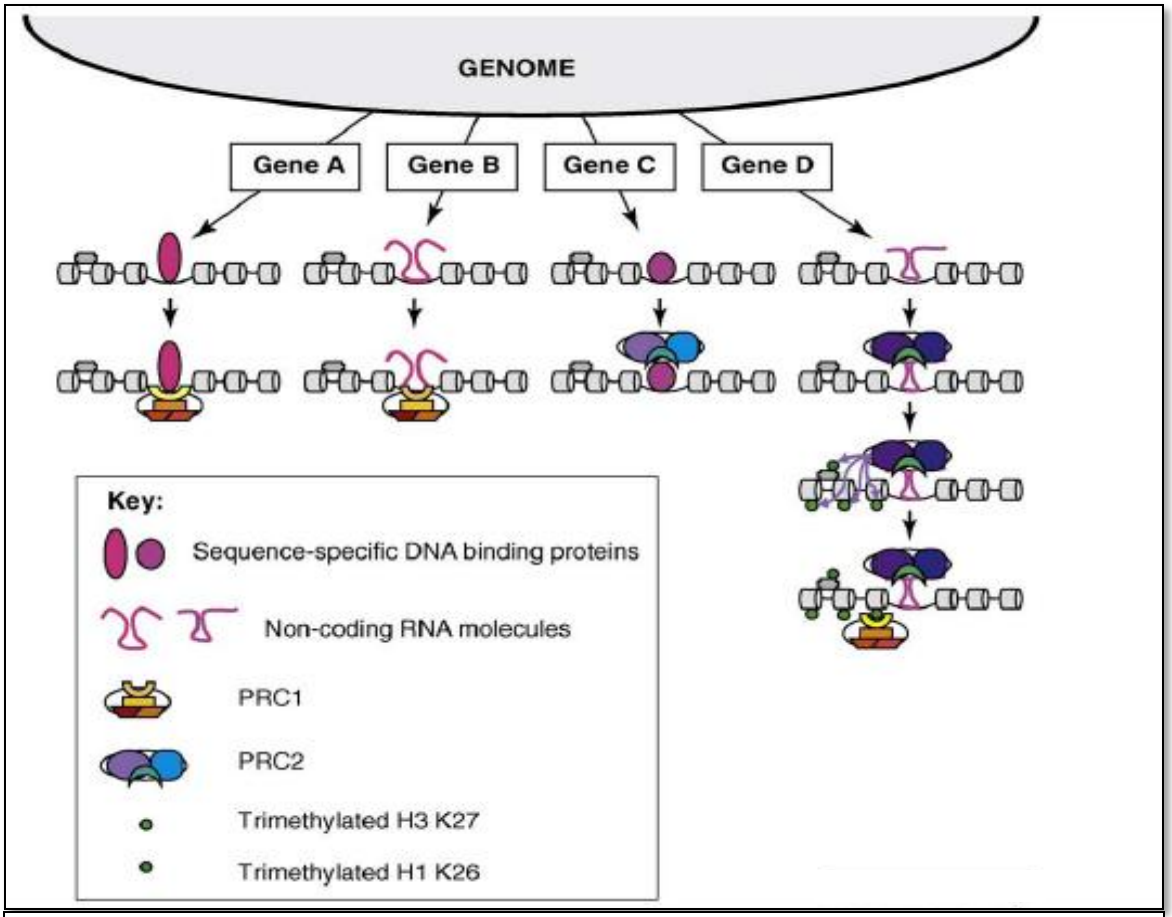


Figure 5: Speculative models for PcG protein complex recruitment to target genes. PcG proteins could be recruited by sequence-specific DNA binding proteins, non-coding RNA molecules, or a combination of the two as depicted. For the scope of this paper, the focus is on the mechanism in Gene D. The recruitment of PRC2 results of H3K27, which is well-studied. A second methylation target is depicted, H1K26 (see figure), but the role of this modification is not fully understood. As shown, H3K27me3 is suggested to be directly responsible for recruitment of PRC1. Gray cylinders represent nucleosomes and the gray bar is histone H1. Other interacting proteins are identified (see legend). Taken from Kappola, (2009).

family complex, specifically through the chromodomain of the chick homeobox (Cbx) family protein. This property has given rise to the model that methylation of H3K27 by PRC2 is required for recruitment of PRC1 to target genes (17).

In Figure 5, gene D shows a mechanism of the proposed cooperatively-binding PcG protein model. The model retains its popularity even though some evidence suggests that PRC1 binds chromatin independently of PRC2, as shown in gene A of Figure 5. Another element of the figure suggests non-coding RNA sequences as a second possible binding site for PcG proteins. More frequently, it is believed that sequence-specific DNA binding proteins such as MBPs initiate chromatin remodeling processes following DNA methylation.

The methylation of R sites by protein arginine methyltransferases (PRMTs) is performed through a common catalytic methyltransferase domain that consists of a highly conserved core region, as well as subdomains important for binding to the methyl donor and substrate (13). A comparison of domain location among various PRMTs is shown in Figure 6. The individual PRMT family members differ in distinctive N-terminal regions of variable length and combinations of other conserved domains (13). These domains vary from dual PRMT domains in some enzymes to Zn fingers, F-boxes, NosD, SH3 domains, and myristoylation domains (13).

5. Histone Demethylases:

More recently, the identification of histone demethylases (HDMs) has shown that histone methylation is not lost due to removal of histones from DNA, but in fact actively removed without disruption of nucleosomes. For example, as stated earlier, H3K27me3 has been suggested to repress transcription of *HOX* genes. The discovery of the UTX

demethylase, which is specific for H3K27me3, has revealed that removal of the trimethylation is under active control (18). UTX is required for activation of *HOX* genes and anterior-posterior identity (18). Interestingly, UTX has been suggested to interact with the CREB-binding protein (CBP), the H3K27-specific HAT. Following the demethylation of H3K27, this residue can be subsequently acetylated by CBP. This HDM-HAT association presents an example of combinatorial control as proposed by the histone code hypothesis. The idea that one single PTM can yield a particular transcriptional state has become very unlikely. Rather, combinatorial histone modifications, along with DNA methylation, more likely lead to discrete transcriptional states.

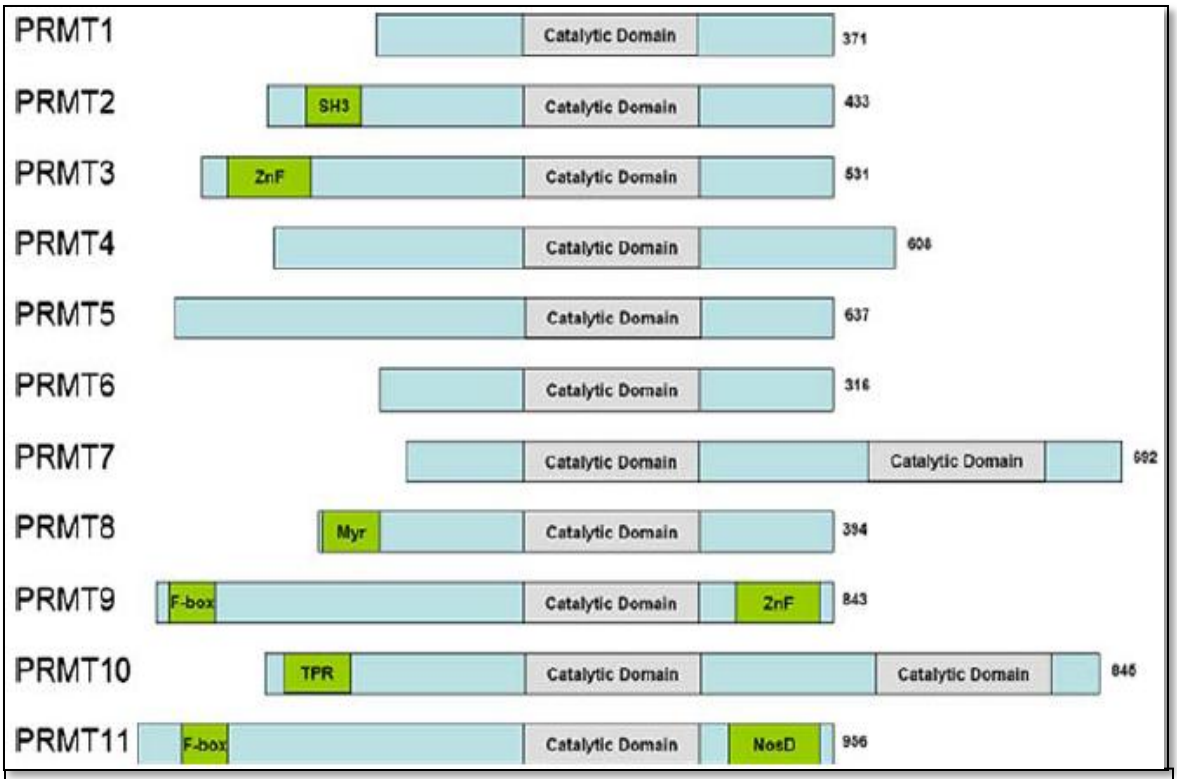


Figure 6: The human protein arginine methyltransferase (PRMT) family. The different members are indicated as boxes and the protein length by the number of amino acids. Only the longest isoforms are shown. All PRMTs have at least one conserved catalytic domain (gray box). Different additional domains are highlighted in green: *SH3*, *ZnF* (zinc finger), *Myr* (myristoylation), *F-box*, *TPR* (tetratricopeptide), *and NosD* (nitrous oxidase accessory protein). Taken from Wolf (2009).

It is important to note that there are other mechanisms known to alter gene expression, such as ATP-dependent chromatin remodeling and the incorporation of histone

variants within nucleosomes. An epigenetic mechanism implies the inheritance of a chromatin state from mother to daughter cells. ATP-dependent chromatin remodeling merely changes the position of nucleosomes or their densities. Then, during replication, the gaps are filled and nucleosome positions might be altered. Therefore, these changes are not known to be permanent or inherited. This is different from PTMs and histone variants, which really change the nucleosome composition. Detailed reviews of the mechanisms not discussed are referenced (7, 19).

6. DNA Methylation:

Methylation of cytosine (C) in CpG repeat-rich elements is considered to be the one of the most important epigenetic traits in the regulation of transcriptional repression in mammals. Methylation within promoter regions attracts proteins called methyl-CpG-binding domain proteins (MBPs), which act to prevent transcription factors from binding to promoter sequences (7). These proteins can also recruit histone modifying enzymes that alter nearby chromatin. DNA methylation is therefore normally implicated in marking sites for chromatin compaction and gene silencing. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), particularly DNMT3A and DNMT3B, as well as DNMT1, which are described in further detail below (11). Sites of methylation within the C residues are shown in Figure 7.

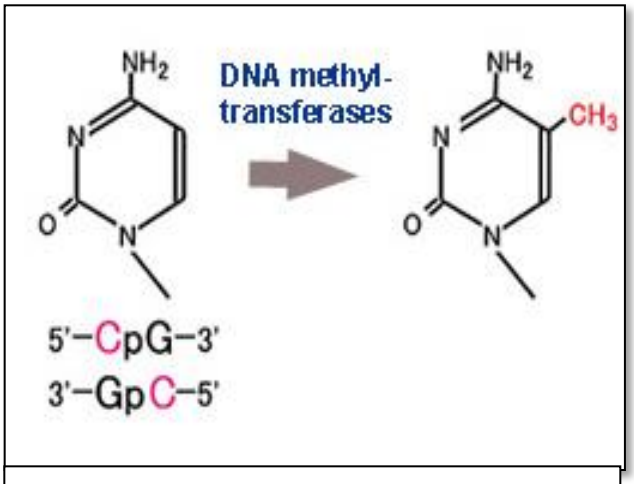


Figure 7: Methylation by DNA methyltransferases at CpG islands. DNMTs attack the 5' carbon of the cytosine residue. Taken from Taylor (2006).

7. CpG Islands:

CpG "islands" are called islands because they are usually found within stretches of DNA that have very high frequencies of C-G dinucleotide repeats. They also occupy approximately 60% of human gene promoters (11). Figure 8 shows an example of CpG island methylation between normal sites of transcription and transcriptionally repressed DNA sequences. As shown, the aberrantly methylated sites are often observed in diseases such as cancer. Also, the crucial areas affected by high frequency CpG sites are in promoter regions and transcription start sites, supporting that the methylation defects are indicative of changed transcription states of the affected genes.

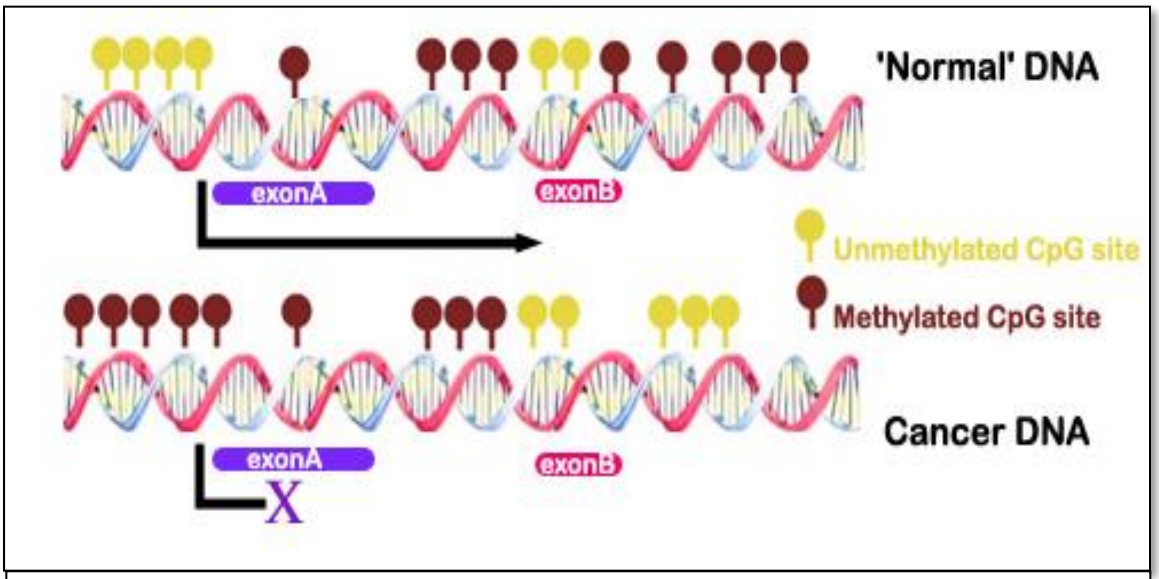


Figure 8: Tumor cells are characterized by hypermethylation of CpG islands. A typical CpG methylation pattern in DNA sequences of a normal (top) and a cancer cell (bottom). Yellow circles are unmethylated C's, and maroon circles are methyl-C's. DNA double helix is shown as white and pink alternating strands. In cancer cells, the distributions of unmethylated and methylated C's are changed. Taken from Taylor (2006).

8. DNA Methyltransferases:

As shown in Figure 7, the specific site of methylation within DNA sequences occurs at the 5 position on C residues, which is nearly always accompanied by a 3' G residue. The specific enzymes that catalyze this reaction are appropriately called DNA methyltransferases (DNMTs). The mechanism of methylating DNA involves the DNMT

catalytic domain to induce base flipping, everting the C residue out of the helix to insert into the active site of the enzyme. This allows association of the base with the methyl donor, S-adenosyl-L-methionine (SAM), and the formation of 5-methylcytosine (20, Figure 7). Although the mechanism is similar for different DNMTs, they appear to have different responsibilities in various developmental stages within organisms. DNMT1 is responsible for the maintenance of correct methylation while the roles of DNMT3A and DNMT3B are to establish the *de novo* methylation patterns in embryos (19).

DNMTs are widely conserved among eukaryotes, and there are three major types used for regulation of normal DNA methylation. The major function of DNMT1 is in the maintenance of methylation patterns, therefore its function is essential in cellular environments such as S-phase of the cell cycle and in the initiation of genome wide methylation in early development. The importance of DNMT1 has been revealed by targeted mutation of the *DNMT1* gene. In a past study, embryonic stem (ES) cell lines were used to create homozygous mutant *DNMT1* cells by gene targeting (21). The mutant cells were viable and showed no deviation from normal growth rate or morphology, but Southern blot analysis revealed that DNA methylation was reduced to about one-third that of normal cells (21). The introduction of the *Dnmt1* mutation into a mouse germ-line however resulted in a recessive lethal phenotype. Homozygous embryos were stunted, showed delayed development, and did not survive past mid-gestation (21). These experiments suggest that although there is only a reduced amount of DNA methylation in *DNMT* $^{(-/-)}$ ES cell lines, the role of DNMT1 in organisms is essential for development and embryonic viability.

Two other DNMTs; DNMT3A and DNMT3B, are also important in the

methyltion of DNA within an organism. Similar to the transgenic *Dnmt1* experiments in mice, the targeted disruption of one or both of Dnmt3a and Dnmt3b results in embryonic lethality (19). In addition, germ-line mutations of *DNMT3B* have been associated with the Immunodeficiency, Centromeric Instability, and Facial Abnormalities (ICF) syndrome (22). Patients suffering from ICF exhibit mild facial anomalies, variable reductions in serum immunoglobulin levels, as well as chromosomal abnormalities in lymphocytes (22). The loss of methylation at specific centromeric regions and profound chromosomal structural changes that results from ICF indicates that DNMT3A and DNMT3B are critical for normal cellular function.

9. Correlation between DNA and Repressive Histone Methylation:

It is widely accepted that both DNA methylation and histone modifications have important contributing factors in the regulation of transcriptional control (7, 16). It is believed that the methylation of CpG islands within promoter regions of DNA is probably functionally equivalent and a specialized regulatory addition that exploits repressive histone methylation and/or deacetylation specifically in mammals. As outlined above, these PTMs usually contribute to the condensation of chromatin and repression of transcription. It has been reported that the methylation sites on the DNA attracts HMTs that cause these PTMs via associated methyl-DNA binding proteins (MBPs) (8, 11). One of the latest reported epigenetic mechanisms linked to non-methylated CpG islands is that they are frequently enriched for histone PTMs linked to active transcription such as H3K4 di- and trimethylation (23). Researchers analyzed the genome-wide distribution of a protein in question, namely CXXC finger protein 1 (Cfp1), using high-throughput DNA sequencing of immunoprecipitated DNA (ChIP). The Cfp1 protein, part of the Set1 H3K4

methyltransferase complex, showed association to 81% of non-methylated CpG islands (23). Additionally, ChIP sequencing with antibodies recognizing methylated H3K4 showed that 93% of Cfp1-bound CpG islands possessed the specific PTM (23). The introduction of shRNA directed against *Cfp1* reduced levels of the protein in mouse brain cells, which correlated with a near complete loss of H3K4 methylation across the genome. These studies suggest the HMT Cfp1 is recruited to non-methylated DNA CpG sites. Subsequently, the methylation of H3K4 is promoted, leading to transcriptional activation. As this and other experiments suggest, there is no single modification linked to a specific cellular response, but rather combinations of various modifications. As another example, both DNA methylation and histone modifications play integral roles in the development of organisms, described next.

B. Developmental programming:

In early development, genome-wide changes in methylation patterns allow organisms to maintain specific transcriptional states necessary for pluripotency (24). This pattern is established by demethylation of DNA and methylation of specific histone sites that repress transcription of many developmental genes (24). As the organism progresses through successive stages of development, histone modifications and DNA methylation become more differentiation-specific. The patterns of methylation are established based on the distinct cell lineages that occur in the various areas within the organism. An outline of epigenetic patterns of DNA methylation and important histone modifications during early development is presented in Figure 9.

During embryonic development, the patterns of global histone modifications change drastically (Figure 9). The genome-wide removal of DNA methylation in very

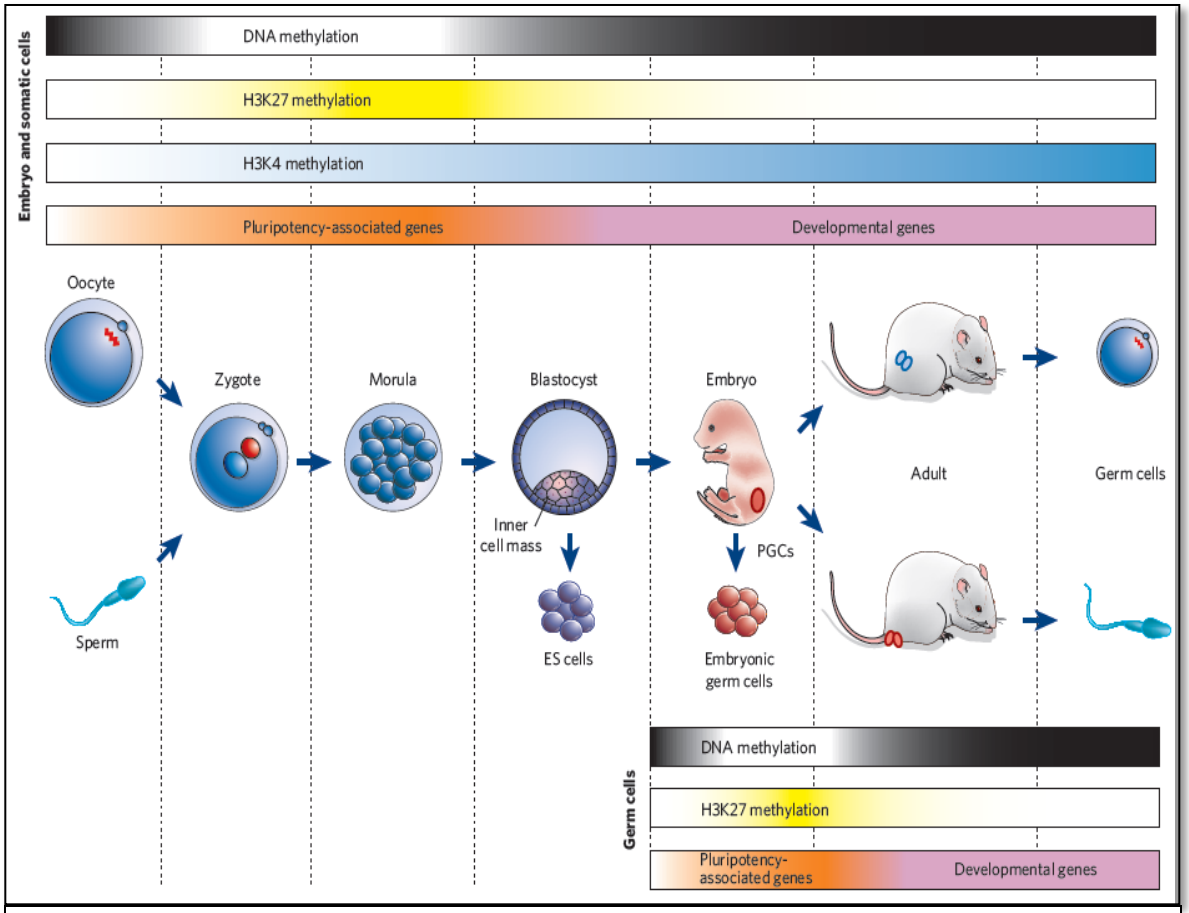


Figure 9: Epigenetic gene regulation during mammalian development. Key developmental events are shown together with global epigenetic modifications and gene-expression patterns. Darker sections within each bar represent higher frequencies of expression. See text for details. Taken from Reik (2007).

early development occurs upon fertilization. As the zygote passes through the morula stage and into a blastocyst, DNA methylation patterns are re-established by DNMT1 (24). The increase in H3K27 methylation during this same developmental stage helps temporarily regulate transcription repression while DNA methylation patterns are being re-established (24). This specific combination of methylation patterns allows expression of pluripotency-associated genes to preprogram embryonic stem (ES) cells preceding development into the embryo. In early phases of development, H3K4 methylation increases. As H3K4 methylation increases, the activity of pluripotency-associated genes goes down, while a large number of developmental genes are up-regulated. At later stages of development, H3K4me3 levels drop, while higher levels of DNA methylation and H3K27me3

are found. In the beginning there is not much transcription, while boosts of transcription occur during gastrulation and specification of tissues. Later during development, only specific subsets of developmental genes are expressed that regulate differentiation. Differentiation is also accompanied by chromatin compaction and silencing.

It is imperative that the epigenetic mechanisms described above work together to perform positive regulation of transcription. Of course, there are instances that result in altered epigenetic modifications. These irregular changes are frequently associated with the misregulated methylation of both DNA and histone sites. These changes have the potential to give rise to very detrimental cellular states, and can have very negative physiological effects. As we will see later, irregular epigenetic modifications contribute to diseases such as cancer, as well as various neurological disorders.

C. Implications for disease:

The development of genetic diseases has historically been credited to inherited mutations in the germ line or direct changes in DNA sequences in the cells of an individual during its life. These mutations affect the expression of particular genetic information, and the ultimate effects are responsible for diseases including cancer, as well as neurological disorders such as schizophrenia. The study of epigenetics has shown that the aberrant methylation of DNA and modifications on histones can mimic the effects of DNA mutations. But how is the dysregulation of the epigenome carried out?

1. Environmental exposure:

As described previously, the agricultural fungicide vinclozolin is just one example that has provided evidence that various environmental factors induce altered epigenetic marks, making these individuals susceptible to future disease. In many of these

instances, epigenetic changes are inherited mitotically in reproductive cells, thus presenting a model by which environmental effects on the epigenome can induce long-term effects on gene expression (16). Numerous animal studies indicate that both prenatal and early postnatal environmental factors, such as the example using vinclozolin, result in altered epigenetic programming and consequent
risk of disease.

2. Role of miRNA:

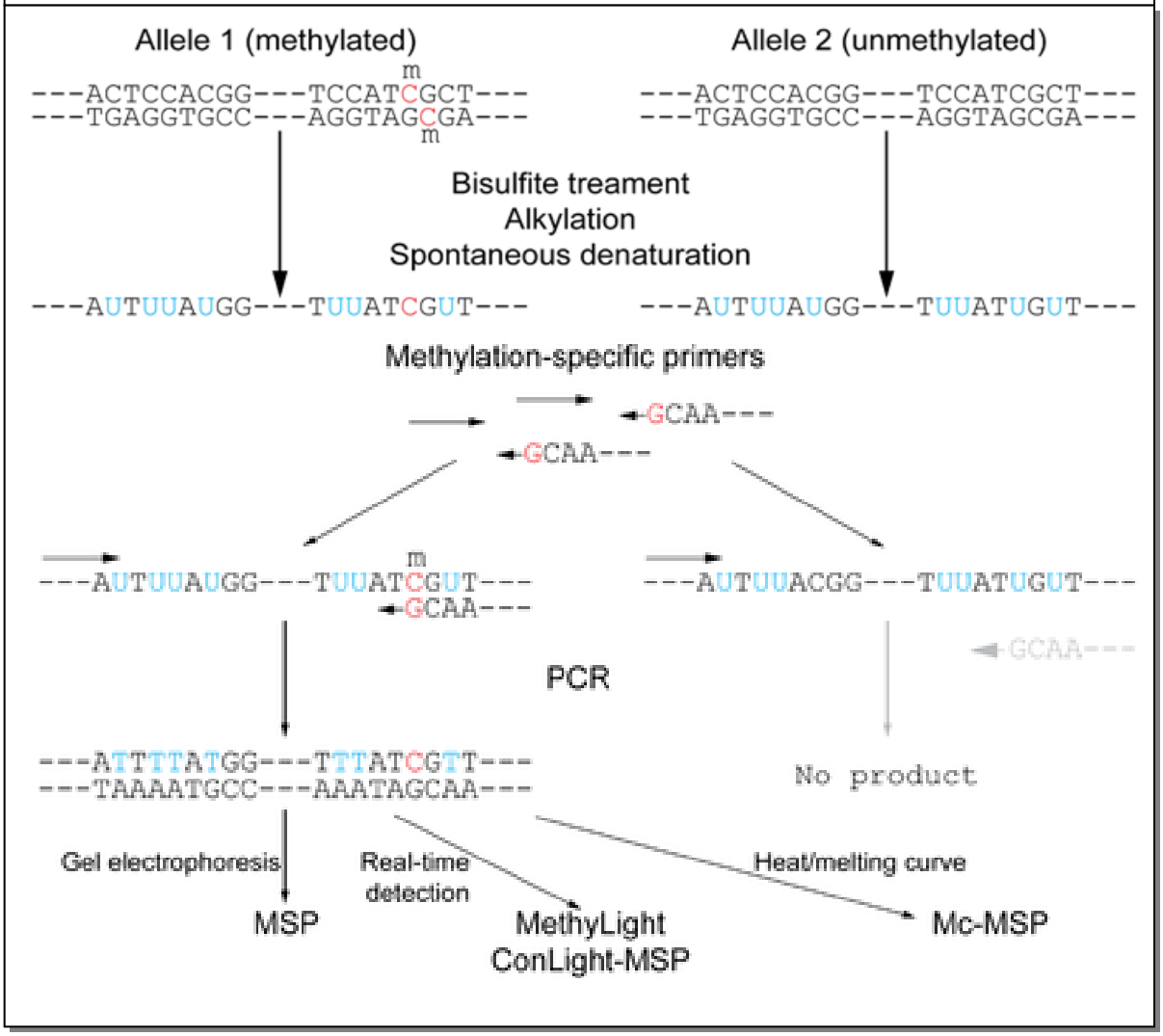
Small, non-coding RNA sequences called microRNAs (miRNAs) have been extensively studied, and have been found to effectively induce posttranscriptional silencing of target genes (11). Most miRNAs have the ability to do this by sequence-specific base pairing with 3' untranslated regions of target messenger (mRNA), resulting in the degradation of the target mRNA or inhibition of translation (25). miRNAs function to influence several biological processes including cellular differentiation, apoptosis, and cell proliferation. Through recent studies involving miRNA molecules, they appear to control epigenetic regulatory mechanisms by targeting such enzymes as DNMT3A and DNMT3B, responsible for DNA methylation, as well as EZH2, responsible for histone methylation (26, 27). miRNAs are not the sole effectors of epigenetic regulation, being themselves targets of regulation by epigenetic mechanisms (28).

3. Aging:

As stated above, there are specific enzymes responsible for the methylation of DNA and histones at various stages within the life of cells. Unlike the highly proficient capabilities of enzymes like DNA polymerase to check and correct errors during DNA replication, methyltransferases lack the components to provide such efficient correction

methods. This has been proposed to lead to random epigenetic "drift", and is consistent with the observation that an increase in an individual's age has a direct and linear relationship to the increase in aberrant DNA methylation within the organism (29).

Figure 10: Methylation-specific polymerase chain reaction (MSP). Once bisulfite treatment modifies all unmethylated cytosine residues into uracil, primer pairs are designed to be "methylated-specific," including sequences complementary to unconverted 5-methylcytosines. Quantification of methylation is determined by the ability of the specific primer to achieve amplification. Taken from AbsoluteAstronomy.com (2010).



An example of this correlation between epigenetic methylation and aging has been seen in studies of estrogen receptors in vascular tissue, based on the finding that the epigenetic DNA methylation of promoter sequences increases with age (30). In the study, coronary atherosclerotic tissues showed significantly higher methylation levels

within the *estrogen receptor β (ERβ)* promoter sequence than normal arterial tissues. Additionally, human aortic endothelial and smooth muscle cell lines of varying age were cultured *in vitro*. DNA methylation can be quantified by a procedure called methylation-specific polymerase chain reaction (MSP). This is a novel PCR method, in which bisulfite treated DNA chemically modifies all unmethylated C residues into uracil, as shown in the diagram in Figure 10. Primers designed to distinguish between modified and unmodified DNA were used to initiate the normal PCR process, giving a quantitative measure of the amount of methylation within a specific sequence. This second step is shown in the bottom portion of Figure 10 (30). MSP analysis showed that the older cell lines showed higher levels of *ERβ* promoter methylation (30). Furthermore, when the cell lines were treated with 5-aza-2'-deoxycytidine (DAC), a known DNA demethylating agent, the demethylation resulted in increased ERβ expression. The cell lines were also treated with the deacetylase inhibitor trichostatin A (TSA), and the increased expression of ERβ receptors was further enhanced (30). These results illustrate the unique reversible capability of deleterious epigenetic methylation, a key therapeutic factor that will be discussed in detail later.

Studies of monozygotic twins have also showed evidence that epigenetic methylation is relative to the aging process of an organism. These "identical" twins have exactly the same DNA at birth, but as they grow, they experience a substantial rate of discordance. The different types of disease within twins have ranged from diabetes and autism in young to heart disease and cancer in old (29). In one study of monozygotic twin girls, one had developed a duplicated section of spinal vertebrae in the posterior portion of her body. This duplication is similar to a known mutation in mice at the *axin inhibitor (axin)*

locus (31). *Axin* encodes an inhibitor of the Wnt-signaling pathway, and the mutant dysregulates embryonic axis formation in mice by the formation of bifurcated tails. Coding sequences for the human gene, *AXIN1*, in each twin revealed no mutation (31). Consequently, researchers examined methylation near the promoter sequence of the gene. In the twin with the spine defect, significantly higher levels of methylation were present in the promoter region. Through *in vitro* experiments, the promoter of the *AXIN1* gene was demonstrated to contain a CpG island that, through methylation, inhibits its activity (31).

There is rarely one singular molecular cause of disease development, but instead several biological factors must usually do wrong to induce such a state. These essential factors include aberrant DNA methylation and histone modifications. Recent research has begun to explain how epigenetic methylation has contributed to the detrimental cellular fates, particularly in the development of cancer (11). Additionally, the correlation of increased DNA methylation with the development of neurological disorders has become increasingly apparent (32). It is essential, as we will see, to understand the mechanistic actions that induce such states in order to develop treatment methods. In the following, I will give a few detailed examples for the experimental confirmation of DNA and histone methylation defects in cancer and neurodegenerative diseases. I also will discuss the interpretation of the observed defects in relation to the disease.

II. Examples of Epigenetic Contribution to Human Diseases:

A. Cancer:

1. Tumorigenesis:

In 1971, Alfred Knudson proposed a theory that is referred to as the "two-hit model," which predicts that both alleles of a gene must be inactivated in order to induce a phenotypic consequence of cancer-related genes (11). The genetic mutation within a promoter region or first exon of a particular gene on one allele would constitute the first hit. Knudson suggested that this includes the mutation or deletion of coding regions within essential cancer regulators, tumor suppressor genes and proto-oncogenes. Although the mutation of one allele of a gene usually maintains a stable state of expression, it is now recognized that the second hit in the model can occur from methylation on an essential coding or regulatory region of the remaining allele (19). These epigenetic changes can work in conjunction with the original genetic mutations that, in combination, initiate tumorigenesis (11).

Recent studies have shown evidence of the important link between epigenetic methylation and cancer formation in an organism. For example, there are instances where epigenetic aberrations precede DNA mutation, generating the initial mechanism (or first hit, according to Knudson) in the initiation of cancer (19). Specific examples, as described below and listed in Table 1, provide support that in these cases, epigenetic DNA and histone methylations correlate with mutations that are linked to a cancerous outcome (19). In the following, I will give a few examples of epigenetic aberrations linked to hereditary diseases or cancers and discuss their molecular etiology.

a) O^6-methylguanine-DNA methyltransferase:

K-ras, a key player in early signal transduction pathways, encodes a proto-oncoprotein essential for normal tissue signaling. The most common *K-ras* mutation is based on G to A transitions in multiple codons within its coding sequence. This is an oncogenic mutation found in about half of colorectal carcinomas (33). In animal models, the G to A transition is induced by the chemical carcinogen, *N*-methyl-N-nitrosourea (*N*-MNU), which creates the modified nucleotide O^6-methylguanine. Read as adenine by DNA polymerase, this modified base causes the G to A transitions. It is important to note that there is not a targeted mutation in *K-ras* here, but rather mutations occurring all over the genome, including the target. The particular result is a random outcome from exposure to the drug in the animal model.

In normal cells, a DNA repair protein, O^6-methylguanine-DNA methyltransferase (MGMT), is responsible for correcting modified bases such as O^6-methylguanine. *In vivo* the hypermethylation of the promoter sequence of the *MGMT* gene, though, decreases expression of the repair protein, resulting in uncontrolled *K-ras* mutations. In a particular study, a series of 244 colorectal lesions and 179 carcinomas were analyzed, and approximately 71% showed hypermethylation of *MGMT* in relation to *K-ras* mutations (33). The amount of methylation was measured using MSP (Figure 10).

In addition, reverse transcription (RT)-PCR revealed that in 19 colorectal adenomas in which *MGMT* methylation occurs, little or no detectable *MGMT* mRNA was found. In 10 other colorectal adenomas with no CpG methylation defects in the *MGMT* regulatory regions, researchers detected normal levels of *MGMT* mRNA. This suggests that the aberrant CpG methylation of the *MGMT* gene decreases mRNA expression, which is likely to be causal for subsequent *K-ras* mutations and the formation of

colorectal carcinomas.

The experiments conducted here were very straight forward, and the reported results were qualitatively represented. The group provided good evidence supporting that the specific epigenetic aberrations in *MGMT* regulatory regions reduces its expression and ability to control subsequent mutations on the *K-ras* gene, which codes for the important proto-oncogene and tissue signaling protein K-ras.

b) Cyclin-Dependent Kinase Inhibitor 2A/B:

The dysregulation of cell cycle control is a problem observed in many neoplasms. Two regulatory genes, *CDKN2A* and *CDKN2B*, which encode proteins p16 and p15, respectively, function in the inhibition of the phosphorylation of a key cell cycle regulator and proto-oncogene, retinoblastoma (Rb). Rb controls the activity of the activator E2 transcription factor (E2F) protein by binding to it and both blocking activation and recruiting a deacetylase enzyme that represses its target genes. Phosphorylation of Rb causes release of this protein from E2F, and thus activation of E2F target genes (34). The deletion or specific point mutations of these genes results in uncontrolled cell cycle progression (34), and is present in various forms of hematological malignancies. Based on the results of several studies, however, it has become apparent that the formation of these malignancies is more frequently due to hypermethylation of their promoter sequences (35). Using Southern blot hybridization with probes for the 1st exon of either *p15* or *p16*, researchers were able to analyze both CpG island methylation and deletion of promoter sequences. In any of 87 cases of acute myeloid leukemia (AML), the group observed no homozygous deletions of either *p15* or *p16* genes (35). However, it was found that hypermethylation of *p15* occurred in 88% of adults and 67% of children with AML.

Only one patient showed hypermethylation of both *p15* and *p16*.

The group next examined inactivation of *p15* and *p16* in another type of hematological malignancy, namely non-Hodgkin's lymphoma (NHL). In 25 cases, *p15* was inactivated by homozygous deletion in a single sample, while hypermethylation of *p16* was observed in 12 samples (35). An interesting observation made by this group was that in low-grade NHL cases, hypermethylation of *p16* occurred 9% of the time, while in high-grade NHL cases, hypermethylation of this gene occurred 83% of the time (35). This suggests that hypermethylation of important cell regulating genes may be a key trigger in the progression of hematological malignancies.

The analyses of *p15* and *p16* hypermethylation in this paper were performed on cell lines of various hematological malignancies. Although all of the results indicated significant hypermethylation in the promoter sequences of *p15* or *p16* genes, some experiments lacked convincing qualitative results due to the size of samples analyzed. Perhaps the researchers could have analyzed a greater number of samples, which may have strengthened their conclusions. Another method of testing the physiological effects of hypermethylated CpG islands of particular genes is the introduction of a known demethylating agent, 5-aza-2'-deoxycytidine (DAC).

c) Ras Association Domain Family 1 Isoform A:

The allelic loss of human chromosome 3p21.3 is correlated with the formation of many types of cancer, including lung and breast cancers (36). This particular chromosomal region has thus become a popular target for the search for new tumor suppressor genes. In such studies, the *Ras association domain family 1 isoform A (RASSF1A)* was found in this region. Indeed, it was found to be located here upon genomic sequencing

(36). The gene encodes a protein with a currently unknown function. Evidence points to its potential as a Ras-signaling protein, possibly functioning in DNA damage response. RASSF1A is expressed in normal lung and breast epithelial cultures. Using RT-PCR, researchers showed highly reduced expression of RASSF1A in both lung and breast can cer cell lines (36). By sequencing sodium bisulfite-modified DNA from eight lung can-

Table 1. Genes subject to promoter sequence hypermethylation

Gene	Function	Sequence affected	Form of cancer induced
MGMT (O^6-Methylguanine DNA methyltransferase)	DNA repair of adducts (O^6-methylguanine)	CpG islands within promoter sequence	colorectal carcinoma
CDKN2A (Cyclin-dependent kinase inhibitor 2A)	p16/p14 expression	CpG islands within promoter sequence	non-Hodgkin's lymphoma, Burkitt's lymphoma
CDKN2B (Cyclin-dependent kinase inhibitor 2B)	p15 expression	CpG islands within promoter sequence	acute myelogenous leukemia, acute lymphocytic leukemia, Burkitt's lymphoma
RASSF1A (Ras association domain family 1 isoform A)	unknown (possible roles in DNA damage, cell cycle arrest, apoptosis)	CpG islands within promoter sequence	lung cancer, breast cancer
hMLH1 (human mutL homologue 1)	DNA mismatch repair	CpG islands within promoter sequence	colorectal cancer, endometrial cancer

cer cell lines, MSP analysis (Figure 10) showed methylation of nearly every CpG dinucleotide within the promoter region. In addition, genomic DNA removed from a large number of primary non-small cell lung carcinomas, resected primary breast cancers, or 22 breast cancer cell lines showed abnormally high levels of CpG methylation in the promoter region of *RASSF1A* (36). In contrast, no methylation of the gene was found in 104 nonmalignant lung tissue samples (36). These studies showed that the hypermethylation of the *RASSF1A* promoter sequence correlated to breast and lung cancers. In these studies, the hypermethylation of CpG islands in the otherwise normal *RASSF1A* locus

caused phenotypes similar to those observed in patients with chromosomal deficiencies. This underscores the importance of proper DNA methylation levels and patterns in normal cellular behavior.

d) Human mutL Homologue 1:

DNA mismatch repair is an important cellular checking system that helps prevent deleterious genetic mutations and subsequent disease states within an organism. There are at least five genes required for DNA mismatch repair (37). The mutation of genes within the repair system, specifically *human mutL homologue 1 (hMLH1)*, gives rise to hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, which exhibits high risk to develop colon cancer in the affected individuals. Studies on the *hMLH1* locus have identified regulatory regions that were particularly sensitive to aberrant DNA methylation. Sequence analysis showed that the promoter region of *hMLH1* is high in CpG dinucleotides (37), similar to all previously discussed genes. To detect possible mutations in the sequence, the entire gene was sequenced from genomic DNA of two tumor cell lines (37), the colon tumor cell line SW48 and the endometrial tumor cell line AN3CA. Both cell lines showed abnormally low levels of hMLH1 expression. There were no mutations present within any regions of the *hMLH1* gene. The authors next tested for changes in DNA methylation as a likely factor in the reduced expression of hMLH1 in cell lines.

A combination of restriction analysis with DNA methylation sensitive endonucleases and PCR assays was used to examine the promoter region of *hMLH1* in SW48 and AN3CA cell lines, along with four control cell lines (37). Researchers observed that hMLH1 promoter DNA extracted from the two cancer cell lines was resistant to digestion

using the methyl-DNA sensitive restriction endonuclease, *HpaII*, but were sensitive to digestion by the restriction endonuclease *MspI*. The second endonuclease used, *MspI*, was not DNA methylation sensitive. In all cases, the promoter region of control cell lines was sensitive to digestion by *HpaII* and *MspI* (37). The results of the PCR assay suggest that the lack of digestion in tumor cell lines is due to DNA methylation on important promoter sequences, which may also explain the lack of protein expression of hMLH1. These findings present an alternative to the previously held idea that all DNA mismatch repair inactivation was influenced by inherited DNA mutations.

2. Sporadic forms of cancer:

Evidence for the genetic predisposition to certain cancers exists, specifically because germ-line mutations of the DNA of an individual are inherited by future generations. These genetic predispositions to cancers are referred to as familial forms of cancer. There are instances in which non-familial cases of these cancers result, and the underlying cause has been suggested to involve epigenetic silencing. The genes predicted to play roles in sporadic cancer formations are described below and outlined in Table 2.

a) von Hippel-Lindau:

The *von Hippel-Lindau (VHL)* gene encodes the tumor suppressor pVHL protein. The protein blocks the metabolic pathway leading to vascularization, and its inactivation is considered to be a major contributing factor to renal cell carcinoma (RCC) (38). The pVHL protein acts as a substrate recognition component of an E3 ubiquitin ligase complex. Under normal oxygen conditions, a family of transcription factors called hypoxia inducible factor (HIF)-α subunits are hydroxylated, recruited by pVHL to the E3 complex for ubiquitylation, and subsequently degraded (38). Under low oxygen conditions or by

the inactivation of the *VHL* gene, the HIF-α subunits are stabilized. This induces the transcription of several hypoxia responsive genes, such as vascular endothelial growth factor, glucose transporter 1, and prolyl hydroxlase family member Egln3 (38). These genes induce the highly vascular phenotype of RCC.

The non-familial nature of RCC in patients led researchers to look for the specific cause(s). Examination of the first exon of the *VHL* gene was conducted using Southern blot analysis with restriction enzymes sensitive to methylation. The results revealed specifically high methylation on CpG sites within the promoter region (39). To determine if methylation occurred prior to cell line culture and metastasis, investigation of DNA was performed on cell lines from early stages of cancerous tissue samples including primary tumor and metastatic lymph node cells from six patients (39). The same intensity of DNA methylation was seen throughout the various cell lines, and similar hypermethylation was also found between the primary tumor and metastasized lymph node from individual patients. These results indicated that the methylation precedes both culture and metastasis of RCC, making it a likely early event during tumorigenesis rather than a secondary event.

RNase protection analysis uses a probe to detect RNA expression levels within cells. In this study it showed that all cells with heavily methylated *VHL* promoters did not express pVHL, while normal kidney cells and unmethylated but mutated cancerous *VHL* cell lines showed significant expression of *VHL* mRNA (39). The difference suggested that in contrast to the generation of a nonfunctional protein generated from a mutated gene, aberrant methylation of promoter regions prevented *VHL* transcription altogether.

b) Breast Cancer 1, Early Onset:

When molecular geneticists discovered that there were no mutations identified in many of the sporadic cases of *Breast Cancer 1, Early Onset (BRCA1)*, they were perplexed. Germ-line mutations in the BRCA1 gene are responsible for about 50% of familial breast cancer cases (40). The gene encodes a protein that acts in DNA repair through association with the RAD51 and BARD1 proteins. The dysregulation of other repair mechanisms that gave rise to cancers, specifically *hMLH1* and *p16* (discussed above) led to studies showing aberrant epigenetic silencing of the aforementioned genes in tumors. This led researchers to conduct similar experiments on expression of the *BRCA1* gene (40).

MSP analysis revealed that the *BRCA1* promoter was not methylated in normal lymphocytes, breast, ovary, lung, colon, and liver. In comparison, examination of a series of breast cancer xenografts in immunodeficient mice revealed complete methylation of the *BRCA1* promoter, specifically at multiple CpG sites (40). RT-PCR showed that BRCA1 transcripts were not detectable in methylated xenografts. In the same study, methylated promoter regions were detected within primary breast carcinomas. These methylated regions occurred in concert with mutations of the *BRCA1* sequence (40). These findings support the idea of synergistic effects contributing to an enhanced deleterious state, because methylation was observed more commonly in tumors of 2 cm or greater. The presence of epigenetic methylation defects was also observed in several primary ovarian carcinomas (40). Results indicated that in about one-third of sporadic cases of ovarian cancer, *BRCA1* promoters are hypermethylated (40).

c) Liver Kinase B1:

Liver kinase B1 (LKB1), also known as *STK11*, is a gene that when mutated, results in Peutz-Jeghers syndrome (PJS). PJS is characterized by mucocutaneous pigmentation and the predisposition to tumorigenesis in the gastrointestinal tract, breast, testis, ovary, and cervix (40, 41). The broad range of defects in various tissues occurs because the *LKB1* gene codes for a widely expressed serine/threonine kinase with function in many tissues. As a monomer, LKB1 localizes to the nucleus, but binding to its interaction partners STRAD and MO25 cause it to be exported into the cytoplasm and function as a fully active kinase complex (41). There are several phosphorylation targets of LKB1 with distinct roles. AMP-activated protein kinases (AMPKs) are critical for the regulation of cellular metabolic pathways during starvation, hypoxia, and ischemia. Also, AMPK-related microtubule affinity regulating kinases (MARKs) are LKB1 substrates that control cell polarity through specific effects on tubulin dynamics. Although most cases of PJS are due to inherited mutations of *LKB1*, there are many sporadic cases, which historically were attributed to somatic mutations (40).

Table 2. Genes involved in sporadic forms of cancer

Gene	Function	Sequence affected	Type of cancer induced
VHL (von Hippel-Lindau)	pVHL, substrate recognition component of E3 ubiquitin ligase complex	CpG islands within promoter sequence	RCC (renal cell carcinoma)
BRCA1 (breast cancer 1, early onset)	encodes tumor suppressor protein, repair protein	CpG islands within promoter sequence	breast cancer 1, early onset, ovarian cancer
LKB1/STK11 (liver kinase B1/serine-threonine kinase 11)	LKB1 protein kinase has role in cell growth, metabolism, cell polarity	CpG islands within promoter sequence	PJS (Peutz-Jeghers syndrome), predisposition to gastrointestinal tract, breast, testis, ovary, and cervix cancers

An alternative mechanism for the inactivation of *LKB1* is by promoter hypermethylation, an event that generally occurs more frequently than somatic mutations. In a series of experiments, researchers used MSP to screen the DNA methylation patterns of 51 cancer cell lines (40). Only four displayed aberrant methylation within the *LKB1* promoter region, and these same cell lines did not have any detectable levels of LKB1 protein. Exposure to 5-aza-2'-deoxycytidine (DAC) restored LKB1 expression, indicating that the promoter methylation was directly responsible for the loss of protein expression (40). Next, sporadic tumors with the histological subtypes typical of PJS were studied, and *LKB1* methylation was present in five of 11 papillary breast carcinomas (40). These observations indicate that promoter hypermethylation, a frequent event in sporadic cases of cancer formation, is a likely alternative pathway that acts as Knudson's second hit to directly cause the initiation of tumorigenesis.

3. HMTs and Cancer Aggressiveness:

The Polycomb group transcriptional repressor and HMT, EZH2, has been specifically found to be upregulated in metastatic prostate cancer, the second leading cause of cancer-related deaths in adult males (42). An initial experiment using a statistical technique called significance analysis of microarrays (SAM) helped researchers compose a list of both upregulated and downregulated genes in metastatic prostate cancer, and the most upregulated gene was *EZH2*. Through a DNA microarray analysis, comparisons of EZH2 expression in metastatic, localized, and benign prostate cancers, and a significant increase in expression was observed in metastatic prostate tissue specimens (42). Next, the evaluation of EZH2 expression in a range of over 400 different prostate tissues revealed that the intensity of expression increased as prostate neoplasia progresses. To

disrupt the role of EZH2 in progression of prostate cancer, RNA interference (RNAi) was used by the creation of several siRNA molecules targeted to the *EZH2* mRNA transcript (42). The induction of these oligonucleotides into a metastatic prostate cancer cell line caused significant downregulation of EZH2 expression after 48h, as well as a marked inhibition of cell growth of the prostate cancer cell line (42). These and additional experiments identified high levels of EZH2 as a molecular marker of prostate cancer progression and metastasis. This repressive enzyme has additionally been connected to breast cancer aggressiveness, as well as development of bladder tumors (14).

The deregulation of expression of the *MMSET* gene, also known as *Nuclear Receptor-binding SET Domain 2 (NSD2),* is associated with the Wolf-Hirschhorn syndrome, a disease characterized by a malformed facial appearance, delayed growth and development, and intellectual disabilities (43). This gene contains a SET domain that is found in many histone methyltransferases and determines their enzymatic activity. It has become understood that mechanistically, NSD2 has a transcriptional repressor activity, with increased H4K20 methylation and loss of histone acetylation (43). *NSD2* is associated with the aggressiveness of tumors in various forms of cancer. NSD2 expression was examined in 40 different tumor types, including four hematological malignancies and 36 solid tumors, by analysis of publicly available gene expression data (43). Overexpression was observed in all four blood cancer types, as well as in 12 of 36 solid tumors of various cancer types. Additionally, NSD2 has been shown to be highly overexpressed in advanced stages of several carcinomas as compared to earlier stages. The idea of epigenetic mechanisms having association with the formation and growth of cancer has found much experimental support, as shown by the previous examples. These studies paved the way

for investigations of the possible roles of epigenetic defects in other diseases. Mounting evidence supports that the dysregulation of epigenetic histone and DNA methylation is linked to the development of various neurological disorders such as schizophrenia, Alzheimer's disease, and Rett syndrome. I will summarize and discuss these findings in the following.

B. Neurological diseases:

1. Schizophrenia:

Schizophrenia is a severe, chronic, and generally disabling disease that affects 1% of the world's population. It is technically a form of psychosis, a type of illness causing severe mental disturbances that disrupt normal thought, speech, and behavior. As described earlier, the sequence analysis of genomic DNA from monozygotic twins discordant for diseases was one of the earlier findings that suggested epigenetic methylation changes was linked to disease-specific gene expression changes. Twins discordant for schizophrenia have shown differences in DNA methylation patterns of certain genes, providing a possible epigenetic mechanism for the psychosis phenotype (32).

Studies of schizophrenia and bipolar disorder have revealed alterations in the expression of particular genes, which most likely contribute to the general symptoms of the disorders. Both *glutamic acid decarboxylase (GAD67)* and *reelin* mRNA are reduced in GABAergic interneurons of patients with schizophrenia, compared to normal interneurons (32). Reelin, secreted from the interneurons, surrounds dendritic spines and axon segments of pyramidal neurons and acts as an extracellular matrix protein to promote dendritic protein synthesis (44). The *GAD67* gene encodes an enzyme that catalyzes the decarboxylation of glutamate to γ-aminobutyric acid (GABA). GABA is secreted from GABAergic neurons to provide an inhibitory input to pyramidal neuron dendrites, somata, or axon segments (44, 45). The phenotype of interneurons of schizophrenic patients includes a reduction of the amount of dendritic spines and associated neurophil hypoplasticity. This phenotypic change likely leads to altered synaptic activity that is responsible for the typical cognitive dysfunction and thought disorders in schizophrenic patients (45).

In a study proposing a possible role of Dnmt1 in the downregulation of *reelin* and *GAD67* expression in SZ patients, mouse cortex cultures were maintained *in vitro*. Immunohistochemical stainings showed that Reelin and GAD67 are coexpressed with Dnmt1 in the same neurons (32). Researchers then treated primary neuronal cells with methionine, an agent that has been shown to cause an increase in methylation of CpG islands within the promoter region of the *reelin* gene. RT-PCR showed the significant decrease in expression of the Reelin and GAD67 proteins. There was also a very slight decrease in Dnmt1 expression, but the level was assumed as not significant (32). To examine whether hypermethylation caused the decrease of protein levels, neuronal cells were again treated with methionine for different time periods. After bisulfite treatment, MSP was used to detect methylated sites. The results indicated that after 36h of treatment, methionine induced considerable methylation on CpG sites within the *reelin* promoter (32). This hypermethylation was in direct correlation with the decrease in Reelin levels, as the most significant decrease of Reelin expression occurred after 36h of methionine exposure. Antisense oligonucleotides for Dnmt1 were transfected into the cortex cells, and analyses showed a decrease in Dnmt1 transcript and protein levels (32). This led to an immediate increase of *reelin* mRNA levels. Researchers next tested if antisense oligo Dnmt1 treatment would also affect methionine-induced downregulation of Reelin. In fact, the combined treatment, analyzed after 72h, showed that the methionine-induced downregulation of Reelin and GAD67 was blocked by Dnmt1 downregulation (32). This was probably due to the fact that methionine relies on the enzymatic function of Dnmt1 which introduces the methyl groups to promoters of its target genes.

More recent studies have revealed that an increased expression of localized

DNMT1 in GABAergic interneurons of adult human brains causes hypermethylation of *Reelin* and *GAD67* promoter regions, resulting in the downregulation of these genes (45). These increased levels of DNMT1 were analyzed by RT-PCR allowing a precise quantification of DNMT1 expression levels, and Laser Assisted Microdissection (LAM), a method used to determine the specific GABAergic interneuron subtypes involved (45). It was found that the increased levels of DNMT1 within specific layers of the prefrontal cortex (PFC) of schizophrenic patients were directly related to decreases in both reelin and GAD67 protein levels. Additionally, other genes expressed in the GABAergic interneurons, such as *GAD65*, showed normal expression patterns despite the presence of CpG islands in their promoters. The researchers proposed that the lack of hypermethylation of this gene is due to differences in the accessibility of the CpG island to DNMT1. They suggested that histone modifying enzymes or chromatin remodeling proteins are critical for DNMT1-targeted DNA methylation. This proposed model is discrepant from the more commonly accepted model that methyl-CpG-binding proteins (MBPs) actually read the methylated cytosine residues first, which then leads to chromatin-mediated silencing by associated HDACs. Therefore, while it is quite likely that other chromatin-linked mechanisms contribute to the regulation of CpG methylation, the proposed model requires further experimental confirmation.

2. Alzheimer's Disease:

As discussed earlier, the incidence of cardiovascular disease has been shown to correlate with a person's age, with a convincing argument for aberrant epigenetic DNA methylation as an underlying mechanistic cause. A similar relationship between a person's age and development of Alzheimer's disease (AD) has been reported, suggesting

that epigenetic mechanisms might also play some role in the development of the disease. AD is the most common form of dementia that affects over 6% of individuals 65 or older (46). Studies of AD until recently have revealed little about the pathophysiology of the disease. This is most likely because clinical investigations are usually conducted on patients in advanced stages of the disease. Through the investigation of human postmortem brain samples, peripheral leukocytes, cell culture studies, and transgenic animal models, researchers have begun to examine the likely roles that epigenetic histone modifications and DNA methylation have in the development of AD.

The common neuropathological properties of the affected brain regions in AD are the loss of neurons and synapses, neuritic plaques, and neurofibrillary tangles composed of tau protein and Amyloid Precursor Protein (APP) (46). The *Microtubule-associated Protein Tau (MAPT)* gene encodes various tau protein isoforms. Tau proteins help stabilize microtubules and promote tubulin assembly. The hyperphosphorylation of tau protein results in neurofibrillary tangles when *MAPT* gene expression becomes altered. The *APP* gene can encode several proteins, which are regulated by specific secretases that cleave the precursor at various sites. The proteolytic cleavage of *APP* into Aβ42 is abnormally high in Alzheimer's patients, and this increase is associated with the formation of extracellular plaques within affected neurons (46). The combination of the dysregulation of *APP* and *MAPT* genes leads to impairments in cognitive functions that interfere with daily life functioning (46). This often leads to what is referred to as early onset Alzheimer's disease (EOAD). Aside from these two important regulatory genes, there have been additional studies providing evidence that several other genes have possible roles in the etiology of AD (46, 47).

To investigate the more common form of the disease, namely late onset Alzheimer's disease (LOAD), researchers performed an analysis on postmortem brains and lymphocytes of individuals with AD. The investigation was conducted by mass spectrometry using bisulfite-modified methylated DNA fragments from the analyzed brain tissues and lymphocytes (47). Initially, genome-wide DNA methylation levels were compared between individuals with AD and control samples, and no significant differences were found in post-mortem brain tissues or in lymphocytes (47). To see whether the methylation patterns at individual genes or CpG islands of genes that previously were implicated in AD are different, they next compared the methyl-C distribution patterns of AD patients to the ones of an average that was generated from the methyl-C residues of all non-disease samples. Significantly more of the AD patient's genes contained more abnormal DNA methylation patterns than control patterns (47).

Of all genes analyzed, the single one that was observed not only to have aberrant methyl-C distribution, but also a hypermethylated CpG island was the Apolipoprotein E (APOE)-encoding gene (47). APOE is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. It has been recently found that APOE is considered to be the main candidate for LOAD (37). In the study of post-mortem brain tissues, DNA methylation patterns within the CpG island of *APOE* were close to 100% in all AD individuals. The impact of DNA methylation on APOE activity is currently unknown, but increased methylation patterns have been observed in LOAD patients compared to controls (47), indicating that this protein may contribute to disease risk.

Another interesting idea is that as a person ages, epigenetic "drift" (pg. 20) causes more frequent aberrant modifications among DNA CpG sites. The study that previously

reported the genome wide DNA methylation analysis on postmortem brain tissue additionally described a trend towards an increase in DNA methylation of *APOE* among Alzheimer's patients in comparison to control samples (47). The pattern appeared strongest within the promoter sequences of *APOE* and other genes believed to have roles in the etiology of AD. Although these experiments provide interesting observations comparing methylation patterns between AD and healthy brain tissue, a better understanding of epigenetic involvement in AD will most likely emerge from conducting research on earlier stages of the disease. This will likely reveal possible epigenetic contribution to the development of the disease.

3. Rett Syndrome:

Rett syndrome (RTT) is a severe neurological disorder exemplified by such phenotypic attributes as cognitive impairment, language dysfunction, motor abnormalities, and seizures (48). Mutation of the X-linked *Methyl-CpG-binding Protein 2 (MECP2)* gene was found in patients affected by RTT. Acting as a methyl-CpG-binding domain protein (MBP), MeCP2 assists in transcriptional repression by directly binding to methylated sites of DNA, and then brings in histone modifiers such as HDACs. It is important to note that as we more commonly understand it, the DNA methylation is upstream of repressive histone modifications. MeCP2 has been shown to be required for normal neuronal development and function, as well as necessary for normal synaptic activity, learning, and memory (48).

It has been shown in numerous experiments that chemical agents have the potential to alter levels of DNA methylation within a cell, and this next experiment adds narcotics to the list of potential effectors. A group of researchers used cocaine treatment in

male rats to determine the role of MeCP2 in the regulation of the *Cyclin-dependent Kinase-like 5 (Cdkl5)* gene. Mutation of *Cdkl5* has been associated with infantile spasms and mental retardation, as well as contributing to an "early-onset seizure" variant of RTT (48). In the experiment, researchers treated animals with cocaine or with saline for a 10 day period. Using RT-PCR, the animals injected with cocaine showed a 23% decrease in *Cdkl5* mRNA expression within the striatum of the brain as compared to the control group (48). Previous experiments had shown that MeCP2 expression was induced in the striatum by cocaine treatment (49). Using bisulfite mapping, analysis of striatum genomic DNA from rats treated with saline or cocaine for 10 days revealed a significant difference in the percentage of methylation. Although 59% of DNA was methylated in saline-treated rats, 82% of CpG methylation in the *Cdkl5* gene was observed in cocaine-treated animals (48).

The repressive characteristics of MeCP2 rely on initial methylation by DNMTs, and are carried out in part by the recruitment of associated HDACs. Some genes have been found to be activated by DNMT inhibitors, while others are activated by HDAC inhibitors or by both agents (48). To test the regulation of *Cdkl5* by DNA hypermethylation, 5-aza-2'-deoxycytidine (DAC), a DNMT inhibitor, and TSA, an HDAC inhibitor, were both administered to rat neuronal cultured cells. Following treatment of DAC, demethylation of CpG sites did occur within the *Cdkl5* gene, and this was correlated with a 3- to 4-fold induction of *Cdkl5* expression (48). *Cdkl5* expression was similarly induced in response to TSA treatment. Based on previous reports that *Mecp2* expression was induced in rat brain by the serotonin-elevating agent cocaine (49), researchers evaluated the expression of *Mecp2* and *Cdkl5* in neuronal cells in response to serotonin. Treatment

with serotonin added directly to the culture medium induced both an increase in the expression of *Mecp2* and a 2-fold decrease in *Cdkl5* expression (48).

To test whether *Cdkl5* expression could be controlled by overexpression or silencing of MeCP2, a construct allowing the expression of MeCP2-GFP fusion protein was introduced into rat neuronal cells. Following induction of the fusion protein expression, the cells were analyzed after three days for the correlation between MeCP-GFP overexpression and *Cdkl5* expression levels by cell imaging and RT-PCR (48). Compared to parental control cells, the transfected neuronal cells exhibited a 2.4-fold increase in expression of *Mecp2*. This overexpression was associated with a 3.6-fold repression of *Cdkl5* expression (48). Next, two different *Mecp2* siRNAs were transfected into rat neuronal cells in order to silence endogenous *Mecp2* expression. A direct repression of MeCP2 correlated with the upregulation of the *Cdkl5* gene (48). These series of experiments strongly suggests that MeCP2 acts as a transcriptional repressor towards *Cdkl5*, and provides a specific example of a novel epigenetic mechanism that results in the altered expression of genes, which may be responsible for the contribution to disease.

Even though the study of epigenetics and its involvement in the development of many diseases is still in its infancy, a very promising field of therapy has been created. The most intriguing aspect of epigenetics is its reversibility, a factor that has stimulated the greatest interest in new treatment methods.

III. Therapeutics:

When compared to direct genetic mutation, epigenetic alterations have begun to show a high range of therapeutic potentials for a rapidly increasing catalog of associated diseases. To date, there are a few methods of epigenetic therapy that are most popular, including DNMT inhibitors and HDAC inhibitors. The FDA approval of three cancer-related, epigenetically targeting drugs has also provided hope in a promising future of epigenetic therapy.

A. DNMT Inhibitors:

Although the inhibition of DNMTs may be expected to decrease the overall DNA methylation levels and result in the reactivation of many genes, the use of specific chemical agents has shown to specifically target aberrantly methylated tumorigenic cells. The demethylating agent 5-azacytidine was first synthesized as a possible chemotherapy agent, and has since been FDA-approved under the name Vidaza (50). The deoxy derivative 5-aza-2'-deoxycytidine (DAC), known as Decitabine, has shown to be even more effective. Both agents work by becoming incorporated into the nucleic acids of cells in the S-phase of the cell cycle due to their analogous properties to nucleosides. The DNMTs become covalently linked to the nucleoside analogues, creating enzyme-DNA adducts, with a consequent cellular depletion of DNMTs (50).

These demethylating agents have been shown to work by inducing apoptosis or differentiation on various forms of cancer cells. The experimental use of Decitabine in many of the studies reviewed in this paper showed significant changes in DNA methylation patterns, as well as an increase in expression of the genes being repressed in cancer cells. Decitabine has also been shown to induce differentiation of leukemia cells, as well

as apoptosis in a Burkitt's lymphoma cell line by reactivating the pro-apoptotic gene *DAPK1* (50). Azacitidine has been shown to induce apoptosis in Myelodysplastic syndrome (MDS) derived cells, and is also currently being studied in the treatment of other types of cancer.

The use of increased concentrations of these agents can cause toxic side effects. They are chemically unstable in water when under high concentrations, because the degradation products cause suppression of growth and proliferation of blood cells (19). Nevertheless, these agents are still effective at low concentrations. Another DNMT inhibitor, Zebularine, is currently in clinical trials and has shown promise in being much less toxic to normal cells. A recent study using Zebularine has shown to cause demethylation and reactivation of the *p16* gene in human bladder tumor cells grown in nude mice (51). Nevertheless, Decitabine to date is the most effective DNMT inhibitor, and the combination treatment with HDAC inhibitors, which are described below, shows even better efficacy. A summary of the discussed DNMT inhibitors is shown in Table 3.

Table 3. Some current DNMT inhibitors of potential clinical relevance

Chemical Name	FDA Approval?	Treatment potential
Azacitidine	Yes, as Vidaza	approved for MDS; currently in trials for various lymphomas
Decitabine	Yes, as Dacogen	approved for MDS; currently in trials for cervical cancer; promising results in lung cancer, leukemia, and Burkitt's lymphoma
Zebularine	No	human bladder cancer, Burkitt's lymphoma

B. HDAC Inhibitors:

The use of drugs for the inhibition of HDACs is a method of reducing epigenetic histone methylation contributing to various disease states. The basic mechanistic approach for these inhibitors is to interfere with the catalytic domain of HDACs, blocking substrate recognition and preventing the deacetylation of nucleosomal histones that usually are found at expressed genes and in other regions of the genome. In addition to current clinical trials of various forms of cancer, a wide range of HDAC inhibitors has shown to reduce *in vitro* cell growth of multiple myeloma and induce apoptosis in both myeloma cell lines and primary patient cells (52).

Table 4. Some HDAC inhibitors of potential clinical relevance

Chemical Name	FDA Approval?	Treatment potential
Vorinostat	Yes, as Zolinza	approved for CTCL
Trichostatin A	No	breast cancer, atherosclerosis
Belinostat	No	peripheral T-cell lymphoma, CUP, MDS
Romidepsin	Yes, as Istodax	approved for CTCL; clinical trials in peripheral T-cell lymphoma

The first HDAC inhibitor approved for treatment of a hematological malignancy was Vorinostat, marketed as Zolinza, for the use in treatment of cutaneous T-cell lymphoma (CTCL) in 2006 (52). Current clinical trials are under way for the combination of Vorinostat and other agents in various cancer treatments. Another HDAC inhibitor shown to have high anticancer effects is Trichostatin A (TSA). However, it is currently not in clinical trials because of several severe side effects (50). TSA also showed very

significant results in the study of atherosclerotic cell lines discussed earlier on aging. The treatment on these cells, in comparison to cancer cells, showed surprisingly little toxicity.

Belinostat, another HDAC inhibitor, is currently in phase III clinical trials for several forms of cancer treatment, including peripheral T-cell lymphoma, cancer of unknown primary site (CUP), and MDS (in combination with Vidaza) (52). It works by inhibiting proliferation of cancer cells and inducing apoptosis, similarly to other HDAC inhibitors. Belinostat has also shown promising results when treating aggressive ovarian xenograft tumors, in prostate cancer, and in suppression of the growth of bladder cancer cells *in vivo* and *in vitro* (50).

Romidepsin is another form of HDAC inhibitor, and has shown *in vivo* antitumor activity in various human tumor xenografts, as well as many human cancer cell lines (50). In November of 2009, Romidepsin was approved by the FDA for treatment of CTCL, and is currently in clinical trials for other forms of cancer, including peripheral T-cell lymphoma. A compound similar to Romidepsin, known as Aplidin, is currently in stage II clinical trials for treatment of solid and hematological malignant neoplasms such as multiple myeloma, T-cell lymphoma, and myelofibrosis. In addition to the few described here, there are several other HDAC inhibitors in clinical trials, and the therapeutic field of epigenetic treatment appears to only be getting more prominent. A summary of the HDAC inhibitors discussed are available in Table 4.

An area that is likely to see more growth is in the use of DNMT inhibitors in combinational therapy with HDAC inhibitors, largely because both DNMTs and HDACs can cooperatively contribute to the reduced DNA and histone methylation that cause silencing of important tumor suppressor genes. Another form of combination treatment is

the use of these DNMT and HDAC inhibitors with conventional chemotherapeutic treatments. Clinical trials are actually currently under way for combination treatment of many of the aforementioned chemical agents with chemotherapy.

C. Epigenetic Treatment of Brain Disorders:

The chemical agents discussed above have recently begun to be studied on brain disorders, in conjunction with the recent discoveries of DNA methylation and association with such disorders as schizophrenia, Alzheimer's, and Rett syndrome. A commonly used HDAC inhibitor, Valproic acid (VPA), is known for its function as an anti-convulsant mood-stabilizing drug. Its HDAC inhibiting function may have other actions on brain cells including enhanced brain repair in neurodegenerative disorders by promoting neurotrophin production in astrocytes (53). Similarly, DNMT inhibitors have been used to determine the effects on brain disorders for possible future treatment options.

As described earlier, the repression of *reelin* mRNA expression is a direct result of promoter sequence hypermethylation that results in the cognitive impairments observed in schizophrenics. A recent study showed that *in vitro*, the DNMT inhibitor Azacitidine reversed *reelin* promoter hypermethylation and induced a 60-fold increase in mRNA levels (53). VPA has also been shown *in vivo* to reverse hypermethylation of both *reelin* and *GAD67* genes, as well as reverse behavioral changes in *reeler*$^{(-/-)}$ mouse models (54). Another very interesting study involved the concept of nicotine self-medication of schizophrenic patients with a smoking addiction. Researchers showed that nicotine administration decreases *DNMT1* mRNA levels, and increases GAD67 expression through activation of nicotinic acetylcholine receptors on cortical and hippocampal GABAergic interneurons (55).

Alzheimer's disease is a progressive neurodegenerative disorder resulting in severe dementia, affecting an increasing proportion of aging populations. The mechanism of the development of AD is described in a previous section. The potential role of DNA methylation in the etiology of AD is controversial, as there are published studies showing increased DNA methylation on promoter regions of important genes such as *APP* in AD, as well as other studies that identify decreases in DNA methylation.

A study using the HAT inhibitor curcumin has been shown to interfere with the formation of amyloid plaques in AD disease rat models (53). This agrees with the recent study revealing that the risk of developing the disease is significantly reduced in a population within a rural community in India that consumes large quantities of curcumin (56). Therefore, the role of environmental factors including diet, maternal care, and exposure to various agents such as certain heavy metals may contribute to the increased susceptibility of the disease.

IV. Conclusion:

The development of genetic disorders has historically been attributed to inherited mutations in the germ line or soma of an individual during its life. These mutations affect the expression of particular genetic information, and these defects are ultimately responsible for diseases including cancer, as well as neurological disorders such as schizophrenia, Alzheimer's disease, and Rett syndrome. The study of epigenetics has shown that the aberrant methylation of DNA and modifications on histones can mimic the effects of DNA mutations.

The recent support for environmental factors causing epigenetic alterations has created an interesting idea about how an individual's behavior can affect their disease

susceptibility. The idea that some of these alterations in the epigenetic programming of an individual can be transmitted to the next generation show that the life-style decisions, but also the treatment of an individual, could affect subsequent generations. This is something that has not been considered very often in treatment options and requires more careful research. Therefore, studies of the transgenerational effects of epigenetic therapeutics must certainly be considered to effectively and safely expand the therapeutic arena of epigenetic treatments in the future without causing adverse effects in future generations.

References:

1. Berger, S.L., et al. An operational definition of epigenetics. Genes Dev. Vol. 23, pp. 781 – 83. (2009).

2. Chang et al. Transgenerational epigenetic imprinting of the male germline by endocrine disruptor exposure during gonadal sex determination. Endocrinology. Vol. 147, No. 12, pp. 5524 – 5541. (2006).

3. Anway, M.D. et al. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science. Vol. 308, pp. 1466 – 1469. (2005).

4. Costa et al. Reviewing the role of DNA (cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. Epigenetics. Vol. 2, No. 1, pp. 29 – 36. (2007).

5. Skinner, M.K. What is an epigenetic transgenerational phenotype? Reproductive Toxicology. Vol. 25, pp. 2 – 6. (2007).

6. Davey, C.A., et al. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J. Mol. Biol. Vol. 319, pp. 1097 – 1113. (2002).

7. Campos, E. and Reinberg, D. Histones: Annotating Chromatin. Annu. Rev. Genet. Vol. 43, pp. 559 – 599. (2009).

8. Kouzarides, Tony. Chromatin Modifications and Their Function. Cell. Vol. 128, pp. 693 – 705. (2007).

9. Strahl, B.D. and Allis, C.D. The language of covalent histone modifications. Nature. Vol. 403, pp. 41 – 45. (2000).

10. Rottach, A. et al. DNA methylation-mediated epigenetic control. J. Cell. Biochem. Vol. 108, pp. 43 – 51. (2009).

11. Sharma, S., et al., Epigenetics in cancer. Carcinogenesis. Vol. 31, No. 1, pp. 27 – 36. (2010).

12. Li, B. et al. The role of chromatin during transcription. Cell. Vol. 128, No. 4, pp. 707 – 719. (2007).

13. Wolf, S. The protein arginine methyltransferase family: an update about function, new perspectives and the physiological role in humans. Cell. Mol. Life Sci. Vol. 66, pp. 2109 – 2121. (2009).

14. Albert, M. and Helin, K. Histone methyltransferases in cancer. Semin. Cell Dev. Biol. doi:10.1016/j.semcdb.2009.10.007 (2009).

15. Schlesinger, Y. et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for *de novo* methylation in cancer. Nature Genet. Vol. 39, No. 2, pp. 232 – 236. (2007).

16. Widschwendter, M. et al. Epigenetic stem cell signature in cancer. Nature Genet. Vol. 39, No. 2, pp. 157 – 158. (2007).

17. Kerppola, Tom K. Polycomb group complexes – many combinations, many functions. Trends in Cell Biology. Vol. 19, No. 12, pp. 692 – 704. (2009).

18. Wang, J. et al. The histone demethylase UTX enables RB-dependent cell fate control. Genes and Development. Vol. 24, pp. 327 – 332. (2010).

19. Jones, P. and Baylin, S. The fundamental role of epigenetic events in cancer. Nat. Rev. Genet. Vol. 3, pp. 415 – 428. (2002).

20. Bestor, TH. The DNA methyltransferases of mammals. Hum. Mol. Genet. Vol. 9, No. 16, pp. 2395 – 2402. (2000).

21. Li, E. et al. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell. Vol. 69, Iss. 6, pp. 915 – 926. (1992).

22. Xu, G. et al. Chromosome instability and immunodeificiency syndrome caused by mutations in a DNA methyltransferase gene. Nature. Vol. 402, pp. 187 – 191. (1999).

23. Thomson, J. P. et al. CpG islands influence chromatin structure via the CpG-binding protein Cfp1. Nature. Vol. 464, pp. 1082 – 1087. (2010).

24. Reik, Wolf. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature. Vol. 447, pp. 425 – 432. (2007).

25. He, L. et al. MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. Vol. 5, pp. 522 – 531. (2004).

26. Fabbri, M. et al. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc. Natl. Acad. Sci. USA. Vol. 104, pp. 15805 – 15810. (2007).

27. Friedman, J.M. et al. The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. Cancer Res. Vol. 69, pp. 2623 – 2629. (2009).

28. Handel, A. et al. Epigenetics: molecular mechanisms and implications for disease. Trends in Molecular Medicine. Vol. 16, No. 1, pp. 7 – 16. (2009).

29. Gilbert, S. Ageing and cancer as diseases of epigenetics. J. Biosci. Vol. 34, No. 4, pp. 601 – 604. (2009).
30. Kim J. et al. Epigenetic changes in estrogen receptor beta gene in atherosclerotic cardiovascular tissues and in vitro vascular senescence. Biochim. Biophys. Acta. Vol. 1772, pp. 72 – 80. (2007).

31. Oates, N.A. et al. Increased DNA methylation at the AXIN1 gene in a monozygotic twin from a pair discordant for a caudal duplication anomaly. Am. J. Hum. Genet. Vol. 79, No. 1, pp. 155 – 162. (2006).

32. Noh, J. et al. DNA methyltransferase 1 regulates reelin mRNA expression in mouse primary cortical cultures. PNAS. Vol. 102, No. 5, pp. 1749 – 1754. (2005).

33. Esteller, M. et al. Inactivation of the DNA repair gene *O^6-Methylguanine-DNA Methyltransferase* by promoter hypermethylation is associated with G to A mutations in *K-ras* in colorectal tumorigenesis. Cancer Research. Vol. 60, pp. 2368 – 2371. (2000).

34. Lodish, H. et al. Molecular cell biology. W.H. Freeman and Company. 5th ed. (2004).

35. Herman, J. et al. Distinct patterns of inactivation of *$p15^{INK4B}$* and *$p16^{INK4A}$* characterize the major types of hematological malignancies. Cancer Research. Vol. 57, pp. 837 – 841. (1997).

36. Burbee, D. et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. Journal of the National Cancer Institute. Vol. 93, No. 9, pp. 691 – 699. (2001).

37. Kane, M. et al. Methylation of the *hMLH1* promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Research. Vol. 57, pp. 808 – 811. (1997).

38. Wright, T.M. and Rathmell, W. K. Identification of Ror2 as a hypoxia inducible factor (HIF) target in von Hippel-Lindau (VHL)-associated renal cell carcinoma. J. Biol. Chem. (2010).

39. Herman, J.G., et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc. Natl. Acad. Sci. USA. Vol. 91, pp. 9700 – 9704. (1994).

40. Esteller, M. et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J. Natl. Cancer Inst. Vol. 92, pp. 564 – 569. (2000).

41. McCabe, M. et al. Homozygous deletion of the STK11/LKB1 locus and the generation of novel fusion transcripts in cervical cancer cells. Cancer Genet. Cytogenet. Vol. 197, No. 2, pp. 130 – 141. (2010).

42. Varambally, S. et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. Vol. 419, pp. 624 – 629. (2002).

43. Kassambara, A. et al. MMSET is overexpressed in cancers: Link with tumor aggressiveness. Biochem. Biophys. Res. Commun. Vol. 379, Iss. 4, pp. 840 – 845. (2009).

44. Niu, S. et al. The reelin signaling pathway promotes dendritic spine development in hippocampal cells. J. Neurosci. Vol. 28, Iss. 41, pp. 10339 – 10348. (2008).

45. Costa, E. et al. Reviewing the role of DNA (Cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. Epigenetics. Vol. 2, No. 1, pp. 29 – 36. (2007).

46. Chouliaras, L. et al. Epigenetic regulation in the pathophysiology of Alzheimer's disease. Prog. Neurobiol. doi:10.1016/j.pneurobio.2010.01.002. (2010).

47. Wang, S. et al. Age-specific epigenetic drift in late onset Alzheimer's disease. PloS ONE. Vol. 3, Iss. 7, pp. e2698. (2008).

48. Carouge, D. et al. CDKL5 is a brain MeCP2 target gene regulated by DNA methylation. Neurobiol. Dis. doi:10.1016/j.nbd.2010.02.014. (2010).

49. Cassel, S. et al. Fluoxetine and cocaine induce the epigenetic factors MeCP2 and MBD1 in adult rat brain. Mol. Pharmacol. Vol. 70, pp. 487 – 492. (2006).

50. Kristensen, L.S. et al. Epigenetics and cancer treatment. European Journal of Pharmacology. Vol. 625, pp. 131 – 142. (2009).

51. Yoo, C.B. et al. Zebularine: a new drug for epigenetic therapy. Biochemical Society Transactions. Vol. 34, Pt. 6, pp. 910 – 912. (2004).

52. Smith, E.M. et al. The potential role of epigenetic therapy in multiple myeloma. British Journal of Haematology. Vol. 148, pp. 702 – 713. (2009).

53. Narayan, P. and Dragunow, M. Pharmacology of epigenetics in brain disorders. British Journal of Pharmacology. Vol. 159, pp. 285 – 303. (2010).

54. Dong, E. et al. Histone hyperacetylation induces demethylation of reelin and 67-kDa glutamic acid decarboxylase promoters. Proc. Natl. Acad. Sci. USA. Vol. 104, pp. 4676 – 4681. (2007).

55. Satta, R. et al. Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons. Proc. Natl. Acad. Sci. USA. Vol. 105, pp. 16356 – 16361. (2008).

56. Chandra, V. et al. Incidence of Alzheimer's disease in a rural community in India: the Indo-US study. Neurology. Vol. 57, pp. 985 – 989. (2001).