White Paper

The Message in the Marks: Deciphering Cancer Epigenetics

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Cancer is a complex disease manifestation. At its core, it remains a disease of abnormal cellular proliferation and inappropriate gene expression. In the early days, carcinogenesis was viewed simply as resulting from a collection of genetic mutations that altered the gene expression of key oncogenic genes or tumor suppressor genes leading to uncontrolled growth and disease (Virani, S et al 2012). Today, however, research is showing that carcinogenesis results from the successive accumulation of heritable genetic and epigenetic changes. Moreover, the success in how we predict, treat and overcome cancer will likely involve not only understanding the consequences of direct genetic changes that can cause cancer, but also how the epigenetic and environmental changes cause cancer (Johnson C et al 2015; Waldmann T et al 2013).

Epigenetics is the study of heritable gene expression as it relates to changes in DNA structure that are not tied to changes in DNA sequence but, instead, are tied to how the nucleic acid material is read or processed via the myriad of protein-protein, protein-nucleic acid, and nucleic acid-nucleic acid interactions that ultimately manifest themselves into a specific expression phenotype (Ngai SC et al 2012, Johnson C et al 2015). This review will discuss some of the principal aspects of epigenetic research and how they relate to our current understanding of carcinogenesis. Because epigenetics affects phenotype and changes in epigenetics are thought to be key to environmental adaptability and thus may in fact be reversed or manipulated, understanding the integration of experimental and epidemiologic science surrounding cancer and its many manifestations should lead to more effective cancer prognostics as well as treatments (Virani S et al 2012).

Cancer as an Epigenetic Disease

Epigenetic changes are central to normal development. Terminal differentiation of cell types, whether they be ES cells to skin cells or brain cells, is largely controlled via a tightly orchestrated program of gene expression changes that are under careful, yet adaptable, epigenetic control (Khavan DA 2010). One only has to look at the explosive work and progress surrounding stem cell culture and its technologies ((iPSCs, piPSCs) to observe just how important the expression of a handful of key genes is to powering fundamental changes in cellular phenotype and launching a whole new science arena surrounding cellular reprogramming and its manifestations (Mali P et al 2011, Takahashi K, Yamanaka S 2006). Furthermore, research from flies to humans has shown how factors such as environmental stresses, age and adverse lifestyle changes can influence gene expression transgenerationally through epigenetic changes (Daxinger L et al 2010, Jirtle RL 2007). For example, investigators using epidemiological research that focused on the children of mothers that had experienced starvation and famine during the Dutch famine of WWII (1944-1945) showed that these children exhibited increased rates of coronary heart disease and obesity compared to those whose mothers had not been exposed to famine (Heijmans BT et al 2008).

It is no wonder, then, that with such power emboldened within epigenetic change, that its role in disease, particularly cancer, is also prominent. The epigenetic origins of cancer have been discussed for many years now (Esteller M et al 2002, Ho M-W 1998). Changes in methylation and chromatin structure abound in cancerous tissue when compared to normal tissue, thus implicating epigenetic change as a possible culprit (Jaenisch R (2003). However, mountains of research have shown that distinct genetic mutations are also found in cancers and that certain mutations in particular genes can also cause cancers; thus, the genetic basis for carcinogenesis is also very apparent (McLean MH et al 2014).

But are the epigenetic and genetic changes in cancerous tissues really the cause, or are they merely a symptom? This question has dogged epigenetic and genetic cancer research for decades. Fortunately, a new paper recently helped cement the contention that epigenetic change itself can cause cancer (Yu DH et al 2014). In their paper, the researchers demonstrated that, in mice, when a key cell cycle regulatory gene, p16, was silenced via methylation, 27% of the animals developed not one but numerous different cancers while the controls did not, and even of those that received only one copy of the gene, 5% developed cancers (Yu DH et al 2014). Because this introduced change was the only change between the two groups of mice, these results indicated that methylation of p16 was responsible for the observed increase in carcinogenesis (Yu DH et al 2014), thereby directly linking epigenetic change with carcinogenesis in a testable system.

If epigenetic change is causing cancer, then research examining at least some of the myriad of epigenetic influencers should also show strong links to cancer. Indeed, research examining environmental exposure to various compounds from hormone mimics to toxins, various forms of physical, environmental, and metabolic stress, unhealthy dietary factors, smoking, lack of sleep and even aging have all demonstrated a link to an increase in cancer incidence, such that it is now generally appreciated that epigenetic changes contribute to carcinogenesis and that there are links between environmental, dietary, age, and lifestyle factors that increase risks of the acquiring the disease (Feinberg AP et al 2014, Sigurdardottir LG et al 2013, Thompson CL 2010 2012, D. Yu et al, 2014, Virani S et al 2012, Jaenisch R et al 2003).

While carcinogenesis is certainly a disease that is capable of being largely governed by epigenetic change, epigenetic change by itself is not always be sufficient for preventing carcinogenesis (Feinberg AP 2004, Plass C 2002). Moreover, genetic research associated with both inherited and acquired forms of cancer surely indicate that cancer can also be a disease resulting from a genetic component as well (Ciriello, G et al 2013). Today, most scientists agree that cancer is a disease that benefits from both acquired epigenetic as well as genetic changes, and in order to prevent it as well as treat it, we need to understand the mechanisms driving these changes (Benavente CA et al 2015; Trietsch MD, et al 2015, Johnson C et al 2015).

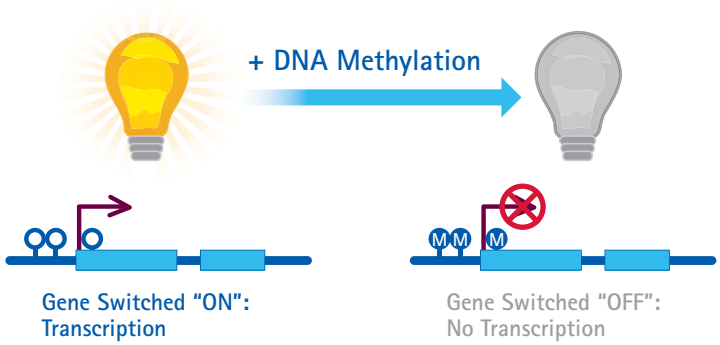
Mechanisms of epigenetic change

Among the many arenas of epigenetic research, three subjects stand out as playing substantial roles in the control of epigenetic gene expression and inheritance. These are DNA methylation, histone modification and, more recently, RNA-associated gene silencing events and/or modification, now called RNA epigenetics (Liu N et al 2015).

DNA Methylation

Research into the placement, control, maintenance, and inheritance of DNA methylation is one of the longest running and most examined epigenetic research arenas. The methylation of cytosine on its fifth carbon to form 5-methylcytosine is the principal form of DNA methylation found in mammalian cells. In humans and other vertebrates, methylation is governed by a family of DNA methyltransferases termed DNMT1, DNMT2, DNMT3a/3b. The enzymes catalyze the transference of a methyl group (CH_3) from S-adenosylmethionine (SAM) to the 5-carbon (C-5) position of cytosine in genomic DNA. In addition, effective methylation requires the services of additional DNA-binding, methyl-CpG binding domain proteins called MeCP2 and MBD1-4 (Bogdanovic O 2009, Bird A 2002). Cytosine methylation is required for the allele-specific expression of imprinted genes, for the transcriptional repression of retrotransposons in both germ and somatic cells, and for X chromosome inactivation in females (Ooi SKT 2008). Moreover, the level of methylation varies by cell type and tissue type but is generally considered high in vertebrates, including mammals (Bird A 2002).

Expression repression occurs because, when the cytosine is methylated, the position of the methyl group in the major groove of the DNA double helix interferes with transcription factor binding and subsequent gene expression from the site. In the DNA sequence, when cytosine (C) and guanine (G) align next to each other in sequence, researchers term this dinucleotide pairing a CpG sequence to distinguish it from the CG nucleotide pairing associated with double stranded pairing and hydrogen bonding. CpG dinucleotide sites are a focus of much research because they are often found in clusters termed "CpG islands" that are associated with gene promoter regions, where, when methylated, the corresponding gene is silenced. CpG dinucleotide sites are also found in repetitive sequences such as tandem and interspersed repeats, and in distal gene regulatory elements. It is estimated that about half the normally expressed genes or tissue-specific genes have CpG islands. (Virani S et al 2012; Jabbari K et al 2004). In normal somatic mammalian cells, some estimates have shown that 70-80% of the CpG sites are methylated (Jabbari K et al 2004). In ES cells, the percentage of CpG methylation is lower and 5-methyl cytosine is found in non-CpG sites as well (Lister R et al 2009; Lister R, Ecker JR 2009). As expected, in adult tissues, DNA methylation analysis of purified DNA from many different individuals has revealed that most CpG sites outside of promoters are heavily methylated, while CpG islands in germ-line cells and in promoters of active somatic genes are unmethylated, therefore allowing for gene expression. (Lister R, Ecker JR 2009).



Interestingly, in cancer, the orderly pattern of methylation seen in somatic cells is highly disrupted. In general, the level of methylation is often much lower in cancerous cells (Virani, S 2012; Feinberg AP et al 1983). In addition, many CpG islands show evidence of being hypermethylated in promoter regions and hypomethylated in distal regulatory regions and repetitive element sites –just the opposite of that of normal non-diseased tissues—presumably leading to inappropriate expression or repression of a number of different genes and gene families (Virani S et al 2012).

Furthermore, CpG profiles (maps of methylation patterns) of cancerous tumors are often heterogeneous. A single tumor may display different methylation profiles when sampled from different sites within it, and cells extracted from these areas often show differing levels and pathways of carcinogenesis (Virani, S 2012; Sartor et al 2011). In addition to aberrant gene expression, hypomethylation impacts DNA and heterochromatin stability.

Methylation at particular sequences is thought to help provide stability to specific chromosomal regions such as those at the points of sister chromatid association (Virani S 2012). Disruption of this methylation contributes to the unpacking of the heterochromatin and increased DNA instability, leading to an increase in chromosome rearrangements and multiple gene translocations. This potential of demethylation was exemplified by experiments conducted with DNMT1(-) cells. DNMT1 appears to be responsible for the maintenance of established patterns of DNA methylation, while DNMT3a and 3b seem to mediate establishment of new or de novo DNA methylation patterns. The DNMT1 knockout cells demonstrated a high degree of chromosomal rearrangements as well as gene duplications and other issues related to aberrant chromatin, thus indicating the importance of DNMT1 and methylation to overall chromosomal integrity (Virani S 2012, Chen et al 1998).

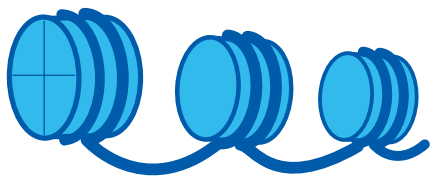
Finally, the hypomethylated state found in cancerous tissue can lead to the expression of normally long-repressed genes and genetic elements such as retrotransposons. Two of the most studied retrotransposons are LINE-1 and Alu. Normally, both of these elements are silenced via methylation, but in many cancerous tissues, including lung, prostate, pancreatic, urothelial and hepatic cancers, they are expressed and worse, found to be transposed, influencing the expression of key genes (Miousse IR et al 2014, Virani S 2012). The expression of these elements is associated with genomic instability and thought to contribute to the neoplastic phenotype when present (Miousse IR et al 2014, Virani S 2012, Daskalos et al 2009).

Furthermore, while cancer cell DNA is generally considered hypomethylated compared to normal cells, there are regions that are abnormally hypermethylated in many cancers. These changes seem to be driven by perturbation of DNA methyltransferase expression. In many cancers, the expression of these enzymes is greatly enhanced, and this, in turn, appears to govern the level of CpG island methylation which, again, influences the expression of key genes. Among the most studied are the tumor suppressor genes such as PTEN, APC or p53. CpG islands in these genes' promoter regions are often hypermethylated, resulting in the silencing of these tumor suppressors, which then, by various consequences, aids in oncogenesis (Virani S 2012, Hatziapostolou et al 2011).

The demethylation of DNA is equally as important to epigenetic change and is an active area of intense epigenetic research as well. As with normal DNA methylation, DNA demethylation is also critical for proper epigenetic control and development. The two processes reflect opposite sides of the same reactive processes and so often share DNA methyltransferase enzymes such as the DNMTs. However, DNA demethylation also utilizes an entirely different family of enzymes called the ten-eleven translocation (TET) family of 5-mC hydroxylases (TET1, TET2, and TET3) to remove methyl groups via oxidation reactions, and evidence suggests that TET proteins affect multiple fates beyond mere demethylation as well. TET proteins and their activities or inactivities are known to be important in modulating gene expression or repression as well as tumor suppression and DNA reprogramming. Because they represent a major modulator of DNA methylation, they are currently being researched as possible protein therapy treatments in cancer pathology (Carvalho AT 2014, Pastor WA 2013, Deplus R 2013, Lian CG 2012).

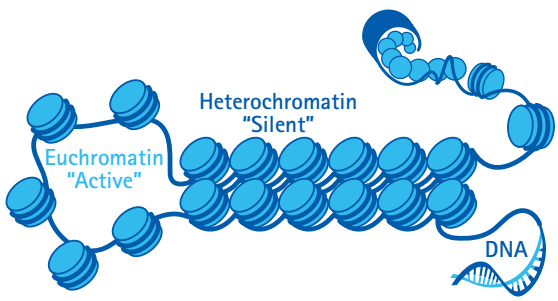
Chromatin Remodeling and Histone Modifications

The major DNA packaging proteins in eukaryotes are the histone proteins. Remarkably, the nearly two meter long genetic material making up a typical human cell's DNA is compacted and neatly organized into an exquisite three-dimensional, multilayer unit comprised primarily of nucleic acid materials and DNA binding histone proteins. Together, they constitute chromatin, and the basic building block of chromatin is the nucleosome. Each nucleosome consists of a protein octomer containing pairs of each of the four core histone proteins H2A, H2B, H3 and H4 (Waldmann T et al 2013, Luger K et al 1997). These histone bundles are further compacted and organized into stacked or ordered arrangements of nucleosomes, giving rise to the principal forms of chromatin termed euchromatin and heterochromatin. Visually, when stained, euchromatin appears lighter in color, airy and lightly packed. Euchromatin houses transcriptionally active genetic material. In contrast, heterochromatin stains much more darkly and densely because of its higher protein content and because it houses the transcriptionally inactive genetic material.



A nucleosome consists of an octamer of histone proteins tightly associated with DNA. Accessibility to regional DNA sequences is regulated by post-translational modifications to these histone proteins, which in turn represent epigenetic events.

These states of chromatin are dynamic within cells and transitions from one to another state are largely governed by the protein modifications inflicted upon the histones that bind the DNA together. These changes to histones are post-translational, and thus they are true epigenetic changes. Such post-translational modifications (PTMs) are catalyzed or constructed by



a horde of different proteins, including histone-modifying enzymes and ATP-dependent remodeling complexes. The PTMs ultimately affect not only the way the histone proteins bind the wound DNA, but also how they interact with each other and with the other proteins and nucleic acids that form chromatin universe within each cell's nucleus (Henikoff S 2008, Sha K 2008, Virani S 2012). Thus, because of their unique position in chromatin and the fact that the state of chromatin is dynamic, histone proteins, their modifications, and the control of those modifications are major epigenetic research arenas particularly as they relate to the role that histone PTMs play in cancer prognosis, oncogenesis, and treatment (Waldmann T et al 2013, Fullgrabe J et al 2011).

Histone Modification and Epigenetic Change

PTMs of histones are many, ranging from acetylation to sumoylation. As we have discussed, the PTMs of histones regulate the structure of chromatin as well as the accessibility of the DNA to binding proteins, transcription factors, remodelers and the like (Bannister AJ et al 2011), which, in turn, regulate DNA transcription, repair, recombination, replication, and chromatin architecture. As such, it is no wonder that histone PTMs are essential to proper regulation and expression of genes.

Table of Histone PTMs*

Chromatin Modification	Residues Modified	Result	Functions Regulated	Histone Protein Target
Lysine Acetylation	Lysine	Lysine-Ac	Transcription, Repair, Replication, Condensation	H2A (K5), H2B (K12, K15), H3 (K9, K14, K18, K23, K27, K56), H4 (K5, K8, K12, K16, K91)
Lysine Deacetylation	Lysine-Ac	Lysine-Ac to Lysine	Transcription	H4 K16
Lysine Methylation	Lysine-Ac	Lysine-Me1, Lysine-Me2, Lysine-Me3	Transcription, Repair	H1K26, H3K4, H3K9, H3K36, H3K79, H4K20, H3K27. H4K59
Lysine Demethylation	Lysine-Me1-3	Lysine	Transcription, Repair	H3K4, H3K36, H3K9
Arginine Methylation	Arginine	Arginine-Me1, Arginine-Me2	Transcription	H3 (R2, R17, R26), H4R3, H3R8
Phosphorylation	Serine, Threonine	phospho-Serine, phospho-Threonine	Transcription, Repair, Condensation	H1S27, H2AS1, H2AS139, H2BS14, H2BS33, H3T3, H3S28, H3S10, H3T11, H4S1
Ubiquitination	Lysine	Ubiquitinated Lysine	Transcription, Repair	H2AK119, H2BK120, H2BK123
ADP ribosylation	Glutamic Acid	ADP-glutamic acid	Transcription	H2BE2ar1
Deimination	Arginine	Arginine to Citrulline	Transcription	H3, H4
Proline Isomerization	Proline	Proline-cis configuration to Protein-trans configuration	Transcription	H3P30, H3P38
Sumoylation	Lysine	Sumoylated Lysine	Transcription	H4, H2AK126, H2BK7, H2BK6
Biotinylation	Lysine	Biotinylated Lysine	Transcription, Repair	H2AK9, H2AK13, H3K4, H3K9, H3K18, H4K12

*Adapted from Kouzarides T 2007

The most commonly studied and some of the best understood histone modifications are acetylation, methylation and phosphorylation (Waldmann T et al 2013, Virani S 2012, Kouzarides T 2007). In contrast with direct DNA methylation, which is associated with gene repression and the suppression of transcription, histone methylation is associated with both transcriptional activation and repression (Virani S 2012). Methylation on histones occurs at both arginines by protein arginine methyltransferases (PRMTs) and lysine amino acids by lysine methyltransferases in the tail regions of histone H3 and histone H4 and can be multi-layered (Yoshimatsu M et al 2010, Virani S 2012). The methylation or demethylation reactions are catalyzed by a large number of histone methyltransferases or demethylases, and research has shown that the delicate regulation of these enzymes is disrupted in cancer, thereby influencing the dysregulation of gene expression seen in many cancers.

Acetylation of histones, however, is associated with gene activation (Virani S 2012). Acetylation of histones occurs on lysine amino acids and, in doing so, alters their charge and decreases their hold on the DNA, thereby allowing increased access to DNA expression machinery and fostering gene expression (Virani S 2012). Acetylation is controlled by histone acetyltransferases (HATs) or histone deacetylase (HDACs). HATs are associated with a more permissive chromatin state and an increase in gene expression, whereas HDACs are associated with gene repression because they remove acetyl groups from histones (as well as other non-histone proteins), thereby shutting down transcription. Like methylases, HAT and HDAC normal function is often perturbed in cancers, leading to aberrant phenotypes or behaviors.

Example histone modifying enzymes deregulated in various cancers*

Histone modifying enzyme	Target histone modification	Cellular function influenced/changed	Cancers types with deregulation
PRMT6	Histone H4	Enhanced expression; transcriptional activation primarily; influences RNA processing, DNA replication increased proliferation, cell cycle control	Bladder, Lung, Breast
PRMT1	Histone H3	Enhanced expression; transcriptional activation primarily; influences RNA processing, DNA replication increased proliferation, cell cycle control	Bladder, Lung, Breast
MLL	Histone H3	Transcriptional activation; gene fusions	Leukemias
Enhancer of Zeste 2 (EZH2)	Histone H3	Transcriptional repression; increased tumor aggressiveness; increased cellular proliferation	Multiple tumor types
DOT1	Histone H3	DNA repair; increased gene rearrangements	Leukemias
JMJD6	Histone H4	Increased cell proliferation; increased invasiveness	Breast
P300	Histones & other proteins	Loss of tumor suppression	Colon, stomach, intestinal
CBP	Histones & other proteins	Loss of tumor suppression and gene activation	Leukemias and multiple cancers
HDAC1	Histone H4	Overexpression, hypoacetylation	Breast, prostate, colorectal

*Adapted from data taken from Lee YF et al 2012, Cao W et al 2012, Virani S 2012, Esteller M 2006

Accordingly, histone PTM profiles have been found to be different in cancer tissues when compared to normal tissues of age-matched subjects, though it is still unclear whether these changes are causative or merely a symptom of already defective programming (Waldmann T et al 2013). Histone PTM research reveals that a number of histone PTM changes are common in cancer tissues, for example, the widespread loss of acetylation on histone H4, at lysine 16 commonly referred to as H4K16ac, or the loss of the tri-methylated histone H4 on lysine 20 termed H4K20me3 (Fraga MF et al 2005, Waldmann T 2013). Other changes are more restricted to only certain cancers, while still others can even be used to classify a single cancer type into distinct levels of carcinogenesis, such as been shown in prostate cancer, where the actual PTM pattern of methylation at histone 4 lysine 20 is used to classify the level of tumor progression (Behbahani TE et al 2012, Waldmann T et al 2013).

As research into histone modifications and their role continues, it often raises more questions than answers; however, scientists using new methods such microarray analysis and mass spectrometry are making substantial progress and actually connecting the specific PTM epigenetic profiles with the expression analysis of the histone modifying enzymes in the target cells, thereby constructing a global picture of cancer epigenetics in particular cancers (Leroy G et al 2013).

Table of specific histone modifications altered in cancer cells lines*

Histone modification	Results obtained (Leroy G et al 2013)
H3K18acK23un	Reduced in 293 kidney and H1229 lung lines
H3K27me3	Elevated in SAOS bone line
H3K27me3	Elevated in MDA-MB231 and MCF7 breast lines
H3K36me1 and H3K36me2	Elevated in HL60 leukemia line
H3K4me3	Elevated in HCT116 colon line
H3K9me3	Reduced in NB4 and HL60 leukemia lines
H3K9me3 and H3K9me3K14ac	Elevated in MDA-MB231 and MCF7 breast lines
H4K16ac	Reduced in MDA-MB231 and MCF7 breast lines
H4K20me2	Reduced in PC3 prostate line
H4K20me3	Reduced in H1229 lung line

*Adapted from Leroy G et al 2013

In an extension of similar research but instead looking at genomic expression profile data from thousands of tumor samples and using mathematical analyses, researchers developed methods that allowed tissue-independent classification of tumors solely based upon their genetic and epigenetic alternations (Ciriello, G et al. 2013). The aim of such research was to better correlate the epigenetic profiles with cellular phenotypes to allow for better prognosis, treatments and recovery from cancers. Furthermore, research funded under the United States National Institutes of Health (NIH)-sponsored ENCODE (Encyclopedia of DNA Elements) project and its Roadmap Epigenomics Project continue to extend this vision. These projects are aimed at characterizing functional features in DNA, including epigenetic marks such as histone modifications, chromatin accessibility and DNA methylation (Souza E 2015). From that research, a table of prominent histone modifications has emerged that advances understanding of histone PTMs and their influence, changes and effects both during normal health and in diseases like cancer.

Table of prominent histone modifications*

Histone modification or variant	Putative functions
H2A.Z	Histone protein variant (H2A.Z) associated with regulatory elements with dynamic chromatin
H3K4me1	Mark of regulatory elements associated with enhancers and other distal elements, but also enriched downstream of transcription starts
H3K4me2	Mark of regulatory elements associated with promoters and enhancers
H3K4me3	Mark of regulatory elements primarily associated with promoters/transcription starts
H3K9ac	Mark of active regulatory elements with preference for promoters
H3K9me1	Preference for the 5′ end of genes
H3K9me3	Repressive mark associated with constitutive heterochromatin and repetitive elements
H3K27ac	Mark of active regulatory elements; may distinguish active enhancers and promoters from their inactive counterparts
H3K27me3	Repressive mark established by polycomb complex activity associated with repressive domains and silent developmental genes
H3K36me3	Elongation mark associated with transcribed portions of genes, with preference for 3′ regions after intron 1
H3K79me2	Transcription-associated mark, with preference for 5′ end of genes
H4K20me1	Preference for 5′ end of genes

*Adapted from Dunham, I 2012

Noncoding RNAs: RNA-driven epigenetic change

RNA, long thought of as just the molecule that carries the genetic information stored in the gene to machinery to convert it into proteins, is emerging as a major player and source of epigenetic regulation and bioactivity. Moreover, because RNA biogenesis typically precedes protein synthesis, RNA is emerging as an early control point worth examining for many diseases including cancer, and scientists realize that expression profiling must include both protein and RNA expression examinations to be complete. It is not just mRNA that is a target for therapy and study. Growing evidence demonstrates that other forms of RNA, namely noncoding RNAs (ncRNAs), are involved in the regulation of genome organization and gene expression, and indeed, that such noncoding RNAs may be the key to our understanding and controlling of cellular processes in both health and disease (Morris KV et al. 2014).

Noncoding RNAs (ncRNAs) constitute a significant fraction of the transcriptome; some estimates ascribe up to 1% of the genome to encoding noncoding RNA (Birney E et al. 2007, Xie C et al. 2014). Noncoding RNAs are expressed from specific genes like mRNA but not translated into protein. Multiple forms of ncRNAs have been discovered, covering a wide range of sizes and functions, and they can perform functions not only within the nucleus and in concert with DNA structure and function, but also outside the nucleus in the cellular compartment. Additionally, because they are not translated, they affect gene expression largely at the transcription and post-transcriptional levels (Cech TR et al. 2014, Amaral PP et al. 2008), Mattick JS et al. 2006).

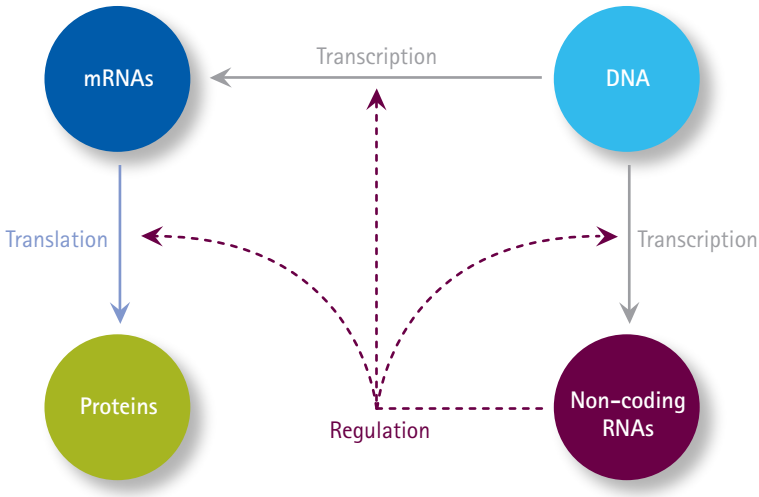


Table of ncRNA types and their function

Abbreviation	Name	Function
aRNA, asRNA	Antisense RNA	RNA message transcription attenuation; message degradation
cis-NAT	Cis-natural antisense transcript	Gene regulation
crRNA	CRISPR RNA	Resistance to parasites, probably by targeting their DNA
gRNA	Guide RNA	mRNA nucleotide modification
lncRNA	Long noncoding RNA	Regulation of gene transcription, epigenetic regulation
miRNA	MicroRNA	Gene regulation
piRNA	Piwi-interacting RNA	Transposon defense, maybe other functions
scaRNA	Small Cajal body-specific RNA	Type of snoRNA; Nucleotide modification of RNAs
siRNA	Small interfering RNA	Gene regulation
SL RNA	Spliced Leader RNA	mRNA trans-splicing, RNA processing
SmY	SmY RNA	mRNA trans-splicing
snoRNA	Small nucleolar RNA	Nucleotide modification of RNAs
snRNA	Small nuclear RNA	Splicing and other functions
TERC	Telomerase RNA Component	Telomere synthesis

Noncoding RNAs can be divided roughly into two distinct groups based upon their sequence size: Short ncRNAs (sncRNAs) which have sequences typically of less than 30 nucleotides in length, and long ncRNAs (lncRNA) which have much longer sequences, from 200 bp to several kilobases in size. Short ncRNAs are further divided into three classes. The three classes of short noncoding RNAs are microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs).

The three sncRNA differ primarily in their size, RNA processing, target choice, and interacting targets. For instance, piRNAs are classed as such because only they bind the Piwi family of RNA binding and cleaving proteins. MiRNAs are expressed from endogenous genes within the genome, whereas siRNA gene sources can be either endogenous or exogenous in origin. For example, siRNAs can arise from endogenous genes such as natural anti-sense transcripts, pseudogenes, and hairpin RNAs, but were originally discovered as silencing RNAs adopted from exogenous origins such as viruses, or transposons (Carthew RW et al. 2009, Golden DE et al. 2008, Mattrick JS et al. 2006). Long ncRNAs (lncRNAs), so far, only have one class and arise from endogenous genes and represent one of the largest number of regulatory RNA molecules discovered. Finally, both sncRNAs and lncRNAs have been shown to modulate gene expression; however, they operate via different but classical epigenetic pathways including chromatin modification, DNA methylation regulation, histone modification regulation, and direct gene silencing (Cech TR et al. 2014).

Short ncRNAs

Epigenetically, short ncRNAs, in the simplest terms, typically operate by binding to a specific target RNA by complementarity and subsequently via interaction with specific protein complexes (RNA-induced silencing complexes (RISCs) complexes) and either, induce message degradation, mediate cleavage or simply block translation in the case of some miRNAs. MiRNA targets tend to be endogenous gene mRNAs whereas siRNAs targets tend to be either mRNA or ncRNA in nature and expressed from various sources. In all cases the identities of the genes to be silenced are specified by the small RNA component, which recognizes each target by traditional Watson-Crick base pairing. Built in to such a RNA-driven gene silencing system, then, is adaptability and reprogrammability. Changing cellular environments or new genomic threats can easily and efficiently be accommodated simply by changing out the miRNAs, or responding adaptively to the threat or stress (Carthew RW et al. 2009). In addition both miRNAs and siRNA share common RNA processing systems, including Dicer enzymes, that initially process their precursors and Ago proteins which coordinate the silencing process (Carthew, RW et al. 2009).

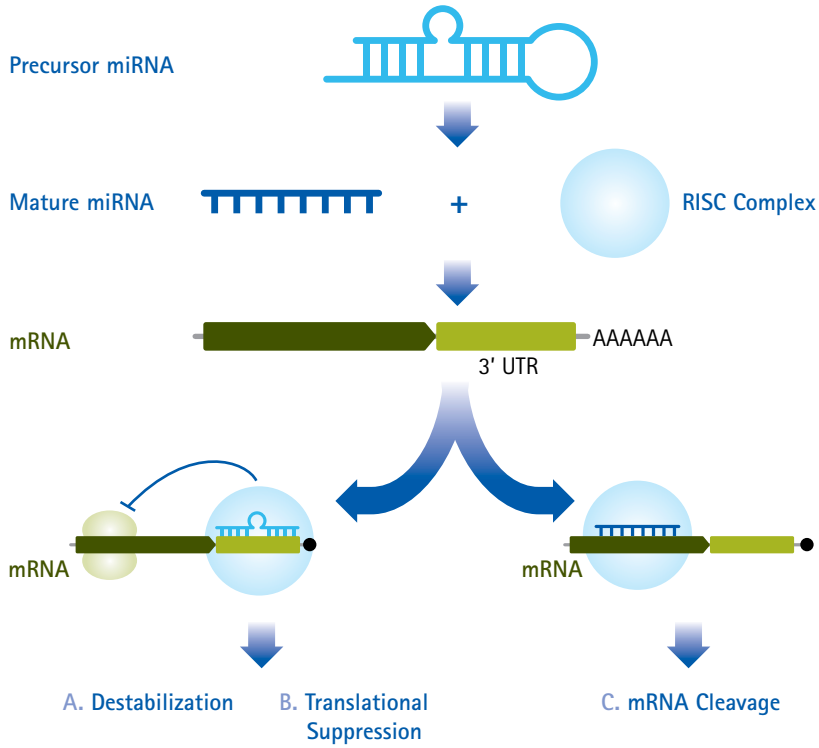


Table of discrete miRNAs found expressed in various cancers*

Description	miRNAs
Glioblastoma multiforme	446
Ovarian serous cystadenocarcinoma	589
Colon and rectum adenocarcinoma	347
Kidney renal clear-cell carcinoma	376
Lung squamous-cell carcinoma	439
Breast invasive carcinoma	419
Uterine corpus endometrioid carcinoma	498
Bladder urothelial carcinoma	507
Head and neck squamous-cell carcinoma	463
Lung adenocarcinoma	472

*Adapted from Jacobsen, A et al. 2013

In cancer, miRNAs have been a major focus of research. Findings from cancer-specific expression profiles studies have found a variety of miRNAs that are associated with various forms of cancers, though the discrete sets differ between cancer types and even between different stages of tumor progression (Jacobsen, A et al. 2013).

In addition, researchers discovered that, in most cancers, miRNAs seem to operate in a negative expression fashion, typically decreasing the expression of its target. However, positively associated pairs were also found (Jacobsen, A et al. 2013).

Finally, researchers examined similar data to find miRNA-mRNA pairings that were active across multiple cancer types and thus may regulate common cancer traits. For instance, using a combination of statistical and rank-based scoring, scientists found that the target interaction between miR-18a, a member of the mir-17-92 cluster, and the transcription factor ZBTB4, first studied in breast cancer, was found in all cancer types and perhaps is relevant for all cancers, not just breast. This finding provides an opportunity to understand and learn how to treat and re-regulate aberrant common sncRNA pathways in hopes of discovering new and effective ways to predict and treat many different cancer types.

Table of top ten inferred recurring negative miRNA–mRNA associations*

miRNA–mRNA Pair	REC Score (the more negative the more likely the association)
miR-29B:NREP	-19.6
miR-18a:ZBTB4	-15.4
miR-30b:SEC23A	-15.4
miR-31:STK40	-15.1
miR-141:ZEB1	-15.1
miR-29a:TDG	-14.8
miR-22:RBMX	-14.0
miR-200c:CNRIP1	-13.7
miR-141:CNRIP1	-13.3
miR-29a:TAF11	-12.6

*Adapted from data taken from Jacobsen, A et al. 2013

Long ncRNAs

Long ncRNAs operate slight differently from sncRNA, as typically, lncRNAs form complexes with specific nuclear and chromatin-modifying enzymes that are then directed by the lncRNA to specific sites where the complex acts to modify chromatin structure or gene expression (Mercer TR 2013). As in the case of sncRNAs, research has shown that lncRNAs impact the regulation of many targets through a variety of cellular processes such as transcriptional regulation, RNA processing and modification, and methylation-mediated gene silencing. Like sncRNAs, lncRNA activity has been linked with human disease, including neurological, immunological, and metabolic diseases, as well as cancer (Wu Z et al. 2014, Cech TR et al. 2014).

Sample lncRNAs associated with cancer*

lncRNA	Associated cancer type	Function in cancer
HOTAIR	Breast, liver, colon, stomach, nasopharynx, esophagus, skin	Gene silencing
PCAT-1	Prostate, colon	Gene silencing
MALAT1	Breast, lung, uterus, pancreas, colon, prostate, liver, osteosarcoma	Protein relocalization of metastasis-associated proteins
MALAT1	Bladder	Promotion of epithelial to mesenchymal transition and cancer progression
H19	Bladder, liver	Promotion of epithelial to mesenchymal transition and cancer progression
CCAT2	Colon	Cancer progression
LET (NPTN intronic transcript 1)	Liver, colon, lung	Tumor suppression regulation
Intronic lncRNAs (PPP3CB,MAP3K14 andDAPK1 loci)	Pancreas	MAPK pathway activation and gene activation

*Adapted from data taken from Shen XH et al. 2014

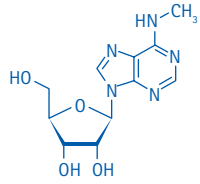
Because lncRNAs constitute one of largest group of regulatory RNAs (most recent NONCODE database entries peg the number at just under 56,000 genes for human; Xie C et al. 2014), understanding the regulation of lncRNA expression is becoming increasingly important as researchers learn more and more about the critical functions and processes that lncRNAs control and the important diseases that they impact. Moreover, research has shown that lncRNAs themselves are often developmentally regulated and expressed in a tissue-specific expression pattern with discrete subcellular distributions, likely indicating their unique importance to gene expression regulation (Gomes AQ et al. 2013).

LncRNAs, unlike many sncRNAs, are expressed via RNA polymerase II-dependent activity and resemble, in many ways, typical protein-coding mRNAs, including having similar processing and splicing mechanisms and exon patterns (Karapetyan A et al. 2013, Xie C et al. 2014 Guttman M et al. 2009, Gerstein MB et al. 2007). Furthermore, lncRNA genes are expressed by many classical transcription factors as protein-coding mRNAs, and, as we have already noted above, many lncRNAs are developmentally regulated and tissue-restricted just like protein-coding RNAs. Thus, the similarity of lncRNA regulatory metabolism to that of protein coding genes makes them subject to the same variety of regulatory and epigenetic influences that we have outlined above including DNA methylation, histone regulation and even silencing.

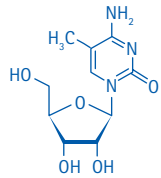
But importantly, because lncRNA gene products themselves are master regulators, the perturbation of their expression can have serious consequences since they themselves are epigenetic operators. For instance, the lncRNA named lincRNA-p21 contains a p53 binding site and serves as a repressor in p53-dependent transcriptional responses (Huarte M et al. 2010). LincRNA-p21 expression is directly induced by p53 upon induction of DNA damage, and loss of lincRNA-p21 affects the expression of hundreds of downstream gene targets normally repressed by p53 including those necessary for p53-dependent apoptosis, thereby implicating lincRNA-p21 dysregulation as key factor in p53-mediated cancers. Finally, because lncRNAs are such powerful epigenetic control agents examining their expression and correlating it to cancer behaviors is allowing scientists to examine lncRNAs not only has markers for particular cancers, but also as potential therapeutic targets for treatments against cancer (Shen XH et al. 2014).

RNA Methylation

It has been known for many years that RNA can be modified post-transcriptionally (Blanco, S et al. 2014). Indeed some 150+ RNA modifications are catalogued (Machnicka MA 2013) and many appear to be related to tRNA and rRNA process refinement. However, two of largest classes of RNA modifications involve RNA methylation on adenosine residues to form N6-methyladenosine (m6A) and methylation on cytosine residues to form 5-methylcytidine (m5C). Because the methylation occurs after the initial transcription, the impact of RNA methylation on gene expression is considered an epigenetic event, and research into the epigenetics surrounding RNA methylation is one of the fastest growing, new epigenetic research arenas.

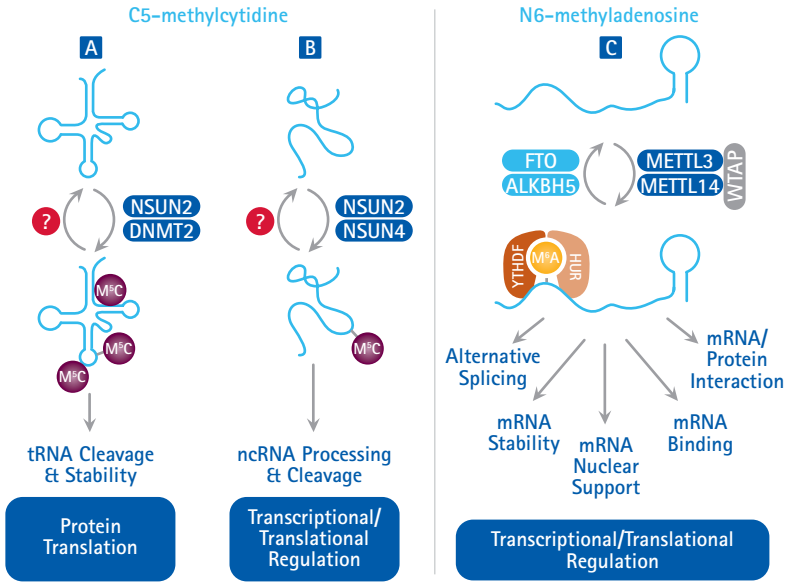


N6-methyladenosine (m6A)



5-methylcytidine (m5C)

Interestingly, N6-methyladenosine has been identified as the most abundant internal modification of messenger RNA in eukaryotes (Wang X et al. 2014, Wei CM et al. 1976). 5-methylcytidine RNA methylation seems to be largely restricted to tRNA and ncRNAs, where it influences tRNA function and processing and/or reading of ncRNA sequences (Blanco S et al. 2014). Until very recently, not much was known about m6A RNA function in mammalian cells, but results gleaned from work done in yeast and plants indicated that m6A RNA methylation was likely to play a critical role in RNA metabolism, cell fate, and perhaps even cell survival. Recent publications have identified m6A modification of mRNA as critical in regulating pluripotency in murine stem cells (Zhao BS et al. 2015). Researchers found that the m6A addition



Regulation and function of RNA methylation :C5-methylcytidine (m5C) is a common modification in (a) tRNAs and (b) other noncoding RNAs (ncRNAs). Processing enzymes catalyze methylation of cytosine-5 but no clear demethylase as been found (a and b). (c) N6-methyl-adenosine (m6A) is an abundant modification in mRNA. Its deposition is dynamically regulated by methylases and demethylases. Methylation levels dictate the fate, processing, interaction of m6A mRNA with other proteins and methylated RNAs . (Adapted from Blanco, S et al. 2014)

reduced the stability of methylated mRNA transcripts in mESCs, and this seems to be a key factor in the maintaining the balance between pluripotency expression and lineage determining expression. This, in turn, makes mRNA methylation important in the orderly differentiation of mESCs (Zhao BS et al. 2015). Fortunately, concurrently with the ability to detect and measure both m6A and m5C RNA methylation has come the ability to study the many enzymes that are critical for their manufacture.

In striking similarity with DNA, researchers have discovered that RNA, too, has enzymes that are methylation-specific writers, erasers, and readers of methylated RNA nucleic acid. Moreover, their expression and functions are essential during embryonic development, as well as for the homeostasis of the reproductive, cardiovascular and central nervous systems. (Blanco S et al. 2014, Fu Y et al. 2014). Furthermore, data from human disease expression studies have linked inappropriate RNA methylation and its effects to a range of human diseases including developmental and neurological disorders, infertility, and obesity, and researchers expect more direct links to other diseases (Fu, Y et al. 2014, Blanco S et al. 2014, McGuinness D et al. 2014, Sibbritt T et al 2013).

Sample of known m6A/m5C methylation enzymes and their relationship with disease*

Gene (Protein)	Affect on m6A/m5C methylation	Disease association
FTO	Demethylation	Attention Deficit Disorder (ADD), Multiple cancers including breast cancer, colorectal cancer, endometrial cancer, pancreatic cancer, prostate cancer, and stomach cancer; epilepsy, neurological disease
ALKBH5	Demethylation	Multiple cancers including bladder cancer
WTAP	Methylation	Leukemia and inflammation
METTL3	Methylation	Multiple cancers, DNA repair disruption, proliferation disorders
METTL14	Methylation	Multiple cancers, cell proliferation and self-renewal
NSUN2	Methylation	Multiple cancers, DNA mutations, proliferation, neurological disorders, growth disorders
TRDMT1	Methylation	Self-renewal, stress tolerance

*Adapted from McGuinness D et al. 2014 & Sibbritt T et al. 2013

Finally, like most epigenetic modifications, recent research demonstrates that RNA methylation, and specifically m6A methylation, is controlled by yet other epigenetic players, including specific miRNAs, and that such regulation is essential pluripotency in stem cells (Chen T et al. 2015, Chhabra R 2015). Thus, it seems that mRNA/ncRNA methylation does indeed represent yet another layer of epigenetic regulation that works in concert with DNA methylation, histone modification, and RNA-driven gene regulation to govern gene expression events.

Concluding Remarks

Epigenetics began as a concept to help explain development, first voiced by Waddington in 1942 as that series of developmental processes that lay between genotype and phenotype (Waddington CH 2012). Today, its definition has been refined, adapted, and finessed into a full-blown scientific research arena. Today, epigenetics refers to changes, changes in phenotype without a change in genotype, but these changes and the mechanisms governing them have a far greater scope and far greater complexities than could ever have been imagined back in 1942.

When the study of epigenetics turns its eyes on to the subject of health, cancer certainly comes to mind as a distinct disease where the power of the environmental change shapes the genotype, and the genotype shapes the phenotype in an ever-evolving circle of adaptation and homeostasis. In this brief review, we have tried to illuminate how the various operators and effectors of epigenetics control and participate in cancer, and we have provided some insight into what today's science understands about how epigenetics and its molecular players all work. Researchers can use this knowledge to help them better understand what the next hypothesis is or what the next experiment might be in their effort to develop more effective cancer treatments and prognoses.

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Appendix A:

Selected Antibodies for Epigenetic Research and Discovery

To search the complete catalog visit: **www.merckmillipore.com/antibodies**

Product Description	Cat. No.
Histone H1	
Anti-acetyl (Lys26) phospho (Ser27) Histone H1.4	06-1372
Anti-acetyl (Lys34) phospho (Ser36) Histone H1	06-1358
Anti-phospho-Histone H1 (Thr165)	06-1370
Anti-phospho-Histone H1, clone 12D11	05-1324
Anti-phospho-Histone H1.B (Ser27)	09-836
Histone H2A	
Anti-acetyl-Histone H2A (Lys5)	07-290
Anti-acetyl-Histone H2A (Lys9)	07-289
Anti-Histone H2A (acidic patch)	07-146
Anti-Histone Macro H2A.1	ABE215
Anti-phospho Histone Macro H2A (Ser137)	09-018
Anti-ubiquityl-Histone H2A , clone E6C5	05-678
Histone H2A.X	
Anti-Histone H2A.X	07-627
Anti-phospho Histone H2A.X (Tyr142)	07-1590
Anti-phospho-Histone H2A.X (Ser139) , clone JBW301	05-636
Anti-phospho-Histone H2A.X (Thr120) , clone 11F5.3	MABE171
Anti-Ubiquityl Histone H2A.X (Lys119)	AB10029
Histone H2A.Z	
Anti-acetyl-Histone H2A.Z (Lys14)	07-719
Anti-acetyl-Histone H2A.Z (Lys10)	07-771
Anti-acetyl-Histone H2A.Z (Lys8)	07-770
Histone H2B	
Anti-acetyl-Histone H2B (Lys120)	07-564
Anti-dimethyl-Histone H2B (Lys5)	07-673
Anti-Histone H2B	07-371
Anti-monoubiquityl Histone H2B (Lys119), clone 7B4	MABE453
Anti-phospho-Histone H2B (Ser14)	07-191
Anti-Ubiquityl-Histone H2B, clone 56	05-1312
Histone H3	
Anti-acetyl Histone H3 (Lys4)	ABE223
Anti-acetyl Histone H3 (Lys9)	ABE18
Anti-acetyl-Histone H3 06-599	06-599
Anti-acetyl-Histone H3 (Lys14)	07-353
Anti-acetyl-Histone H3 (Lys14), clone EP964Y	04-1044
Anti-acetyl-Histone H3 (Lys23)	07-355
Anti-acetyl-Histone H3 (Lys27)	07-360
Anti-acetyl-Histone H3 (Lys56)	07-677-I
Anti-acetyl-Histone H3 (Lys56)	07-677-I
Anti-acetyl-Histone H3 (Lys9)	06-942
Anti-dimethyl Histone H3 (Arg26), Asymmetric	ABE411
Anti-dimethyl-Histone H3 (Arg2), clone 20.2	04-808
Anti-dimethyl-Histone H3 (Lys4), clone AW30	04-790
Anti-dimethyl-Histone H3 (Lys4), clone AW30	04-790
Anti-dimethyl-Histone H3 (Lys79), clone NL59	04-835

Appendix A:

Selected Antibodies for Epigenetic Research and Discovery (continued)

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Product Description	Cat. No.
Histone H3 (continued)	
Anti-dimethyl-Histone H3 (Lys9)	07-441
Anti-Histone H3, K27M mutant	ABE419
Anti-Histone H3.1, clone 1D4F2	MABE952
Anti-Histone H3.1/H3.2	ABE154
Anti-Histone H3.3	09-838
Anti-monomethyl-Histone H3 (Lys36)	ABE244
Anti-monomethyl-Histone H3 (Lys27)	07-448
Anti-monomethyl-Histone H3 (Lys9)	ABE101
Anti-trimethyl Histone H3 (Lys27)	ABE44
Anti-trimethyl Histone H3 (Lys36)	ABE305
Anti-trimethyl Histone H3 (Lys79)	07-952
Anti-trimethyl-Histone H3 (Lys4), clone 15-10C-E4	05-745R
Anti-trimethyl-Histone H3 (Lys9), clone 6F12-H4	05-1242
Histone H4	
Anti-acetyl Histone H4 (Lys12)	06-1352-I
Anti-acetyl-Histone H4 (Lys12), rabbit monoclonal	04-119
Anti-acetyl-Histone H4 (Lys16)	07-329
Anti-dimethyl Histone H4 (Arg3), Asymmetric	ABE441
Anti-dimethyl-Histone H4 (Arg3), Asymmetric	07-213-I
Anti-monomethyl Histone H4 (Lys20)	07-1570
Anti-trimethyl-Histone H4 (Lys20), rabbit monoclonal	04-079
Anti-trimethyl-Histone H4 (Lys20)	07-463
Chromatin-Associated Proteins	
Anti-ATF4	ABE387
Anti-BAF180	ABE70
Anti-Brd4	ABE1391
Anti-Chd2, clone 8H3	MABE873
Anti-CTCF	07-729
Anti-DBC1	ABE1950
Anti-Dnmt1, clone DNM-2C1	MABE306
Anti-DOT1L	07-2066
Anti-E3 ubiquitin-protein ligase UHRF1, clone 7C8	MABE945

Appendix A:

Selected Antibodies for Epigenetic Research and Discovery (Continued)

To search the complete catalog visit: **www.merckmillipore.com/antibodies**

Product Description	Cat. No.
Chromatin-Associated Proteins (continued)	
Anti-EZH2	07-689
Anti-Friend of PRMT1	ABE234
Anti-GABPA	ABE1845
Anti-HDAC1	ABE260
Anti-HDAC10	ABE16
Anti-HDAC11	07-1548
Anti-HDAC3	06-890
Anti-HDAC4	ABE262
Anti-HP1v, clone 42s2	05-690
Anti-JMJD1A	09-823
Anti-JMJD2A	09-809
Anti-JMJD6	09-812
Anti-KLF5 (Krüppel-like factor 5)	07-1580
Anti-LSD1	09-058
Anti-MeCP2, clone 4H7	MABE328
Anti-MLL/HRX, CT., clone 9-12	05-765
Anti-MLL-C	ABE240
Anti-MSL3-like 1	ABE467
Anti-NRF-1	ABE1844
Anti-Nuclear factor 1/C	ABE1387
Anti-P/CAF	AB9962
Anti-p300 CT, clone RW128	05-257
Anti-phospho RNA Polymerase II (Ser2), clone 3E7C7	MABE953
Anti-PRDM14	ABD121
Anti-PRMT1, clone 7D2	MABE431
Anti-PRMT6	ABE124
Anti-RNA polymerase II subunit B1 (phospho-CTD Ser-5), clone 3E8	04-1572
Anti-SIRT1, clone 10E4	04-1557
Anti-Sp1	07-645
Anti-TET2, clone hT2H 21F11	MABE462
Anti-WAC, CT	ABE471
Anti-WHSC1/NSD2, clone 29D1	MABE191

Appendix B:

Selected Kits, Assays and Reagents for Epigenetic Research and Discovery

To search the complete catalog visit: **www.merckmillipore.com/epigenetics**

Product Description	Cat. No.
Chromatin IP Kits	
EZ Magna ChIP® A/G Chromatin Immunoprecipitation Kit	17-10086
EZ-Magna ChIP® A - Chromatin Immunoprecipitation Kit	17-408
EZ-Magna ChIP® G - Chromatin Immunoprecipitation Kit	17-409
EZ-Magna ChIP® HiSens Chromatin Immunoprecipitation Kit	17-10461
EZ-Magna ChIP® HT96 Chromatin Immunoprecipitation Kit	17-10078
Magna ChIP® A - Chromatin Immunoprecipitation Kit	17-610
Magna ChIP® A/G Chromatin Immunoprecipitation Kit	17-10085
Magna ChIP® G - Chromatin Immunoprecipitation Kit	17-611
Magna ChIP® HiSens Chromatin Immunoprecipitation Kit	17-10460
Magna ChIP® HT96 Chromatin Immunoprecipitation Kit	17-10077
Magna ChIP²™ - Human Promoter 244K Microarray Kit	17-1001
ChIP-Seq™ Kits	
Magna ChIP-Seq™ Chromatin Immunoprecipitation and Next Generation Sequencing Library Preparation Kit	17-1010
PureGenome™ Low Input NGS Library Construction Kit	17-10492
ChIP Beads and Reagents	
Magna ChIP® Protein A Magnetic Beads	16-661
Magna ChIP® Protein A+G Magnetic Beads	16-663
Magna ChIP® Protein G Magnetic Beads	16-662
Protein A Agarose/Salmon Sperm DNA	16-157
Protein G Agarose/Salmon Sperm DNA	16-201
Chromatin Preparation and Optimization	
PureEpi™ Chromatin Preparation and Optimization Kit	17-10082
DNA Methylation: Bisulfite Modification	
CpGenome™ Direct Prep Bisulfite Modification Kit (50 reactions)	17-10451
CpGenome™ Direct Prep Bisulfite Modification Kit (200 reactions)	17-10452
CpGenome™ Direct Prep-96 Bisulfite Modification Kit	17-10454
CpGenome™ Turbo Bisulfite Modification Kit	S7847
DNA Methylation–Methylated DNA Isolation and Analysis	
CpG MethylQuest™ DNA Isolation Kit 17-10035	17-10035
CpGenome™ 5-hmC Quantitation Kit 17-10091	17-10091
DNA Methylation–Control DNA	
CpGenome™ Universal Methylated Mouse DNA	S8000
CpGenome™ Methylted and Non-methylated Human DNA Set	S8001
CpGenome™ Methylated Human DNA	S8001M
CpGenome™ Non-methylated Human DNA	S8001U
CpGenome™ 5-mC & 5-hmC Human DNA Standards	S8003
CpGenome™ 5-mC & 5-hmC Mouse DNA Standards	S8004
CpGenome™ 5-mC and 5-hmC DNA Set	S8005
CpGenome™ 5-hmC DNA	S8005H
CpGenome™ 5-mC DNA	S8005M
CpGenome™ Unmethylated DNA	S8005U

Appendix B:

Selected Kits, Assays and Reagents for Epigenetic Research and Discovery (continued)

To search the complete catalog visit: **www.merckmillipore.com/epigenetics**

Product Description	Cat. No.
RNA Binding Protein (RIP) Kits	
Magna Nuclear RIP™ (Cross-Linked) Nuclear RNA-Binding Protein Immunoprecipitation Kit	17-10520
EZ-Magna Nuclear RIP™ (Cross-Linked) Nuclear RNA-Binding Protein Immunoprecipitation Kit	17-10521
Magna Nuclear RIP™ (Native) Nuclear RNA-Binding Protein Immunoprecipitation Kit	17-10522
EZ-Magna Nuclear RIP™ (Native) Nuclear RNA-Binding Protein Immunoprecipitation Kit	17-10523
Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	17-700
EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	17-701
Noncoding RNA ChIRP Kits	
Magna ChIRP™ RNA Interactome Kit - Isolation and characterization of noncoding RNA:chromatin complexes	17-10494
EZ- Magna ChIRP™ RNA Interactome Kit - Isolation and characterization of noncoding RNA:chromatin complexes	17-10495
Magna ChIRP™ Negative Control Probe Set	03-307
Magna ChIRP™ NEAT1 lncRNA Probe Set	03-308
Magna ChIRP™ TERC lncRNA Probe Set	03-309
Related Small Molecules	
SRT1720-CAS 925434-55-5, 10 MG, SIRT1 activator	567860-10MG
InSolution™ SRT1720, HCl - CAS 1001645-58-4, 25 mM solution, SIRT1 activator	530748
HMTase Inhibitor V, UNC0224 - CAS 1197196-48-7, 5 MG	382193-5MG
HMTase Inhibitor II, Chaetocin - CAS 28097-03-2, 500 µg	382191-500UG
JMJD2 Inhibitor, 5-carboxy-8HQ - CAS 5852-78-8, 5 MG	420201-5MG
JMJD Histone Demethylase Inhibitor III, 5 MG	420202-5MG
Histone Methyltransferase EZH2 Inhibitor, DZNep - CAS 120964-45-6, 2 MG	252790-2MG
Related Additonal Products	
AbSurance™ Histone H2A, H2B, H4 Antibody Specificity Array	16-665
AbSurance™ Histone H3 Antibody Specificity Array	16-667
AbSurance™ Complete Core Histone Antibody Specificity Array	16-668
AbSurance™ Pro Histone Peptide Microarray	16-671
Magna GrIP™ Rack (8 well)	20-400
Magna GrIP™ HT96 Rack (for 96 well plates)	17-10071

Appendix C:

TECHNOLOGY HIGHLIGHT

Cancer RNA detection in live cells with SmartFlare™ RNA Detection Probes

Cancer biomarkers have been used to identify transformed cells within heterogeneous tissues and cell lysates only retrospectively. Identifying RNA biomarkers in live cells provides the unique opportunity to analyze the impact of tumor heterogeneity on the behavior of transformed cells and to enrich for unique cells based on RNA markers using fluorescence-activated cell sorting.

Heterogeneity Analysis and Cancer Cell Sorting

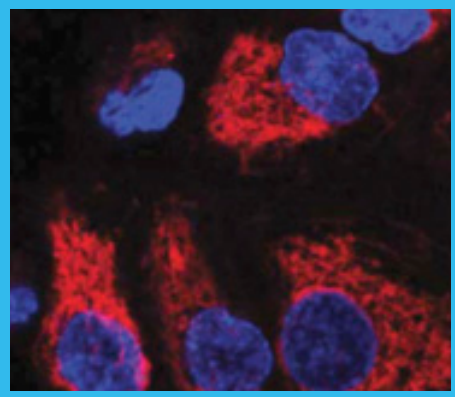
Elucidating the role of cancer biomarkers requires great effort and can be complicated when heterogenous populations produce confounding experimental results. Enrichment of live cells based on RNA expression can be achieved with SmartFlare™ probes and cell sorting. Additionally the sorted cells remain completely viable and unchanged following detection, enabling downstream analyses, such as antibody staining, functional assays and RT-PCR.

Single Cell RNA Analysis

Identifying cells with altered gene expression that exist in a mixed population can be difficult with standard techniques. Most RNA detection methods require lysis of a group of cells which can dilute the message of interest and do not identify which cells were expressing the RNA or to what degree. Performing live cell detection allows for the identification of single cell expression and provides a comparison of relative expression levels within the sample of interest. All of that without destroying or harming your sample.

Finding Needles in Haystacks

Identifying cancer stem cells within mixed populations with the odds stacked against you can be challenging. Cancer stem cells (CSCs) represent a rare subpopulation of a tumorigenic population. SmartFlare™ RNA detection probes can be used to tag live CSCs regardless of surface protein expression. Probes such as those targeting Nanog have been used successfully to target and isolate CSCs from tumorigenic populations.



Live cell imaging of miR-21 RNA in human prostate cancer cells (DU145) using SmartFlare™ RNA Detection Probes (red); DAPI (blue).

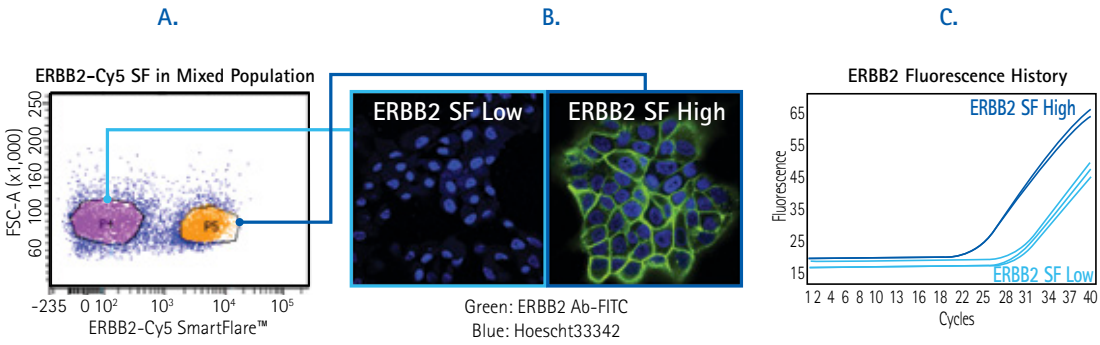
- Live cell detection
- Sequence-based detection of native RNA
- No sample prep
- Open platform
- No toxicity

Appendix C:

TECHNOLOGY HIGHLIGHT

Cancer RNA detection in live cells with SmartFlare™ RNA Detection Probes (continued)

Sorting of mixed cell population based on RNA expression



A mixed population of cells was sorted into subpopulations based on ERBB2 SmartFlare™ probe intensity (A). When the sort products were stained with anti-ERBB2 antibody (B, green), the ERBB2 SmartFlare™-high cells (corresponding to BT474 cells) were positive. The qRT-PCR analysis confirmed the difference in ERBB2 mRNA levels (C).

SmartFlare™ live cell RNA detection probes for human cancer targets

AKT1	CCL5	EPCAM	GRB2	IRAK1	LIN28A	4-Oct	SHC1	TSC2
AKT2	CCND1	ERBB2	GSK3B	IRS1	LTK	PAK1	SMO	TWIST1
AKT3	CD14	ERG	HGF	JAK2	MDM2	PAX6	SOS1	VEGF
APC	CDKN1B	FBXW7	HIF1A	JUN	MET	PTEN	STAT1	
BCL2	c-Myc	FGF2	HRAS	KDR	MUC1	PTGS2	STAT3	
BDNF	CSF1R	FGFR1	HSPB1	KIT	MYC	RAC1	STAT5A	
BIRC2	CTNNB1	FGFR2	ICAM1	KLF4	MYD88	RAF1	TGFA	
BIRC3	CXCR4	FGFR3	IDH1	KRAS	NANOG	RBL2	TLR4	
BIRC5	DICER1	FGFR4	IFNG	KRT19	NGF	RET	TNF	
BTK	EGFR	FN1	IGF1	LGR5	NGFR	RPS6KB1	TP53	

Cell lines with known compatibility

2102Ep	MC3T3-E1 Cl 4	WEHI-3	CCF-STTG1	Hu Astrocytes	Mouse NSC	SK-N-AS
A431	MCF10-2A	10T 1/2	Daoy	Hu Chondrocytes	NCI-H510A	SU-DHL-1
ARPE-19	MDA-MB-231	A172	Daudi	Hu Schwann	NCI-H69	T47D
HCC1806	PC-3	A549	F9	HUVEC	NCI-N87	T98G
HCN1A	RT4-D6P2T	AGS	HDF	INS-1	NIH/Ovcar3	THP-1
Hs578t	Saos-2	BT474	Hec-1-A	Jurkat	PANC-1	U373MG
HT1080	SCC25	BxPC-3	Hepa1c1c7	LNCaP	Rat Astrocytes	U87MG
Hu cardiac myocytes	SK-MEL-28	C33 a	Hepa1-6	MCF-7	RAW 264.7	UMR-106
Hu MSC ES derived	SK-N-SH	CAKI-2	HL-60	MDA-MB-435	RIN-m5F	WI-38
Hu skeletal muscle	U251	Capan1	HT29	Ms Astrocytes	RT4	iPS
H9 Human ES						

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Spain: 901 516 645 Option 1
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Lit No. RF1052ENEU BS-GEN-15-11277 4/2015