Energy Balance and Cancer 11

Nathan A. Berger *Editor*

# Epigenetics, Energy Balance, and Cancer

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# Preface

## Epigenetics, Energy Balance, and Cancer: Impact of Environmentally Induced Genetic Change Without Changing the Genome

Mechanistic studies of energy balance and cancer have largely focused on regulatory effects at the level of metabolic, inflammatory, and endocrine signaling cascades and growth factors, all of which are influenced by genetic interactions with the environment. While genetic effects are hereditary and are primarily determined by the base pair sequence of DNA, recent studies indicate that major effects can be determined also by epigenetic factors that modify gene expression without altering DNA base pair sequence. Thus cancer results from aberrations that occur in oncogene activation and/or tumor suppressor gene inactivation, processes frequently shown to be due to genetic alterations in DNA base pair sequence. These sequence alterations in the genome may exist in the germline where they can be associated with hereditary cancers or they may commonly arise in somatic cells due to gene mutations and/or rearrangements where they are associated with sporadic cancers. In addition to modifications in DNA base pair sequences, genetic readout, resulting in neoplasia, may result from a series of biochemical and structural modifications of DNA and/or its surrounding chromatin proteins to activate or silence oncogene or tumor suppressors resulting in drastic changes in cell biochemistry and growth control. These processes by which the genetic readout is altered by chemical modification of DNA and/or chromatin, without changing DNA sequence, are designated as epigenetics. These changes predominantly include methylation of DNA bases, posttranslational modification of chromatin proteins, and synthesis of noncoding RNA capable of altering chromatin tertiary structure and function, as well as stability of gene transcripts. Posttranslational modifications of histones and other chromatin proteins include multiple processes such as methylation, acetylation, phosphorylation, ubiquitination, ADP ribosylation, and many others controlled by a myriad of different enzymes that attach to, respond to, or remove these groups. These

so-called writers, readers, and erasers can be impacted by environmental factors including dietary composition and quantity, physical activity, and others to drastically impact genetic readout.

Although these epigenetic modifications do not alter DNA sequence, their resultant phenotypes are heritable through multiple generations of cell division and may become transgenerational, passing from parent to offspring. Moreover, epigenetic changes in DNA and chromatin can be induced by a variety of exogenous chemical agents including toxins and xenobiotics and by endogenous processes such as inflammation and generation of free radicals. In addition, it is now becoming increasingly clear that biobehavioral phenomenon related to energy balance such as obesity, exercise, caloric restriction, and stress may all change DNA epigenetic marks and may, in fact, affect incidence and pathology of chronic conditions like obesity, diabetes, and cancer. At the same time, epigenetic changes and processes may provide novel targets for precision medicine interventions to prevent or disrupt the linkage between obesity and cancer.

This volume of Epigenetics, Energy Balance, and Cancer will connect the exciting state-of-the-art research activities in epigenetics and energy balance as they both relate to cancer. The reader will obtain a clear understanding of the multiple processes involved in epigenetic modification of DNA and chromatin, which aspects of energy balance induce these changes, how they affect chronic diseases such as obesity and diabetes, and how these changes impact cancer in general and in specific organ systems. The reader will be introduced also to consideration of how epigenetic changes may impact cancer prevention and control and how they may serve as therapeutic targets.

As in the past, we are fortunate to have chapters contributed by leading authorities from around the world for this volume on Epigenetics, Energy Balance, and Cancer. We extend our sincere thanks to all for their efforts and contributions in preparing this volume. In Chap. 1, Andrew D. Kelly and Jean-Pierre J. Issa (Fels Institute for Cancer Research and Molecular Biology, Lewis Katz School of Medicine at Temple University) describe the multiple mechanisms of epigenetic modification and how they relate to cancer. Chapter 2, written by James J. Morrow and Peter C. Scacheri (Case Western Reserve University), describes the higher-order impact of gene enhancer arrangements in chromatin and how enhancer dysfunction may contribute to cancer. Chapters 3–7 then describe how different environmental processes impact the epigenome to contribute to cancer. Thus Chap. 3, written by Francine H. Einstein (Albert Einstein College of Medicine), describes early life epigenetic effects on obesity, diabetes, and cancer. Chapter 4, written by Eswar Shankar and Sanjay Gupta (Case Western Reserve University), focuses on how nutrition and lifestyle factors impact epigenetics of cancer, and in Chap. 5, David A. Skaar, Randy L. Jirtle, and Catherine Hoyo (North Carolina State University) describe environmentally induced alterations in the epigenome and how they may affect obesity and especially cancer in minority populations. In Chap. 6, Giuseppe Lippy (Universitaria di Parma, Italy), Elisa Danese (University of Verona, Italy), and Fabian Sanchis-Gomar (Research Institute of Hospital 12 de Octubre ("i+12"), Madrid Spain) review effects of stress and exercise on epigenetics and

cancer. The impact of gut microbiota on cancer is a relatively new and rapidly growing research area whose epigenetic effects are described in Chap. 7 written by Joice Kuroiwa-Trzmielina (Garvan Institute of Medical Research) and Luke B. Hesson (Lowy Cancer Research Centre and Prince of Wales Clinical School, Sydney Australia). The concluding Chaps. 8–11 describe the epigenetic impact of energy balance on cancer in specific organ systems. In Chap. 8, Andrew M. Kaz (VA Puget Sound Health Care System) and William M. Grady (Fred Hutchinson Cancer Research Center) discuss epigenetics in obesity and esophageal cancer. In Chapter 9, Ruifang Li and Paul A. Wade (National Institute of Environmental Health Sciences) discuss epigenetics in obesity and colon cancer. In Chap. 10, David Heber, Susanne M. Henning, and Zhaoping Li (David Geffen School of Medicine, University of California Los Angeles) describe epigenetic effects of energy balance on prostate cancer, and in Chap. 11, Herbert Yu (University of Hawaii Cancer Center) and Melinda L. Irwin (Yale School of Public Health) discuss how physical activity may influence breast cancer through epigenetic mechanisms.

Overall this volume on Epigenetics, Energy Balance, and Cancer provides a state-of-the-art and transdisciplinary description of the rapidly evolving field of epigenetics and its potential role in mediating the impact of energy balance on cancer. It should serve as an important resource for students at all levels and for practitioners in related fields seeking to better understand this important area of evolving science. It should likewise provide important background information for development of research strategies to further interrogate, promote, and/or interrupt these epigenetic regulatory processes as well as new targets for precision medicine.

Cleveland, OH, USA Nathan A. Berger

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# Chapter 1
# Epigenetics and Cancer

**Andrew D. Kelly and Jean-Pierre J. Issa**

**Abstract** Epigenetic characteristics are heritable features, propagated through cell division, that contribute to cellular identity independent of DNA sequence. Such characteristics include DNA methylation, covalent histone modifications, and non-coding RNA-dependent gene regulation. Over the past few decades, epigenetic changes in cancer have become recognized and widely accepted as important contributors to malignant transformation. Such alterations result in a transcriptional program that promotes molecular diversity and provides a selective advantage to cancer cells through tumor suppressor gene silencing and aberrant oncogene activation. Causes of epigenetic aberrations remain under active investigation and include at least stochastic changes associated with aging, mutations in epigenetic modifying enzymes, and altered cellular metabolism through changing the metabolite repertoire. A number of therapies targeting epigenetic modifiers have been approved by the FDA for cancer treatment, and many others are in clinical trials. Ongoing research is focused on better understanding mechanisms contributing to the altered epigenome, how the altered epigenome contributes to malignant transformation, and how epigenetic therapies can be best applied clinically to patients most likely to benefit from them.

**Keywords** Epigenetics • DNA methylation • Histone modification • Non-coding RNA • Epigenetic therapy • Precision medicine

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## Background

Epigenetics can be broadly defined as the biological mechanisms governing cellular identity and heritable phenotypes arising from characteristics other than DNA sequence [1, 2]. These distinct phenotypes arise as a result of epigenetic regulation of gene expression, which falls into several major categories: DNA methylation, histone modification, and non-coding RNA-dependent regulation (Table 1.1).

## *DNA Methylation*

### Characteristics of DNA Methylation

Methylation refers to the biochemical process of adding a methyl group to a molecule. In mammals, DNA methylation occurs predominantly at the five position of cytosine residues within CG dinucleotides and is distributed in specific genomic compartments [3]: CpG islands are regions of high CG dinucleotide density that lie near the promoters of up to 70 % of genes and are unmethylated under normal conditions [3–5]. CpG island methylation status has been causally linked to transcription regulation, where methylated CpG islands lie upstream of transcriptionally repressed genes and unmethylated CpG islands lie upstream of transcriptionally active (or ready) genes (Figs. 1.1 and 1.2a, b) [6]. Under normal conditions, non-CpG island sites have high levels of methylation throughout the genome [7–9]. Methylated CpG islands characterize instances of irreversible gene silencing in adult cells, including X-inactivation, imprinting, and germ cell-specific genes (Figs. 1.1 and 1.2b). Methylation of CpG sites outside of islands is also associated with transcriptional regulation; for example, methylation within gene bodies is positively correlated with expression, methylation within gene promoters is associated with repression, and CpGs are often unmethylated at active enhancers [10, 11]. However, non-CpG island methylation is dynamically regulated and may serve as a rheostat of expression rather than a mechanism of permanent regulation.

### Regulation of DNA Methylation

DNA methylation status is regulated by several enzymes that "write" and "erase" CG methylation, including the DNA methyltransferases (DNMTs) and the ten-eleven-translocation (TET) family of proteins (Fig. 1.1, Table 1.2). There are three DNMTs in humans, all of which require S-adenosyl-methionine (SAM) as a methyl donor. DNMT1 is primarily a "maintenance methyltransferase" due to its affinity for hemimethylated (newly synthesized) DNA; it acts during cell division to copy methylation to each newly synthesized strand [12, 13]. In contrast, DNMT3a and DNMT3b show no preference for hemimethylated DNA and thus are "de novo methyltransferases" able to establish new methylation states on unmethylated DNA [14].

**Table 1.1** Types of epigenetic modifications

| Modification | Functional association |
|---|---|
| *DNA methylation* | |
| Promoters | |
| Promoter CpG islands | Irreversible transcriptional repression |
| Promoter non-CpG islands | Inversely associated with transcription |
| Enhancers | Inversely associated with transcription |
| Gene bodies | Positively associated with transcription |
| Intergenic | Heterochromatin/repeat silencing |
| *Histone modifications* | |
| H3K4me$^{1}$ | Active enhancers and promoters |
| H3K4me$^{2/3}$ | Active promoters |
| H3K9me$^{1}$ | Active promoters |
| H3K9me$^{2/3}$ | Silenced promoters |
| H3K27me$^{1}$ | Active promoters |
| H3K27me$^{2/3}$ | Silenced promoters |
| H3K36me$^{3}$ | Transcribed gene bodies |
| H2B.K5me$^{1}$ | Transcribed gene bodies |
| H4K20me$^{1}$ | Active enhancers and promoters |
| H3K79me$^{2}$ | Active transcriptional elongation |
| H3K9Ac | Active promoters |
| H3K27Ac | Active enhancers and promoters |
| H2A.R3me$^{2}$ | Active promoters |
| H3R2me$^{2}$ | Active transcription |
| H3R8me$^{2}$ | Silenced promoters |
| H3R17me$^{2}$ | Active transcription |
| H3R42me$^{2}$ | Active transcription |
| H4R3me$^{2}$ | Unclear association with transcription |
| *Variant histones* | |
| H2A.X | DNA repair machinery recruitment |
| H2A.Z | Active promoters and DNA repair machinery recruitment |
| macroH2A | Cellular differentiation |
| H3.3 | Active transcription |
| *Non-coding RNAs* | |
| miRNAs | Repression of gene expression via transcript destabilization or translational repression |
| RNAi | Repression of gene expression via transcript destabilization; maintenance of histone and DNA methylation states (shown in yeast) |
| IncRNAs | Regulation of gene expression via interaction with histone modifiers, hybridization with mRNA |

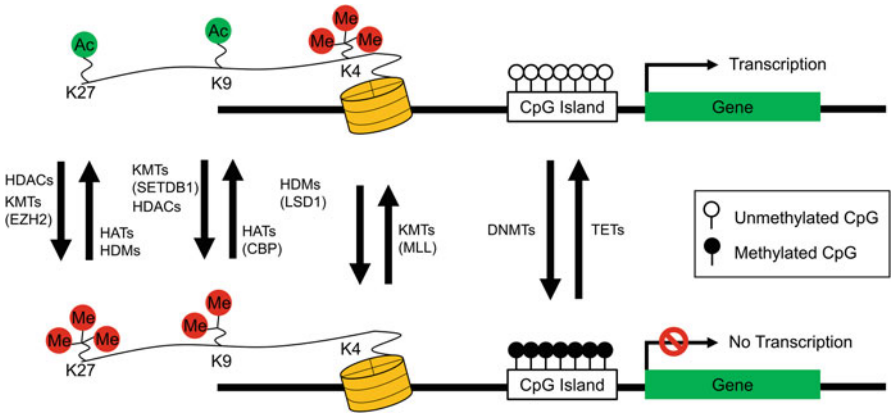


**Fig. 1.1** Model of epigenetic regulation of gene expression at promoters. Epigenetic marks including histone modification and DNA methylation at promoters collectively act as a molecular switch that controls gene expression. *5mC* 5-methylcytosine, *Me* methyl, *Ac* acetyl, *HDM* histone demethylase, *KMT* histone lysine methyltransferase, *HDAC* histone deacetylase, *HAT* histone acetyltransferase, *DNMT* DNA methyltransferase, *TET* ten-eleven translocation family member

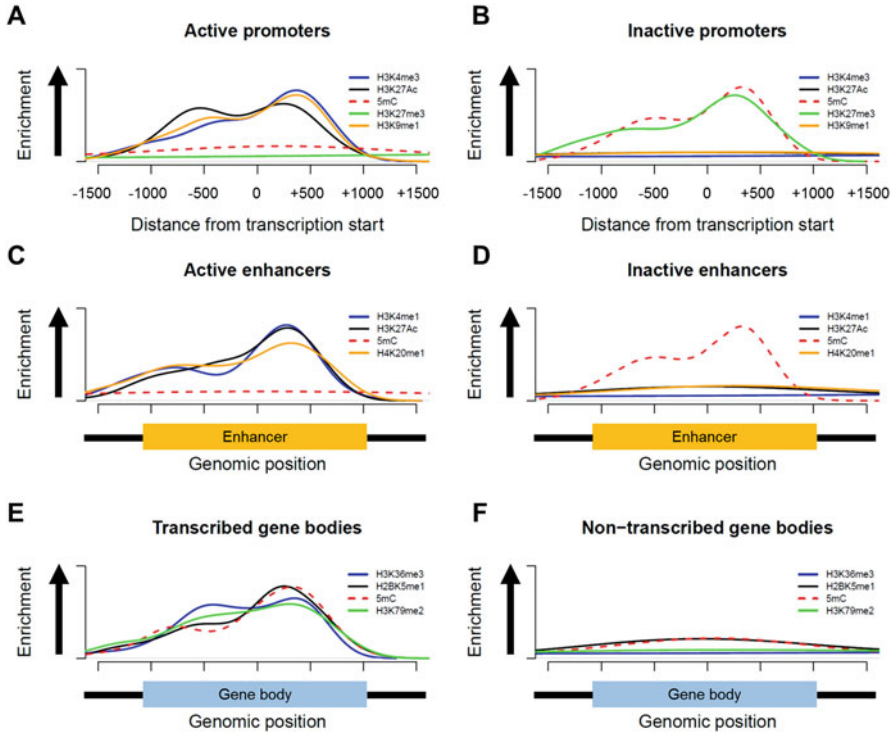


**Fig. 1.2** Epigenetic features in different genomic compartments. Epigenetic modifications present at an active (**a**) and inactive (**b**) promoter; an active (**c**) and inactive (**d**) enhancer; and within an actively transcribed (**e**) and non-transcribed (**f**) gene body as determined by ChIP-seq analysis

**Table 1.2** Epigenetic regulators

| Modification type | Writers | Readers | Erasers | Example genes mutated in cancer |
|---|---|---|---|---|
| DNA methylation | DNA methyltransferases (DNMTs) | Methyl binding domain proteins (MBDs) | Ten-eleven translocation family (TET) | *DNMT3A*, *DNMT3B*, *TET1*, *TET2* |
| *Histone modifications* | | | | |
| Histone acetylation | Histone acetyltransferases (HATs) | Bromodomain and extra-terminal (BET) proteins | Histone deacetylases (HDACs) | *CREBBP*, *KAT3B*, *KAT6A*, *KAT6B*, *BRD1*, *BRD2*, *BRD3*, *BRD4*, *TRIM33*, *PBRM1* |
| Histone methylation | Histone lysine methyltransferases (KMTs) | PHD finger (PHF) and chromodomain-containing (CHD) proteins | Histone demethylases (HDMs) | *MLL1*, *MLL2*, *MLL3*, *EZH2*, *KDM5A*, *KDM5C*, *KDM6A*, *CHD1*, *CHD3*, *CHD4*, *CHD5*, *CHD7*, *CHD8*, *EHMT1* |

The TET family of proteins are critical players in demethylation; to perform their catalytic functions TET enzymes require $Fe^{2+}$ and α-ketoglutarate as cofactors [15]. In the presence of these cofactors, TETs convert 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine. These oxidized bases are poor substrates for DNMT1 and are copied as unmethylated during cell division; some oxidized cytosines may also be converted back to cytosine through a base-excision repair-dependent pathway [15–18].

In addition to proteins involved in the writing and erasing of DNA methylation, there are "reader" proteins that recognize and bind to methylated DNA. These related proteins all contain a methyl-CpG binding domain (MBD), and most of them play a mechanistic role in reducing transcription of methylated gene promoters through direct repression and the recruitment of silencing complexes that modify histones and reduce accessibility to transcription factors [8, 19].

## *Histone Modifications*

### Characterization of Histone Modifications

In addition to DNA methylation, histone proteins play a critical role in the epigenetic regulation of gene expression (Fig. 1.1). Histone tails contain lysine and arginine residues which can be covalently modified, leading to different regulatory effects depending on the specific residue involved and type of covalent modification. Di- and trimethylation at lysine 4 of histone H3 (H3K4me2/3) often occurs near the transcription start site of actively transcribed genes, while monomethylation (H3K4me1) at the same residue is associated with enhancer regions (Fig. 1.2) [20, 21]. Monomethylation at lysine 9 or lysine 27 of histone H3 (H3K9me1, H3K27me1) is also associated with actively transcribed genes, while H3K9me3 and H3K27me3 have been linked to silencing of transcription (Figs. 1.1 and 1.2) [20–22]. Trimethylation of lysine 36 in histone H3 (H3K36me3) can often be found downstream of transcription start sites of actively transcribed genes, as can monomethylation of lysine 5 of histone H2B (H2BK5me1) [20]. Finally, monomethylation of lysine 20 on histone H4 (H4K20me1) is associated with active transcription at both enhancer and promoter sequences, while trimethylation at this amino acid is associated with transcriptional repression in both contexts (Fig. 1.2c, d) [22]. Histone lysine residues can also show acetylation that is generally associated with an open chromatin conformation which allows for active gene expression. In particular, acetylation of lysine 27 on histone H3 (H3K27Ac) at both promoter and enhancer regions has been strongly associated with active transcription [22], as has H3K9Ac. Acetylation of other lysine residues has also been linked to active transcription, although to a lesser degree [22]. Other histone marks at different amino acids include dimethylation of arginine residues (Table 1.1), which are largely associated with active transcription of specific gene sets, although they are not as well characterized as lysine modifications [20, 23–27].

### Regulation of Histone Modifications

As with DNA methylation, histone modifications are established and maintained by specific sets of enzymes with "writing," "reading," and "erasing" functions (Fig. 1.1, Table 1.2). The histone mark writers include the protein families of histone lysine methyltransferases (KMTs) and histone acetyltransferases (HATs), while the erasers include histone demethylases (HDMs) and histone deacetylases (HDACs). The histone mark readers consist of the bromodomain-containing proteins, Tudor domain-containing proteins, chromodomain proteins, MBT domain proteins, and PHD fingers.

KMTs catalyze methylation reactions on specific lysine residues of histone proteins resulting in mono-, di-, or trimethylation states. The vast majority of histone KMTs contain a conserved SET domain and utilize SAM as a cofactor [28, 29]. Recent data have elucidated multiprotein complexes responsible for catalyzing specific histone methylation reactions and highlight the specificity of KMTs for par-

ticular histone marks [30–32]. H3K27 methylation, for instance, depends on the activity of EZH2 within the polycomb repressive complex [31]. Similarly, H3K4 methylation is dependent on the MLL/COMPASS complex [30]. The notable exception to SET domain-containing KMTs is the H3K79-specific enzyme DOT1L, which does not contain a SET domain [33, 34]. Numerous aberrations involving KMTs have been reported in cancer and, as discussed in section "Altering the Cancer Epigenome: Epigenetic Therapies and Precision Medicine Applications," are an active area of investigation for novel therapeutics [35].

Histone acetyltransferases (HATs) catalyze acetylation reactions on specific amino acids of histone proteins, most often on the side chains of lysine residues. There are three major protein families of nuclear HATs including the GNAT, p300/CBP, and MYST families [35, 36]. The HATs are highly conserved and, as with KMTs, are often present in complex with other proteins [37–40]. Importantly, mutations in HATs have been reported in a number of malignancies [35].

The removal of acetyl groups from histone proteins is catalyzed by histone deacetylases (HDACs). There are two major families of HDACs: the classical HDACs and the sirtuins [36]. The HDACs can be further subdivided into four distinct classes. The classical HDACs include HDAC1-10 and comprise all of the HDACs belonging to class I, class II, and class IV. The sirtuin family members are SIRT1-7 and comprise class III. Classical HDACs require $Zn^{2+}$ as a cofactor, while the sirtuins require $NAD^{+}$ [36]. Like HATs and KMTs, in addition to necessary cofactors, HDACs often must act as components of multiprotein complexes to perform their function [41–43]. HDAC1 and HDAC2, for instance, interact with RbAp48 and RbAp46 to form the functional nucleosome remodeling and histone deacetylation (NuRD) complex [42]. Another example is seen in HDAC3 which must complex with AKAP95 and HA95 to deacetylate histone H3 [43].

The final histone mark erasers to discuss are the HDMs, which catalyze demethylation reactions on specific methylated histone amino acid residues. There are two known classes of HDMs in humans: LSD1 which is dependent on FAD, and the Jumonji family of demethylases which, like TET family members, are $Fe^{2+}$ and α-ketoglutarate dependent [44–46]. Histone demethylases appear to act in a substrate-specific manner, although the mechanisms that manifest this specificity are still under investigation [45, 47, 48]. As discussed below, LSD1 and other histone demethylases are currently being studied as potential therapeutic targets in cancer [35, 49–52].

The readers of histone modifications are specific to the type of epigenetic marks present; the set of proteins reading methylation is distinct from those that read acetylation. Acetylation of histones is read by the bromodomain-containing proteins, which are highly conserved and consist of 46 distinct proteins in humans [53]. Although other domains in these proteins are very diverse, the fold region of the bromodomains themselves are highly homologous across different proteins [53]. Importantly, the affinity of bromodomain-containing proteins for specific acetylated lysine residues is not particularly strong, and therefore other interaction domains are likely key to their activities [53]. As with other histone modifying proteins, inhibitors of acetylation readers are being developed and tested for the treatment of certain cancers [54, 55].

An analogous group of proteins involved in reading histone methylation fall into several classes [56]. The Tudor domain-containing proteins, chromodomain-containing proteins, MBT domain proteins, and PHD fingers have all been implicated in reading histone methylation [56]. Tudor and chromodomains bind preferentially to trimethylated lysine residues, while MBT readers bind preferentially to mono- and dimethylated lysines, and PHD fingers act on H3K4me3 modifications [57–68]. Functionally, histone methylation readers act to promote either repression or activation of gene transcription, depending on the specific methylation mark and reader [56]. Taken together, the set of histone modifications and machinery involved underscore the complexity of epigenetic regulation and offer many candidate hypotheses regarding mechanisms of tumorigenesis and approaches for novel drug development.

### Histone–DNA Interactions

Regulation of gene expression depends on the interactions between DNA and the histone proteins around which it is wrapped. Generally, histone modifications dictate the density of nucleosomes and their location with respect to transcription start sites. Nucleosome sliding into or out of transcription start sites is one of the mechanisms that integrate the effects of various histone modifications on gene expression. Furthermore, certain variant histone proteins are associated with different epigenetic states (Table 1.1), including H2A.Z and H3.3 which are associated with active transcription, and H2A.X, which is associated with DNA double strand breaks [69]. There are also complex interactions between DNA methylation and histone modifications. For example, DNA methylated promoters are mechanistically linked to histone deacetylation and are often associated with H3K9me2, while there is a general inverse correlation between DNA methylation and PCG-mediated H3K27 methylation [70]. Recent data extend these findings by suggesting that TET1 preferentially binds to acetylated regions regulated by the histone modifying enzyme HDAC2 and subsequently induces demethylation at promoters of differentiation-related genes. These data highlight both direct and indirect interactions between DNA methylation and histone modifications in specific gene regulation and the importance of cross-talk across epigenetic layers of control [71].

## *Non-coding RNAs*

In addition to DNA methylation and histone modifications, non-coding RNA species comprise another layer of epigenetic regulation of gene expression. MicroRNAs (miRNAs) are short RNA molecules which exert their regulatory effects either through mRNA destabilization or translation inhibition [72–75]. miRNA biogenesis is dependent on several proteins including DICER, DROSHA, and DGCR8. In addition, miRNA regulatory activity requires Argonaute family proteins, which

collectively form a complex known as the RNA-induced silencing complex (RISC) [72, 76]. The downstream effect of this process is modulation of gene expression at the transcript and protein levels. A related mechanism known as RNA interference (RNAi) was discovered in yeast which utilizes the same RISC components to silence genes at the transcript level [77]. Interestingly, RNAi was shown to have critical importance in maintaining histone H3K9 methylation, although it is unclear whether this mechanism exists in mammals [77]. Long non-coding RNAs (lncRNAs) have more recently been identified to exert regulatory effects on gene expression through multiple mechanisms [78–85]. Dosage compensation of the X-chromosome is known to involve accumulation of a lncRNA, *Xist*, and lncRNAs have also been shown to modulate transcript abundances via a sequence complementarity-based "sponge" effect [80, 82, 84, 85]. Recent data have shown interactions between lncRNAs and histone modifying enzymes (e.g., for the HOTAIR lncRNA), and lncRNAs have been found to be important to enhance their function, once again highlighting the cross talk and intricacies of epigenetic regulation [81].

### *Importance of Epigenetic Regulation of Organism Development*

Epigenetic mechanisms are critically important in development and gene regulation throughout life [11]. The importance of DNA methylation in normal development is highlighted by observations that mice deficient in Dnmt3a die shortly after birth, and homozygous Dnmt3b-deficiency is embryonic lethal [14]. In further dissecting epigenetic development mechanisms, it was shown that DNA from differentiated nuclei, transferred to oocytes, undergoes erasure of DNA methylation, and that global DNA methylation undergoes reprogramming in germ cells [86–88]. Epigenetic inactivation of pluripotency genes by H3K9me2 and H3K9me3 has been demonstrated during early embryogenesis [89]. A distinct class of genomic loci, known as imprinted regions, rely on DNA methylation to differentially express transcripts from maternal or paternal alleles, and these imprints are erased and rewritten in germ cells [86]. Well-characterized developmental and cognitive syndromes caused by imprinting defects highlight the importance of accurate epigenetic reprogramming of germ cells. Examples of such disorders include Prader-Willi syndrome and Beckwith-Wiedemann syndrome [90, 91]. Collectively, these data suggest that precise and complex epigenetic switches are required for normal physiology.

## Epigenetic Changes in Cancer

Given the clear importance of epigenetic regulation in normal development and physiology—and in particular, the maintenance of cellular differentiation states—it is not surprising that the epigenome is altered in many cancers. Alterations involving all aspects of epigenetic regulation have been reported, spanning from DNA

methylation to histone modifications and aberrant ncRNA expression. One interesting theme that has emerged is the prevalence of epigenetic aberrations in hematologic malignancies, highlighting their unique biology. In recent years novel drugs have taken aim at faulty epigenetic machinery and hold promise as effective cancer therapies in certain settings. Below we will discuss what is known about epigenetic changes in cancer and how they might be exploited therapeutically.

## *Aberrant DNA Methylation in Cancer*

### Observed DNA Methylation Changes in Cancer

DNA methylation changes observed in cancer have now become widely accepted. The first reports of global hypomethylation in cancer were published in 1979 in rat liver tumors, and subsequently this phenomenon was shown to occur largely in gene bodies and at repetitive DNA elements [92–94]. Hypermethylation of tumor suppressor gene (TSG) promoters, leading to their silencing, has also been well established in human tumors [95–97]. The first report of epigenetic TSG silencing in cancer demonstrated promoter methylation-associated loss of *RB1* expression in retinoblastoma [98]. Shortly after, the same mechanism was implicated in lost *VHL* expression in clear cell renal carcinoma [96]. In addition, p16 expression was shown to be lost via CpG island methylation in cell lines and primary tumors derived from breast, prostate, colon, lung, and renal cancers [95].

Building on these observations, it was discovered that hypermethylation of a distinct subset of genes occurs in some colorectal tumors, defining a CpG island methylator phenotype (CIMP) [99]. CIMP+ tumors in the colon were shown to have distinct and consistent biological characteristics, including an association with *BRAF* mutations, *MLH1*, and p16 methylation, and microsatellite instability [99]. Since the initial report of CIMP+ colorectal cancer, hypermethylator phenotypes have been described in almost all malignancies [100–102]. The consistent nature and tumor-suppressor function of hypermethylated genes in CIMP+ cancers suggest that DNA methylation may play a causal, rather than coincidental, role in cancer formation providing a selective advantage for tumor growth. Of clinical importance, patients with CIMP+ cancers have been reported to have different outcomes in different cancer settings. Patients with CIMP+ colon cancer, AML, or glioma have been reported as having favorable prognoses, while CIMP+ renal cell carcinoma is associated with a relatively poor outcome [100, 101, 103].

In addition to CIMP, extensive data have supported the notion of DNA methylation changes associated with normal aging playing a role in cancer. Genes that gain methylation in cancer are enriched for genes that gain methylation with age [99, 104, 105]. In murine models and human samples, an acceleration of age-related DNA methylation changes has been observed in MDS and AML [104]. Many of these genes are involved in normal development and cellular differentiation [104]. Also of note, many age-associated CpG sites consistently modified in cancer are

associated with specific genomic aberrations, many of which—including *WT1* and *IDH2*—are known to regulate DNA methylation [105]. These observations, along with the observation that nonmalignant clonal hematopoiesis with mutations in epigenetic regulators occurs in healthy aging adults at risk of cancer, further support a causal role for DNA methylation in cancer [106].

#### Causes of Aberrant DNA Methylation in Cancer

Although still under active investigation, some of the causes of altered DNA methylation have been recently elucidated. Specific nonsynonymous point mutations in isocitrate dehydrogenase (*IDH*) 1 and 2 have been observed in AML and in high-grade gliomas [102]. Under normal conditions, IDH1/2 catalyzes the conversion of isocitrate to α-ketoglutarate in the citric acid cycle. However, the R132H mutation in *IDH1* and both the R140Q and R172K mutations in *IDH2* have been shown to alter this enzymatic activity such that isocitrate is converted to 2-hydroxyglutarate (2-HG) [107]. Recent data support the role of 2-HG in promoting hypermethylation through inhibition of TET family members [102, 107, 108]. Not surprisingly, mutations in TET family proteins themselves have also been shown to cause distinct aberrant DNA methylation changes [109–111]. It remains unclear how specific genomic compartments are targeted for hypermethylation in these contexts; however, some recent data suggest that perhaps specific proteins interacting with TET enzymes (e.g., WT1 interactions with TET2) may play a role [112, 113]. In addition to hypermethylation-associated mutations in *IDH1/2* or TET family members, the de novo methyltransferase, *DNMT3A*, is mutated in up to 30 % of AML cases resulting in profound *hypomethylation*, and some recent data suggest that these mutations confer a poor clinical prognosis [114, 115]. Open questions in this area include whether there are other causes of aberrant DNA methylation patterns in cancer and how aberrations in the different epigenetic writers and erasers manifest specific patterns.

### *Aberrant Histone Modification in Cancer*

Changes in the transcriptional program seen in cancer can arise as a result of DNA methylation changes, but, indeed, histone modifying enzymes have also been implicated in a number of cancer settings (Table 1.2). Translocations and mutations involving the HAT, *CBP*, for example, have been observed in certain subtypes of AML, B-cell lymphoma, colorectal, breast, and gastric cancer [37, 116–118]. In particular, *CBP* is a frequent fusion partner in *MLL*-rearranged AML [37, 116]. Another example of a HAT aberration implicated in cancer is the MYST family member MOZ, which forms a fusion product with TIF2 in AML [119]. Recent data have also supported a role for MOZ in leukemia and lymphoma development, implicating translocations that lead to aberrant activation of MYC [120, 121].

HDACs have also been recognized as important epigenetic modulators in cancer [122]. In AML HDACs have been shown to modulate gene expression in response to the known fusion gene PML-RARα [123]. In this disease setting HDACs clearly promote an altered transcriptional program that may be abrogated with HDAC inhibition [124, 125]. Aberrant HDAC interactions with BCL6 have also been reported in other settings, and clinical data using HDAC inhibitors in lymphoma have shown efficacy [126, 127]. There are several HDAC inhibitors approved by the FDA for cancer indications which are described in section "Altering the Cancer Epigenome: Epigenetic Therapies and Precision Medicine Applications."

In addition to acetylation regulators, there have been a number of histone methyltransferases and demethylases demonstrated to play a role in various cancers [35]. Some of the best studied KMTs in cancer include the MLL family of genes, which are fusion partners in a distinct subtype of MLL-rearranged AML [128, 129]. In addition, mutations and other aberrations in the histone methyltransferase *EZH2* have been reported in myeloid, lymphoid, and solid epithelial malignancies [130–134]. Although the precise mechanisms remain under investigation, the H3K79 methyltransferase, DOT1L, has also been reported as essential in MLL-rearranged leukemia, and inhibitors of DOT1L have shown promise in preclinical studies of AML [135–137]. Finally, histone demethylases have recently emerged as mechanistic contributors to cancer phenotypes, with mutations in Jumonji family proteins being reported in several tumor types [35]. The non-Jumonji family demethylase, LSD1, has also been implicated in hematologic malignancies, and its mechanistic role remains under active study [49, 50, 138]. As discussed below, histone methyltransferase and demethylase inhibitors are currently being tested in clinical trials.

The final category of histone modifiers known to be altered in cancer include the reader proteins. The most prominent example of which is in nuclear protein in testis (NUT) midline carcinoma where aberrations involving *BRD3* or *BRD4* are often observed [139]. Mechanistically, it has been shown that fusion genes involving *BRD3* or *BRD4* and *NUT* are critical to maintaining cells in an undifferentiated state [140]. Moreover, inhibition of these fusion proteins was shown to cause cell cycle arrest and differentiation [140]. Although bromodomain-containing proteins are most strongly associated with NUT midline carcinoma, data in other settings have suggested that inhibiting these proteins may dampen the tumorigenic activity of MYC and represent a promising therapeutic avenue [141, 142].

## *Non-coding RNA Expression in Cancer*

Aberrant ncRNA expression has been reported in many cancers and is now widely accepted as one mechanism of tumorigenicity. Since their discovery there have been thousands of articles detailing changes in expression of various miRNAs in cancer [72, 74]. miRNAs can be tumor suppressors or oncogenes, depending on their target transcripts and cellular contexts; oncogenic miRNAs target tumor suppressor mRNAs, while tumor suppressor miRNAs target oncogenic mRNAs [72, 75].

Although miRNAs demonstrate tissue specific activities in the context of cancer, certain clusters have relatively consistent functions as either tumor suppressors or tumor promoters [143]. One example is seen in the oncogenic miR-17-92 cluster, which has been reported as overexpressed in lymphoma, breast, lung, gastric, colorectal, and pancreatic cancers, and was causally linked to inactivation of tumor suppressor genes including *BCL2L11*, *PTEN*, and *CDKN1A* [72].

LncRNAs have a less established role in cancer, but recent data highlight their potential importance [79, 144]. One interesting example is seen with HOTAIR-mediated targeting of the polycomb repressive complex 2 (PRC2) in breast and colorectal cancer [35, 81, 145]. Another example is seen in non-small cell lung cancer and gastric cancer, where the lncRNA ANRIL is upregulated, leading to PRC2 recruitment to tumor suppressor gene loci [146]. These data suggest that lncRNAs can mediate neoplastic phenotypes through a variety of mechanisms, including modulation of other epigenetic regulators.

## Altering the Cancer Epigenome: Epigenetic Therapies and Precision Medicine Applications

In recent years it has become apparent that targeting the epigenome of cancers may be a viable therapeutic strategy [147]. Over the past decade novel epigenetic therapies have started making their way to the clinic (Tables 1.3 and 1.4). The first FDA-approved epigenetic therapy was azacitidine. Along with decitabine, these DNA methyltransferase inhibitors are now approved for certain indications in MDS and AML. More recently, HDAC inhibitors such as vorinostat, belinostat, and panobinostat have been FDA approved for treatment of peripheral cutaneous T-cell

**Table 1.3** FDA-approved epigenetic therapies for cancer

| Compound | Target | Approved cancer indication |
|---|---|---|
| *DNMT inhibitors* | | |
| Azacitidine[a] | DNMTs | AML, MDS |
| Decitabine[b] | DNMTs | AML, MDS |
| *HDAC inhibitors* | | |
| Belinostat | HDAC classes I, II | PTCL |
| Panobinostat | HDAC classes I, II, IV | Multiple myeloma |
| Romidepsin | HDAC class I | CTCL, PTCL |
| Valproic acid[c] | HDAC classes I, IIa | Investigated in multiple cancer types |
| Vorinostat | HDAC classes I, II, IV | CTCL |
| *LSD1 inhibitors* | | |
| Tranylcypromine[c] | LSD1 | Investigated in multiple cancer types |

[a]Approved in the USA for AML in patients with 20–30 % blasts
[b]Approved in the USA for MDS and in Europe for AML in patients aged 65 and older
[c]FDA approved for noncancer indication

**Table 1.4** Investigational epigenetic therapies

| Compound | Target | Status | Clinical trial inclusion |
|---|---|---|---|
| *DNMT inhibitors* | | | |
| Guadecitabine | DNMTs | Phase III | AML, MDS |
| *HDAC inhibitors* | | | |
| Abexinostat | HDAC classes I, II, IV | Phase I | Sarcoma, lymphoma |
| ACY-241 | HDAC6 | Phase I | Multiple myeloma |
| AR-42 | HDAC classes I, II, IV | Phase I | Investigated in multiple cancers |
| CUDC-907 | HDAC classes I, IIb | Phase I | Lymphoma, multiple myeloma |
| CXD101 | HDAC class I | Phase I | Investigated in multiple cancers |
| Entinostat | HDAC class I | Phase I | Investigated in multiple cancers |
| Givinostat | HDAC classes I, II | Phase II | Investigated in multiple cancers |
| Mocetinostat | HDAC class I | Phase II | MDS, urothelial carcinoma |
| Resminostat | HDAC1, HDAC3, HDAC6 | Phase II | Investigated in multiple cancers |
| Ricolinostat | HDAC6 | Phase II | Lymphoma, multiple myeloma |
| *EZH2 inhibitors* | | | |
| CPI-1205 | EZH2 | Phase I | Lymphoma |
| EPZ-6438 | EZH2 | Phase II | Lymphoma |
| *LSD1 inhibitors* | | | |
| GSK2879552 | LSD1 | Phase I | AML, small cell lung cancer |
| *BET inhibitors* | | | |
| CPI-0610 | BRD2, BRD3, BRD4, BRDT | Phase I | Lymphoma, AML, MDS, multiple myeloma |
| TEN-010 | BRD2, BRD3, BRD4, BRDT | Phase I | Investigated in multiple cancers |
| BAY1238097 | BRD2, BRD3, BRD4, BRDT | Phase I | Investigated in multiple cancers |
| OTX015 | BRD2, BRD3, BRD4, BRDT | Phase I | Investigated in multiple cancers |
| INCB054329 | BRD2, BRD3, BRD4, BRDT | Phase I | Investigated in multiple cancers |
| BMS-986158 | BRD2, BRD3, BRD4, BRDT | Phase I | Investigated in multiple cancers |
| FT-1101 | BRD2, BRD3, BRD4, BRDT | Phase I | AML, MDS |
| GSK525762 | BRD2, BRD3, BRD4, BRDT | Phase I | NUT midline carcinoma |
| *IDH inhibitors* | | | |
| AG-881 | IDH1, IDH2 | Phase I | AML, MDS, chondrosarcoma, glioma, cholangiocarcinoma |

(continued)

**Table 1.4** (continued)

| Compound | Target | Status | Clinical trial inclusion |
|---|---|---|---|
| AG-120 | IDH1 | Phase I | AML, cholangiocarcinoma, chondrosarcoma, glioma |
| IDH305 | IDH1 | Phase I | Investigated in multiple cancers |
| AG-221 | IDH2 | Phase I | AML, glioma, cholangiocarcinoma |

lymphoma, and many other HDAC inhibitors are currently in clinical trials for a range of malignancies (Tables 1.3 and 1.4). In this section, we will discuss the state of epigenetic therapies falling into discrete categories based on their targets.

## *DNA Methyltransferase Inhibitors*

DNMT1 inhibition is the principal mechanism by which DNA hypomethylating agents act [148]. There are currently two FDA-approved DNMT1 inhibitors: azacitidine and decitabine. Both of these agents are approved for the treatment of patients with myelodysplastic syndromes and are considered a low-intensity treatment option in some patients with AML. Ongoing clinical trials of the novel DNMT1 inhibitor, guadecitabine (SGI-110), have shown promise in AML and MDS, with a recent phase 1 study showing induced DNA hypomethylation, and clinical responses in 6 of 74 AML patients and 6 of 19 MDS patients [149]. In addition to potential benefits as a monotherapy, DNMT1 inhibitors may also sensitize cancer cells to chemotherapy or immunotherapy [150, 151].

## *HDAC Inhibitors*

The first FDA-approved HDAC inhibitor, vorinostat, has an indication for T-cell lymphoma. Over the past decade, many other HDAC inhibitors have been developed, three of which have also gained FDA approval: romidepsin, belinostat, and panobinostat. In clinical practice, roughly one-third of peripheral T-cell lymphoma patients achieve an objective response to HDAC inhibition, suggesting that although effective in subsets of patients, novel predictive biomarkers are still needed [152]. There are many other HDAC inhibitors of varying HDAC class selectivity currently in clinical trials for treatment of a range of malignancies, and remain a promising therapeutic avenue (Table 1.4).

## *EZH2 Inhibitors*

Recent data showing the reliance of certain lymphomas on EZH2 activity suggested that its inhibition may be a viable therapeutic strategy. There are currently two EZH2 inhibitors in clinical trials, both of which are being tested in lymphoma. EPZ-6438 is currently in a phase I/II trial (NCT01897571), and CPI-1205 is currently being tested in a phase I study (NCT02395601).

## *LSD1 Inhibitors*

The histone demethylase LSD1 was shown to play a crucial mechanistic role in promoting neoplastic transformation in AML, small cell lung cancer, and esophageal cancer, with promising preclinical data on LSD1 inhibition in these settings [50, 51, 138, 153]. As a result the LSD1 inhibitor GSK2879552 is being tested in a phase I study recruiting patients with small cell lung cancer and AML (NCT02034123, NCT02177812). The FDA-approved monoamine oxidase inhibitor, tranylcypromine, has also been shown to inhibit LSD1 and is also being tested in two studies in its capacity as an anticancer agent in AML and MDS (NCT02273102, NCT02261779) [153].

## *Bromodomain and Extra-Terminal (BET) Protein Inhibitors*

Inhibition of the class of histone modification readers, the bromodomain-containing proteins, has been supported by preclinical data as a viable strategy for therapy in certain tumor types, including lymphoma, AML, ALL, and NUT midline carcinoma [141, 142, 154, 155]. More specifically, in a model of multiple myeloma, BET inhibition was shown to downregulate *MYC* transcription, leading to cell cycle arrest and decreased proliferation in vitro, and prolonged survival in vivo [142]. Following successful preclinical data, a number of small molecule compounds were developed and are currently in clinical trials. The majority of these are in hematologic malignancies, although there are several active trials recruiting patients with other advanced solid tumors (Table 1.4).

## *IDH Inhibitors*

Based on observations of recurrent oncogenic mutations in *IDH1/2* seen in AML, novel inhibitors specific for the IDH1 R132H, IDH2 R140Q, and IDH2 R172K mutant proteins were developed and demonstrated preclinical efficacy. Currently there are four mutant IDH inhibitors in clinical trials, all of which are recruiting patients with hematologic malignancies harboring one of the known oncogenic mutations in *IDH1* or *IDH2*. AG-881 inhibits both mutant IDH1 and IDH2 and is

currently in two phase I trials recruiting patients with AML, MDS, chondrosarcoma, glioma, and cholangiocarcinoma (NCT02492737, NCT02481154). AG-120 is selective for mutant IDH1 and is also in two phase I studies of patients with AML, cholangiocarcinoma, chondrosarcoma, glioma, and other advanced tumors with *IDH1* mutations (NCT02073994, NCT02074839). IDH305 is an additional mutant IDH1-selective inhibitor which is being tested in a phase I study of patients with any advanced malignancy harboring *IDH1* R132 mutations (NCT02381886). Finally, AG-221 is a mutant IDH2-selective inhibitor being tested in two phase I studies of patients with AML, glioma, and cholangiocarcinoma harboring *IDH2* mutations (NCT01915498, NCT02273739).

### *Opportunities for Precision Medicine*

It has now been shown that specific genomic or epigenomic states may be associated with differential responses to therapy, and this concept is making its way into clinical trials. For instance, the novel IDH inhibitors described above are selective for oncogenic mutant forms of the protein known to generate 2-HG. Thus, in ongoing phase I studies, patients are selected to possess the specific mutations being targeted. In addition to mutant protein forms, however, distinct epigenotypes may ultimately stratify patients with other genetic backgrounds as likely responders to hypomethylating agents. One published example of this phenomenon is the expression of the miRNA, miR-29b, as a candidate predictor of response to decitabine in AML [156]. Epigenetic biomarkers can also predict response to more classical therapies. For example, MGMT methylation in brain tumors is associated with a better response to temozolomide, and an enhancer DNA methylation signature predicts response to intensive chemotherapy in AML [157, 158]. Ongoing research in this area continues to focus on understanding what genetic and epigenetic patterns may be associated with response to novel epigenetic agents and may ultimately identify integrated genetic/epigenetic predictive biomarkers for precision cancer treatment.

## Conclusions and Future Research

Over the past decade we have seen an explosion in knowledge of the epigenetic determinants of cellular identity, normal physiology, and malignant transformation. Several distinct mechanisms of aberrant DNA methylation and histone modification have been uncovered, and the transcriptional programs altered by these epigenetic phenomena have become better understood. As a result of this basic knowledge, we have started to target the epigenome for cancer treatment; there are currently several FDA-approved epigenetic drugs with cancer indications, and many others are in preclinical or clinical studies. Going forward it will be critical to harness the power of genomic technologies to identify patients most likely to benefit from epigenetic therapies. It seems likely that DNA methylation patterns in conjunction with mutational

backgrounds may identify likely responders to epigenetic therapies in a manner analogous to how somatic point mutations identify likely responders to mutant protein inhibition. Finally, going forward it is important to not only continue developing novel drugs against epigenetic targets but also attempt to rationally combine epigenetic therapies, conventional cytotoxic therapies, other targeted agents, and immunotherapy.

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# Chapter 2
# Epigenetics, Enhancers, and Cancer

**James J. Morrow and Peter C. Scacheri**

**Abstract** The first molecular studies of cancer revealed DNA mutations resulting in activation of proto-oncogenes or loss of function of tumor suppressors. These studies formed the basis for the canonical model of the molecular etiology of cancer: DNA mutations in a small number of protein-coding genes lead to increased rates of cellular proliferation. Subsequent to these landmark studies, technological advances have allowed for more thorough characterization of the molecular changes that occur during malignant transformation. Among these advances has been the discovery that gene enhancer elements are key drivers of gene expression in eukaryotic cells and that enhancer elements can be identified through epigenomic profiling of specific chemical modifications on chromatin. In the past several years, epigenomic profiling studies have revealed that enhancer activity is broadly reprogrammed in cancer cells as they transform from their normal precursors during carcinogenesis. Moreover, this enhancer reprogramming results in activation and suppression of specific transcriptional programs and drives many of the characteristic behaviors of tumor cells. In light of these findings, the classic model of malignant transformation has been broadened to include epigenetic changes in enhancer activity as well as DNA mutations to coding genes. In this chapter, we summarize what is known about normal enhancer function in cellular development and differentiation. Next, we outline the most widely utilized technologies for interrogating enhancer activity across epigenomes. We then describe how these approaches have yielded new insights into tumor biology both through enhancer profiling alone and integration of this information with DNA mutations to the genes that regulate enhancer activity and to enhanc-

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ers themselves. Finally, we describe how these insights have begun to be translated into novel approaches for the diagnosis and treatment of cancer. The goal of this chapter is to give a broad background on both the foundation and the current state of the art of this field to familiarize the reader with the progress that has been made and the exciting new potentials for improved patient care resulting from the study of epigenetic enhancer dysregulation in cancer.

**Keywords** Gene enhancers • Super enhancers • Variant enhancer loci • Chromatin writers • Chromatin erasers • Enhancer dysfunction • Enhancer RNA • eRNA

## Enhancer Function and Signature Epigenetic Features

Gene enhancer elements are short stretches of non-coding DNA that function in cells to regulate spatiotemporal gene expression. Across eukaryotes, enhancers have been shown to mediate dynamic state transitions that occur as cells progress through development and are also important for maintenance of terminally differentiated cells [1–6]. As such, enhancers form the foundation of cellular states. Enhancers regulate gene expression by coordinating transcription factor (TF) DNA-binding activity with assembly of the transcription initiation and elongation apparatuses at target gene promoters. As their name suggests, the collective function of these regulatory elements is to enhance the rate of transcription of target genes in a coordinated fashion. To accomplish this, enhancers physically loop to promoters, forming complex three-dimensional chromatin structures within cell nuclei that dictate the transcriptional output of eukaryotic cellular genomes. Intriguingly, enhancers themselves are also transcribed and this transcription is thought to play a functional role in the regulation of gene expression [7]. It has been proposed that the enhancer RNA (eRNA) produced through enhancer transcription collaborates with enhancer DNA sequences that contain TF-binding motifs to facilitate the interaction of enhancers and promoters with key TFs to drive gene transcription [8]. In light of the fundamental role of enhancers in regulating gene expression, a great deal of knowledge about the molecular underpinnings of cell phenotypes can be gleaned from understanding the binding partners and target genes of enhancers in cell types of interest. However, such studies were historically confounded by several complex aspects of the nature of enhancer function. First, enhancers contain no consensus DNA sequences, making it challenging to infer enhancer location, activity, or function from sequence information alone [9, 10]. Second, enhancers can act over broad ranges of linear genomic distance, independent of location relative to target genes, so enhancers cannot be identified by location within eukaryotic genomes and neither their absolute nor their relative genomic positions are conserved across species. Finally, it has been demonstrated that single genes are often regulated by multiple enhancers and that individual enhancers may regulate more than one gene [11–14].

Recently, a solution to some of these problems emerged from the discovery that active gene enhancers in all eukaryotes are associated with signature epigenetic

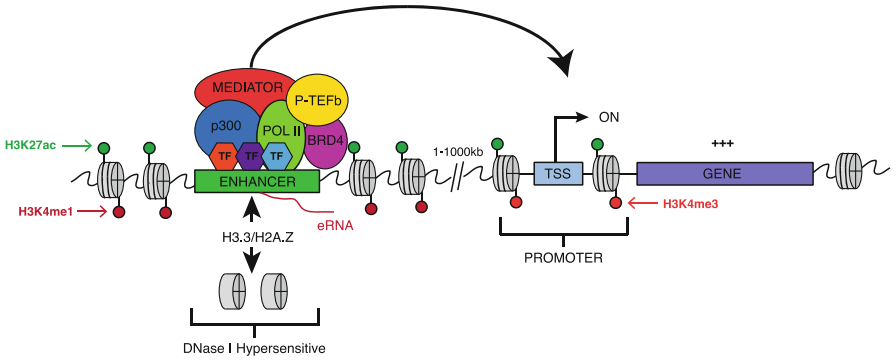


**Fig. 2.1** Epigenetic features of enhancers. Image adapted from Calo & Wysocka Molecular Cell 2013 [15]. Active enhancers are associated with the canonical histone modifications H3K4me1 and K3K27ac. Transcription factor (TF)-binding sites at enhancers are associated with hypermobile nucleosomes that contain the H3.3 and H2A.Z histone variants, making them hypersensitive to digestion by DNase I. Enhancers function by aiding in the assembly of the transcription activation/elongation machinery and physically looping to the promoters of target genes. Promoters are associated with the H3K4me3 histone modification. Enhancers themselves are also transcribed, yielding enhancer RNA (eRNA)

features on chromatin. Principle epigenetic features of active enhancers include specific post-translational modifications to the histone proteins that form nucleosomes, the core structural units of eukaryotic chromatin. Nucleosomes consist of 147 bases of DNA wrapped around protein octamers comprising two copies of each of the four core histone proteins: 2A, 2B, 3, and 4. From these core histone proteins protrude amino acid tails which are covalently modified at specific residues to impart function to associated DNA. These epigenetic modifications make up a "histone code," which can be used to interpret the function of non-coding DNA elements including enhancers (Fig. 2.1). Histone marks associated with active enhancers include monomethylation of lysine 4 on the tail of histone protein 3 (H3K4me1) as well as acetylation of lysine 27 on the tail of the same histone protein (H3K27ac). Additionally, active enhancers are associated with nucleosome-free DNA (i.e. "open chromatin") at the specific regions containing TF-binding sites, allowing TFs to bind DNA at these regions [1, 16–21]. Enhancers can be distinguished from gene promoters by these marks. While both active enhancers and promoters are associated with H3K27ac, promoters have H3K4me3 as opposed to H3K4me1 found at enhancers [1].

A critical aspect of enhancer function is their ability to physically loop to the promoters of target genes. Many of the key factors responsible for enhancer-promoter looping have been identified, including CTCF, ZNF143, and Mediator among others, and understanding the three-dimensional architecture of eukaryotic genomes in normal development as well as in disease states is an area of ongoing research.

Just as enhancers can be associated with activation marks, they can also be associated with inactivating or "silencing" marks. These include methylation of

enhancer DNA [22, 23] as well as specific histone modifications including trimethylation of lysine 27 on histone protein 3 (H3K27me3). Enhancers possessing both H3K27me3 and H3K4me1 have been termed "poised" as they can become activated given the proper stimulus through loss of H3K27me3 [16, 20, 21]. Poised enhancers were first described in embryonic stem (ES) cells. Upon differentiation of ES cells to more mature cell types, poised enhancers can be permanently silenced or activated. These shifts in enhancer activity are the key drivers of cellular development and establish the basis for how specific cell types initiate and maintain their mature phenotypes [24].

Intriguingly, epigenetic processes have been intimately linked to cellular energy balance. It has been demonstrated in a number of studies that epigenetic dysregulation including DNA methylation and histone modifications at promoters and enhancers can alter the metabolic state of cells [25]. Likewise, altered metabolic states can change the epigenetic landscape of cells as well [25]. These findings demonstrate that epigenetic information acts as a key intermediary between cellular metabolism and the information encoded within cellular genomes. As cancer is associated with altered states of both cellular and organism-wide metabolism, it is perhaps not surprising that epigenetic changes, including locus-specific changes in the signature enhancer-histone marks, H3K4me1 and H3K27ac, have been shown to be drivers of tumor formation and progression across cancers. Recent studies have begun to catalogue enhancer changes in various cancers and delineate the specific functional consequences of such enhancer dysregulation to tumor cell biology. In this chapter, we will summarize some of the seminal and emerging work linking epigenetic changes at gene enhancers to cancer risk, development, and progression as well as recent efforts to exploit these changes in the development of targeted epigenetic cancer therapies. To establish a foundation for these studies, we will describe the latest technical approaches employed for cataloguing and characterizing enhancer activity across cell types and outline the current theories for the biochemical basis of regulation of enhancer activity and function. An understanding of these areas will allow the reader to appreciate the mechanisms by which enhancer function may be dysregulated in cancer and how these processes may be exploited for the development of anti-cancer therapies and new approaches to the detection and management of cancer in patients.

## Approaches for Global Enhancer Profiling

The knowledge that enhancers are associated with specific epigenetic states has allowed for the adaptation of genome-scale assays to identify and catalogue enhancers across cellular genomes. Enhancer-associated histone modifications (e.g., H3K4me1 and H3K27ac) can be identified by chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-seq) [10]. This approach

isolates regions of cell genomes containing a given mark through immunoprecipitation of fragmented chromatin using specific antibodies. The DNA associated with these marks can then be identified by high-throughput sequencing strategies that allow the location of the marks within cell genomes to be determined. Genome-wide strategies for identifying open regions of chromatin, like those found at enhancer TF-binding sites, have also been developed. These approaches include DNase-I hypersensitivity sequencing (DHS-seq) [5] as well as an analogous approach known as assay for transposase-accessible chromatin using sequencing (ATAC-seq) that uses the enzyme transposase to incorporate sequencing tags into regions of open chromatin [26]. Like ChIP-seq, these genome scale approaches allow for global characterization of open chromatin within the genomes of cell types of interest. The utility of these approaches increases when they are used in combination. While H3K4me1, H3K27ac, and open regions of chromatin may be found at non-enhancer genomic regions (e.g. promoters), their presence in combination at regions distal to gene transcription start sites (TSSs) has been shown to comprise a chromatin "signature" of enhancer activity [16, 21]. Example output from H3K4me1 ChIP-seq, H3K27ac ChIP-seq, and DHS-seq experiments used to detect enhancers is provided in Fig. 2.2.

More recently, additional assays allowing for global assessment of enhancer activity have been developed. One approach uses levels of eRNA expression as a surrogate measure of enhancer activity. This approach employs a strategy known as cap analysis of gene expression (CAGE) to identify the capped transcripts that result from bidirectional transcription at active enhancers. Quantification of such transcripts allows for genome-wide assessment of enhancer activity across cell types [27]. Another approach known as self-transcribing active regulatory region sequencing (STARR-seq) allows for direct assessment of both enhancer activity and strength of arbitrary sources of DNA [28]. STARR-seq thus enables genome-wide screens for enhancer function.

These strategies have been employed by large international consortia, including the Encyclopedia of DNA Elements (ENCODE) [3], the Roadmap Epigenomics project [29], and the Functional Annotation of the Mammalian Genome (FANTOM5) project [27], to catalogue enhancers across cell types. These large-scale projects as well as myriad studies from individual research groups have elucidated how enhancer function drives cellular differentiation and have allowed for global characterization of enhancer activity in diverse cell types. Such studies have identified roughly 400,000 enhancers in the human genome [3] and it is estimated that approximately 10% of these are active in a given cell type [1]. Additionally, these approaches have been extended to understand the molecular underpinnings of altered cellular states in disease. Indeed, this strategy is shedding new light on many disease processes including auto-immunity [30, 31], hematopoiesis, diabetes mellitus [32], central nervous system disorders [33], and cancer [34] among others.

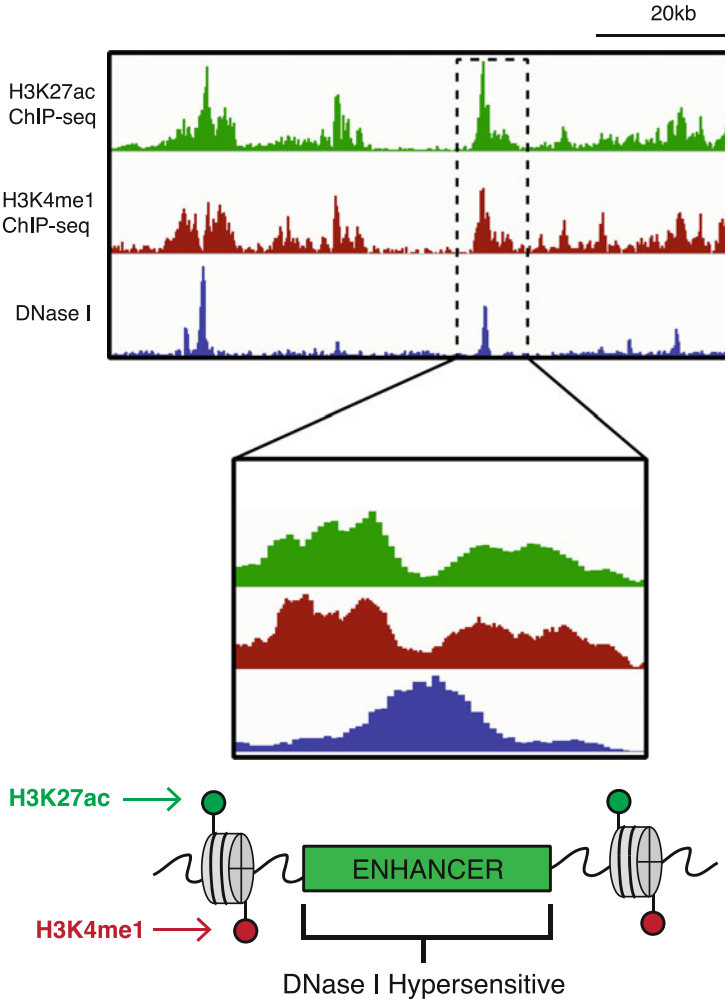


**Fig. 2.2** ChIP-seq and DHS-seq signature of enhancers. *Top panel* shows ~100 kb window of exemplar data from H3K27ac and H3K4me1 chromatin immunoprecipitation sequencing (ChIP-seq) and DNase I Hypersensitivity sequencing (DHS-seq) experiments. *Peaks* represent regions of increased signal, indicating deposition of each mark at specific loci. The *lower panel* shows a zoomed-in view of ChIP-seq and DHS-seq profiles at an active enhancer. Schematic shows modifications corresponding to signal shown at the enhancer locus

## Super-Enhancers, Stretch Enhancers, and Enhancer Clusters

Recent studies have shown that not all regions of enhancer activity are created equal, but that within the enhancer landscape of a given cell type there are stretches of highly active, densely clustered enhancers. A number of approaches for identifying such regions have been developed and, while similar, the regions defined by

these approaches are not identical. Super-enhancers (SEs) are defined as broad regions of the enhancer activity that show disproportionately high ChIP-seq signals for lineage-specifying TFs, enhancer-associated proteins (e.g., mediator), or histone modifications (e.g., H3K27ac). SEs are regulated by lineage-specifying TFs and drive expression of cell-type-specific genes in both embryonic stem cells and differentiated cell types [35, 36]. Stretch enhancers are defined by broad swaths of enhancer-associated histone modifications and multiple regions of open chromatin. Similar to SEs, stretch enhancers and the genes that they regulate are more cell-type-specific than typical enhancers [32]. Enhancer clusters are defined by dense distributions of individual active enhancers within physically interacting chromatin domains. Like SEs and stretch enhancers, these enhancer clusters are bound by cell-type-specific TFs and regulate key cell identity genes [37]. Collectively, these findings suggest that expression of genes that are key in establishing and maintaining cell identity is under the tight transcriptional control of broad regulatory domains containing multiple active enhancers.

## Enhancer Readers, Writers, and Erasers

In light of the essential role of gene enhancers in establishing and maintaining cellular identity described above, full understanding of the molecular underpinnings of normal development and the aberrant cellular states of disease requires rigorous investigation of the molecular processes that establish, maintain, and decommission enhancers as well as the role of enhancers in regulating transcriptional output.

### *Nucleosome Depletion*

A key step in initiating the formation of active enhancers is chromatin decompaction and nucleosome displacement [38]. These processes facilitated through the action of pioneer transcription factors that, in contrast to standard TFs, are able to bind DNA sequences within closed chromatin conformations. These factors include FoxA and GATA family TFs that were originally identified as pioneer TFs essential for liver proper development. Since the initial descriptions of the function of these hallmark pioneer TFs, many other examples have been identified across tissues and cell types [39]. One prevailing model posits that pioneer TFs act to bind closed chromatin and to facilitate both decompaction and recruitment of other key TFs and transcriptional co-activators. Chromatin decompaction is also thought to be dependent on the incorporation of less stable histone variant proteins including H2A.Z and H3.3. However, the temporal relationship between histone changes and TF binding during enhancer activation remains to be fully understood [15]. Another key function in mediating chromatin accessibility at active enhancers is ATP-dependent chromatin remodeling. Two major protein complexes mediating these nucleosome dynamics are SWI/SNF and INO80 [40].

### *H3K4me1*

High levels of H3K4me1 are broadly associated with active, poised, and silenced enhancers, and H3K4me1 is also found at lower levels in promoters of actively transcribed genes [17]. Deposition of H3K4me1 at enhancers seems to precede nucleosome depletion, H3K27ac deposition, and enhancer activation [16, 20, 21, 41–44]. Intriguingly, H3K4me1 seems to persist at locations of active enhancers even after they lose the ability to actively promote transcription of target genes, unlike H3K27ac or Pol II [41, 42]. Thus, it appears that H3K4me1 alone is probably not sufficient to drive target gene expression. Therefore, it has been proposed that H3K4me1 deposition acts to prime enhancers to become active given the proper stimulus, at which time H3K27ac is deposited. In support of this, H3K27ac is nearly always present concomitantly with H3K4me1 at active enhancers [42].

One function of H3K4me1 seems to be to mitigate interactions of marked regions with transcriptional repressors that preferentially bind to the unmethylated form of H3K4 (H3K4me0) including the CoREST complex, DNMT1, and DNMT3L [45–47]. While this function of H3K4me1 as an inhibitor of H3K4me0 readers is well-established, others have hypothesized that H3K4me1 may directly bind to other readers to influence chromatin structure and function at enhancers. However, most H3K4 methyl readers studied to date preferentially bind to the trimethylated form of H3K4. Indeed, a number of H3K4me3-promoter readers have been identified, including TFIID which plays a key role in transcriptional activation [48]. One example of an H3K4me1 reader is the acetyltransferase, TIP60, which preferentially associates with H3K4me1 and acts to facilitate the incorporation of transient histone protein H2A.Z into chromatin [49]. Therefore, H3K4me1 may indeed potentiate chromatin accessibility.

Histone methylation in eukaryotes is achieved through the enzymatic activity of six methyltransferases in the MLL/Set family, all of which contain the catalytic SET domain that has been shown to be capable of mono-, di-, and tri-methylation in in vitro studies [50]. This family is comprised of three subgroups corresponding to homologous proteins in *Drosophila*: SET1a/b homologous to *Drosophila* Set1, MLL1/2 homologous to Trithorax (Trx), and MLL3/4 homologous to Trithrorax-related (Trr). SET1a/b are primarily responsible for H3K4me3 deposition, while MLL3/4 are the primary writers of H3K4me1 [51]. While the mechanism of this specificity remains incompletely understood, one proposed explanation is that SET1a/b require association with CpG dinucleotides found primarily at promoters, while MLL3/4 lack sequence requirements for activity [52–54]. In this model, H3K4me3 deposition by Set1a/b would segregate to CpG-containing promoters, while H3K4me1 deposition by MLL3/4 would occur at both promoters and enhancers, with opportunities for subsequent conversion to H3K4me3 only at promoters. In support of this, levels of MLL3/4 have been shown to correlate with global levels of H3K4me1 deposition [55]. The mechanism by which MLL family proteins are recruited to chromatin are not worked out, although coordination with pioneer TFs [56, 57] and association with non-coding RNAs [58, 59] have been proposed.

Individual studies have also associated MLL1/2 with enhancers [60]. However, the role of these complexes in H3K4me1 globally as well as the mechanism of their specificity is not well understood. Demethylation of H3K4 is achieved through the activity of lysine-specific demtheylase 1A (KDM1A; also known as LSD1) [61] and Jumonji domain proteins including KDM5A and KDM5C [62].

### *H3K27ac*

The two major histone acetyltransferases (HATs) responsible for activation of enhancers are p300 and CBP. p300/CBP are ubiquitously expressed and recruited to chromatin by TFs [1, 10, 63]. H3K27 is one of the main substrates for these HATs [64, 65]. While H3K27ac is broadly used to identify active enhancers, other acetylation marks are known including H3K9ac. Gcn5/PCAF are responsible for H3K9 acetylation [2, 16, 66, 67]. Acetylated lysines are recognized by bromodomains that are present in a broad array of nuclear proteins related to transcriptional regulation. Among these are HATs themselves (e.g., p300, CBP, Gcn5, and PCAF), ATP-dependent chromatin remodelers (e.g., BPTF, WSTF, BRG1, and BRM), TFIID components (e.g., TAF1 and TAFL1), and pause-release factors (e.g., BRD4) [68]. TAF1 is associated with the pre-initiation complex, various HATs and BRG1 are associated with initiation, and BRD4 is associated with elongation [21, 69, 70]. All of these proteins have been shown to broadly correlate with enhancers through ChIP-seq studies. Deacetylation of H3K27ac is thought to occur through the action of HDAC3 in association with the SMRT complex [71].

## Somatic Cancer Mutations to Chromatin-Related Proteins

Mutations in a large number of genes with key functions in mediating enhancer activity have been identified in cancer. Such genes include chromatin writers, transcription factors, chromatin readers, mediators of enhancer-gene interactions, and chromatin erasers. A recent comprehensive study of pan-cancer genome sequencing data from The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) showed that, of the 58 most frequently mutated genes in cancer, 16 (28 %) are chromatin factors [72].

### *Writers and TFs*

As described above, the MLL3/4 complex is responsible for depositing the H3K4me1 mark at regions of enhancer activity. Mutations to MLL3 and MLL4 are frequent in a number of tumor types including breast cancer [73], colon cancer

[74, 75], liver cancer [76], gastric cancer [77], bladder cancer [78], medulloblastoma [79, 80], and non-Hodgkin's lymphoma [81]. Somatic point mutations to *MLL3* and *MLL4* span the gene bodies, but seem to be enriched at conserved motifs of these proteins. While the role of *MLL3/4* somatic mutations as *bone fide* drivers of tumor formation remains to be established, a number of possible mechanisms by which such mutations may lead to tumor formation have been proposed. Possibilities include both gain and loss of function mutations to the catalytic SET domain of MLL3/4, which could affect the rate and extent of H3K4me1 deposition in tumor cells as well as mutations affecting the interaction of MLL3/4 with TFs or other factors that help to coordinate H3K4me1 deposition at specific enhancer regions within a given cell type [82]. Further functional studies are necessary to understand the role of MLL3/4 in both wild-type and mutated forms in the context of specific human cancers.

Lineage specifying TFs play a key role in enhancer writing as they recruit chromatin modifiers to genomic regions containing their binding motifs, thereby providing sequence specificity for these complexes. Somatic mutations to TFs are common across tumor types. Consistent with the known cell-type-specific role of TFs and enhancers, TF mutations seem to segregate with specific cancer types. The pioneer TF, FOXA1, has been shown to be mutated in breast and prostate cancers [83]. RUNX1, a TF whose activity is required for normal hematopoiesis, is commonly mutated in AML [84]. GATA3, a TF critical for development of the luminal breast epithelium, is commonly mutated in a subtype of breast cancer [85].

## *Readers/Chromatin-Looping Factors*

In order for enhancers to regulate expression of their target genes, they must physically contact gene promoters via the formation of chromatin loops. This activity of enhancers is further regulated through higher order chromatin structure that limits the linear genomic area that enhancers can contact in *cis* [86]. Somatic mutations have been discovered in a number of chromatin-looping factors. Looping factors mutated in cancer include MED12 of the mediator complex [87], CTCF, STAG2 [84, 88], NIPBL, SMC1A, and SMC3 [89].

## *Erasers*

Just as chromatin writers can alter enhancer activity in cancer, so too can chromatin erasers. A number of somatic mutations to the key erasers of enhancer histone modifications have been described in cancer. While mutations to the lysine-specific histone demethylase, LSD1, have not been identified, a number of mutations to Jumonji domain proteins that can also demethylate histone lysines are known. These include mutations to KDM5A (JARID1A) and KDM5C (JARID1C), both of which

demethylate lysine 4 of histone protein H3. While mutations to HDACs have been identified, these seem to be rare events with little consequence to epigenetic marks across tumor genomes. These data suggest that altered expression of HDACs is the primary mode of dysregulation of these erasers in cancer [62].

## Enhancer Dysfunction in Cancer

The highly orchestrated function of enhancers allows for a single genome containing ~25,000 genes to create and maintain the hundreds of cell types that comprise the human body. With this in mind, it is perhaps not surprising that enhancer dysfunction underlies the aberrant cellular phenotypes responsible for many human diseases. Cancer, as a disease largely defined by abnormal cellular differentiation, is no exception. Recent studies have demonstrated that in addition to the well-known oncogene and tumor suppressor mutations associated with tumorigenesis, enhancer dysfunction plays a key role in the development of cancer as well. A study comparing the enhancer epigenomes of colon cancer cells relative to their cell type of origin in the colonic crypt found that malignant transformation is associated with a large number of locus-specific gains and losses of enhancer activity across the epigenome. These regions, termed variant enhancer loci (VELs), drive dysregulated gene expression programs that define colon tumors [34]. Subsequent studies of SEs in multiple tumor types have demonstrated that expression of key oncogenes in cancer cells is maintained through aberrant SE activity [90]. Hanahan and Weinberg have proposed that tumor cells acquire hallmark functions that distinguish them from normal cells and lead to tumor formation and progression [91]. SEs have been shown to regulate expression of genes associated with all of these hallmarks in cancer cells. Genes meeting these criteria include Transketolase (TKT) and Enolase 1 (ENO1) that have been implicated in the cancer hallmark of deregulated cellular energetics [36]. The activity of cancer SEs has been proposed to establish feed forward transcriptional loops that lock cells in aberrant transcriptional states, driving tumor formation and maintenance. Indeed, one recent study of SE function in cancer demonstrated that cancer SEs coopt the same regulatory mechanisms that drive normal development to establish and maintain the malignant phenotype of tumor cells [92]. Another study implicating enhancer alterations as drivers of aberrant developmental transcriptional programs in malignancy surveyed open chromatin in tumor cells by DHS-seq. This study demonstrated that cancer cells reactivate regulatory regions of open chromatin normally restricted to ESCs, aberrantly activate open chromatin regions normally restricted to cell types of other tissues, and establish novel regions of open chromatin not found in any normal cell types studied to date [24]. Collectively, the findings indicate that the malignant phenotype is associated with transcriptional reprogramming of cancer cells through genome-wide shifts in enhancer activity.

Since the initial discovery of the role of enhancers in malignant transformation, a number of studies have functionally confirmed enhancers as key drivers of

malignancy across tumor types including acute lymphoblastic leukemia (ALL) [93], diffuse large B-cell lymphoma (DLBCL) [94], acute myeloid leukemia (AML) [95], small cell lung cancer (SCLC) [96], glioblastoma (GBM) [97], medulloblastoma [98], Merkel cell carcinoma [99], and Ewing sarcoma [100].

In addition to playing a role in tumorigenesis, enhancer reprograming has been shown to be a key driver of therapy resistance in cancer. One study demonstrated that endocrine therapy resistance in breast cancer is accompanied by genome-wide chromatin reprogramming including epigenetic changes at enhancers [101]. The authors demonstrated that this reprogramming resulted in hyperactivation of NOTCH signaling and repression of ERalpha signaling. Further, they demonstrate that NOTCH inhibition mitigates growth of resistant breast cancer cells, suggesting that targeted modulation of specific pathways driven by aberrant epigenetic changes at enhancers may be an effective strategy for mitigating acquired drug resistance in breast cancer cells.

## Genetic Alterations to Enhancers

### *Heritable Cancer Risk at Enhancers*

Genome-wide association studies (GWAS) have connected many single-nucleotide polymorphisms (SNPs) to a wide range of phenotypes including risk for cancer and other diseases. Intriguingly, the large majority of phenotype-associated SNPs lie outside of protein-coding regions of the genome. Integration of GWAS data with known TF-binding sites and motifs has shown that genetic variation within TF-binding regions of the genome can be a functional means of phenotype associations with genetic variation across populations [102]. ChIP-seq studies of diverse cell types have shown that phenotype-associated SNPs are twofold enriched for putative enhancers [2]. As part of the ENCODE project, functional regions of the genome defined by chromatin accessibility, TF binding, or other DNA–protein interactions (e.g., histone modifications) revealed that the majority of known GWAS associations are linked to regulatory regions of the genome either directly or through linkage disequilibrium [103].

A large and growing number of GWA studies have identified risk loci within enhancers. One of the most ubiquitously up-regulated genes driving tumor formation is MYC. Multiple risk loci in a gene desert in chromosome 8q24 associated with risk of prostate, breast, and colon cancer have been shown to possess the chromatin marks of putative enhancers and physically loop to the MYC promoter to regulate gene expression in these cancers. Intriguingly, enhancer looping interactions at this locus are tissue-specific and cancer risk SNPs occur in enhancers that are active in the tissue of origin of each cancer, e.g., prostate cancer risk SNPs occur in prostate-specific MYC enhancers, while colon cancer risk SNPs occur in colon-specific MYC enhancers [104]. These findings indicate a close connection between cancer risk and tissue-specific enhancer activity. Two additional studies of the

rs6983267 colon cancer risk SNP in this region demonstrated that this locus possesses the chromatin signature of enhancers, that the risk allele increases binding of the Wnt-regulated TF, TCF4, and that this ultimately leads to increased MYC expression in colon cancer cells [105, 106]. Critical function of this enhancer in colon cancer development was confirmed by mouse studies demonstrating that mice lacking the MYC enhancer containing the human SNP rs6983267 are resistant to the development of intestinal tumors [107]. While these studies have elucidated the role of heritable risk for colon cancer at a single locus, other studies have extended this premise genome-wide. A study identifying variant enhancer loci (VELs) that are gained and lost in colon cancer relative to the colonic crypt cells of origin found that colon cancer VELs are enriched in haplotype blocks containing colon cancer risk variants [34]. An ever-growing number of studies have associated heritable risk for cancer with genetic variation at enhancer elements. This has now been demonstrated in renal cancer [108], prostate cancer [109], ovarian cancer [110], and breast cancer [111–113]. In the case of breast cancer, functional investigation of breast cancer risk SNPs demonstrated that risk variants alter the binding motifs of the pioneer TF, FOXA1, to alter binding affinity at enhancers and affect gene expression [114]. The studies demonstrate the strong functional connection between heritable genetic variation at enhancers, cell-type-specific gene expression, and cancer risk.

## *Enhancers, Energy Balance, Obesity, and Aging*

A number of studies have reported functional associations between enhancers and energy balance, obesity, and age, all of which are risk factors for cancer. GWA studies have linked variants within introns of the *FTO* gene with increased risk for obesity and type 2 diabetes. Two recent studies have demonstrated that these variants alter activity of enhancers at these loci and that these enhancers regulate expression of the homeobox genes *IRX3* and *IRX5* in adipocytes rather than *FTO* [115, 116]. Functional analysis of adipocytes demonstrated that the risk allele results in increased expression of IRX3 and IRX5 during early adipocyte differentiation, resulting in reduced production of energy-dissipating beige adipocytes and increased production of energy-storing white adipocytes [116]. Another study illustrated that the transcription factor CCAAT/enhancer-binding protein (C/EBP) beta is a key regulator of body composition and systemic levels of IGF-1, leptin, and insulin, all of which mediate energy balance. Further, this study demonstrated that C/EBPbeta-deficient mice showed reduced rates of colon adenocarcinoma tumor formation relative to normal controls [117]. Finally, another study demonstrated that longevity is associated with increased enhancer activity at the sirtuin 3 gene (SIRT3) in human populations, confirming that the sirtuin genes are key regulators of longevity and illustrating that enhancer function can be a key mechanism of their regulation [118]. While only one of these studies directly linked enhancer dysfunction to these risk factors and cancer, the findings indicate that enhancers are likely to be keys in mediating a number of cancer risk factors including energy balance, obesity, and aging.

### *Somatic Cancer Mutations at Enhancers*

A fundamental driver of tumor formation is somatic mutation of tumor cell DNA. In the past 15 years, comprehensive sequencing efforts have thoroughly characterized the mutational landscapes of human cancers. These studies have found that many recurrent somatic mutations in cancer affect the 2 % of the human genome that codes for protein [119]. However, the majority of somatic mutations in tumor cells occur in the remaining 98 % of the human genome that does not code for protein [120]. Until recently, it has been difficult or impossible to infer the function of such non-coding somatic cancer mutations. However, the functional annotation of the non-coding genome through large-scale multi-institutional studies including ENCODE and the Roadmap Epigenomics Project has allowed for these analyses on a genome scale. Studies integrating these datasets have been fruitful in unveiling the close connection between genetic and epigenetic alterations in tumor cells. Indeed, a growing number of studies have now revealed that somatic mutations to tumor cell DNA can lead to epigenetic changes in regulatory regions of the non-coding genome with important functional consequence to tumor cell biology. This process is now emerging as a hallmark mechanism of tumor formation and progression that can occur independently or in parallel with coding mutations. The mechanisms by which somatic mutations lead to enhancer dysfunction in cancer are summarized in Fig. 2.3.

Perhaps the most well-studied non-coding somatic mutation in cancer is of a causative "driver" mutation altering the function of the promoter for the telomerase catalytic subunit (TERT) [121]. Signature recurrent mutations in this region result in the creation of new binding motifs for the E-26 (ETS) TFs and ternary complex factors (TCFs), resulting in increased TERT expression in melanoma [122] and other types of cancer [123]. Another recent study has revealed that somatic mutations to CTCF/cohesin-binding sites (CBSs) frequently occur in multiple tumor types including colorectal cancer [124]. CTCF binding has been shown to establish genomic "insulator" regions that help to dictate *cis* interactions of regulatory elements with target genes across the genome by preventing interactions between elements separated by such insulator regions [86]. CTCF often co-binds with cohesin at CBSs and this has been shown to affect genomic stability, gene expression, and epigenetic homeostasis [124]. While the functional consequence of somatic mutations to CBSs in tumor cells has not been well-studied, it is plausible that such mutations would have broad structural and functional consequences on the transcriptional output of tumor cell genomes. Studies of the *TERT* promoter and CBSs have established the premise that non-coding somatic mutations can be functional drivers of oncogenesis.

Despite the finding that causal-inherited cancer SNPs often occur in enhancer elements, the contribution of somatic mutations in enhancer regions to tumor formation has only recently been explored. In one of the first studies investigating the role of SEs in cancer, it was shown that somatic mutations in cancer cells affect SE function either by focal amplification of enhancer sequences at SEs or by bringing

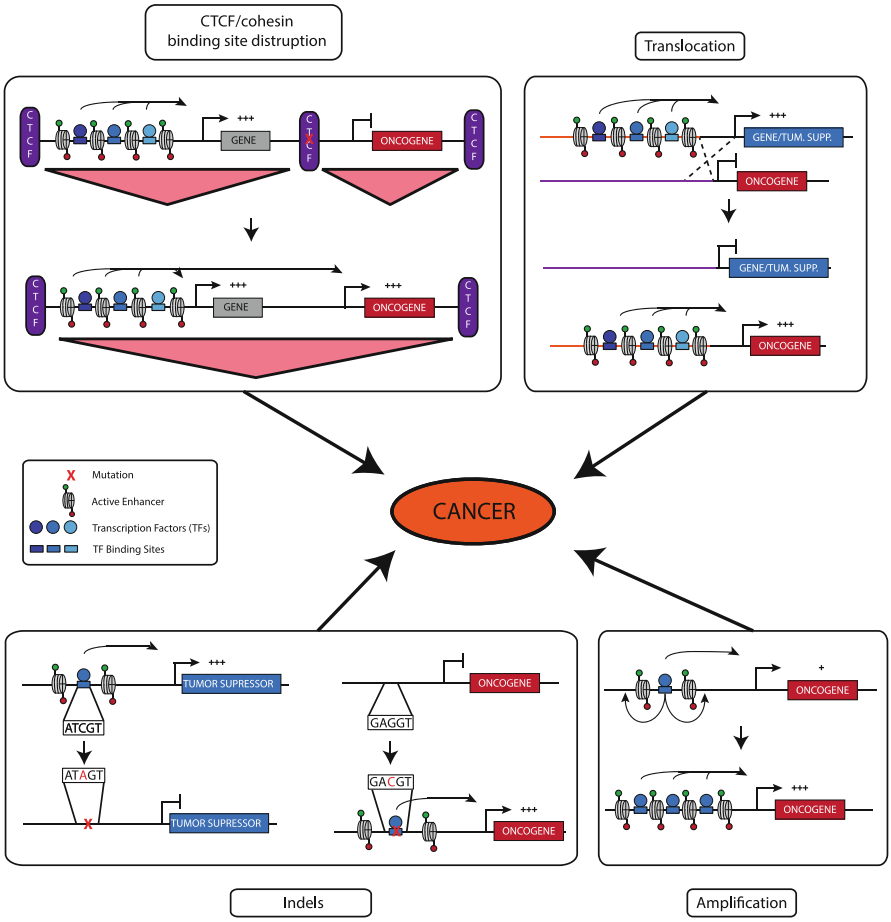


**Fig. 2.3** Mechanisms of somatic mutations leading to enhancer dysfunction in cancer. Each *panel* shows a unique mechanism by which somatic mutations to genomic DNA lead to enhancer dysfunction in cancer. As indicated, these mutations can cause increased expression of oncogenes and/or decreased expression of tumor suppressor genes and thus contribute to the malignant phenotype of tumor cells

active SEs into contact with new target genes via translocations [36]. Both of these genomic mechanisms of enhancer dysregulation in cancer are supported by additional studies. A recent study investigating the mechanism of MYC overexpression in acute lymphoblastic leukemia revealed that recurrent chromosomal duplications occur in T-ALL at a NOTCH1-driven MYC enhancer and that the function of this enhancer is critical for T-ALL development [93]. Translocations have been known to transplant coding genes from their native regulatory context and place them under control of alternate enhancers in cancer cells for quite some time. Indeed, a study published in 1982 demonstrated that in Burkitt cell lymphoma MYC is recurrently translocated from chromosome 8 to alternative locations in the genome [125].

Subsequent studies demonstrated that these translocations place MYC expression under the control of strongly active B-cell enhancers including that of the IgH locus [126–128]. A similar mechanism of somatic mutation commonly occurs in myeloma, resulting in the juxtaposition of MYC and strongly active immunoglobulin enhancers including IgH, IgK, and IgL as well as other enhancers typically targeting genes important for B-cell maturation [129]. Another study in acute myeloid leukemia (AML) demonstrated that rearrangements in the q arm of chromosome 3 result in novel interaction of a GATA2 enhancer with the EVI1 pro-oncogene to simultaneously activate EVI1 and cause halpoinsufficiency of GATA2, resulting in malignant transformation [130]. This mechanism is not limited to tumors of the blood cell compartment, but has also been shown to be active in solid tumors. One recent study in medulloblastoma has shown that the GFI1 family genes, *GFI1* and *GFI1B*, are proto-oncogenes that become activated through somatic structural mutations that place these genes under the control of active enhancer elements including SEs in medulloblastoma cells. Functional studies in mouse models confirmed these genes as *bone fide* oncogenes in this pediatric brain tumor [98].

A number of recent studies integrating data from large-scale sequencing efforts have further investigated the function of non-coding somatic mutations as cancer drivers and revealed a prominent role of insertions and deletions in this regard. One such study interrogating whole genome sequencing (WGS) data from 863 tumors from The Cancer Genome Atlas (TCGA) confirmed the presence of recurrent mutations in the *TERT* promoter and identified other recurrent non-coding somatic mutations in regions upstream of *PLEKHS1*, *WRD74*, and *SDHD* with possible regulatory function [131]. Another study integrating WGS data of 436 patients from TCGA representing eight cancer subtypes with functional genomic data from ENCODE has provided evidence for positive selection of mutations in TF-binding sites. This analysis also validated known recurrent somatic mutations in regulatory regions including the *TERT* promoter. Intriguingly, the study revealed many novel recurrent somatic mutations in regulatory regions including a number of nearby known cancer genes. Two such mutations were assessed functionally and shown to result in reduced enhancer activity following mutation [132]. A third study showed that heterozygous somatic mutations occur in T-cell acute lymphoblastic leukemia (T-ALL) that introduce binding motifs for the MYB TF at a highly recurrent site upstream of the TAL1 proto-oncogene. Introduction of this MYB-binding motif results in the formation of a SE that subsequently drives overexpression of TAL1 [133]. Another study investigating the genomic landscape of 452 chronic lymphocytic leukemia (CLL) cases identified a number of recurrent somatic mutations in non-coding regions including an enhancer located in the vicinity of the B-cell-specific TF, PAX5. These mutations were shown to result in reduced PAX5 expression in CLL [134]. The described studies unequivocally demonstrate the role of somatic mutations altering enhancer activity as functional drivers of cancer.

While translocations forming new enhancer–gene partnerships have been well-described in cancer, other alterations to the three-dimensional chromatin landscape of cancer cell genomes have been less thoroughly explored. However, the advent of genome-scale chromatin structural mapping techniques such as Hi-C has allowed

for such studies across cell types. These studies are particularly powerful when integrated with WGS data. Human developmental disorders such as congenital limb malformations have been associated with structural variants that lead to disruption of normal topological domains (TADs), which normally help to guide enhancer–gene interactions [135]. This strategy is currently being applied to the study of cancer and is likely to reveal new insights into the mechanisms of dysregulation of cancer genomes during malignant transformation.

## Enhancer Alterations As a Therapeutic Target in Cancer

The observation that enhancer function is commonly altered in cancer cell populations has not only yielded unprecedented insight into tumor biology, but has also presented novel opportunities for therapeutic intervention that are just now beginning to be realized. The challenge in translating insights from biological studies of enhancer function in cancer to useful therapeutic strategies stems from the fact that many of the same players driving aberrant enhancer activity in tumor cells are required for normal cellular processes. A major hurdle to developing enhancer-targeted therapies with the potential for real clinical utility is identifying cancer dependencies or therapeutic targets whose activity is uniquely necessary for maintenance of cancer phenotypes, but not required for normal cellular function. One of the first steps in overcoming this hurdle was achieved by several studies that independently identified the H3K27ac reader, BRD4, as a key cancer dependency gene. The first of these studies used an RNAi approach to screen known chromatin regulators for cancer dependencies in acute myeloid leukemia (AML). It was shown that the AML phenotype is uniquely dependent on BRD4 and that BRD4 knockdown resulted in robust anti-leukemic effects [136]. Parallel to these studies, two other groups developed small molecule inhibitors (JQ1 and iBET) of BRD4 that act by binding to BRD4 bromodomains, preventing their interaction with acetylated lysine residues at active promoters and enhancers [137–139]. JQ1 was initially shown to have strong anti-proliferative effects on multiple myeloma and to result in increased survival in mouse myeloma models [138]. iBET was shown to have similar anti-tumor activity against MLL-fusion leukemia [139]. Subsequent to these landmark studies, BRD4 inhibition has been shown to be an effective therapeutic strategy in pre-clinical models of many cancers including lung adenocarcinoma [140], DLBCL [94], medulloblastoma [141, 142], and neuroblastoma [143]. A recent study screening 246 cancer cell lines from 19 types of cancer for in vitro growth inhibition in response to JQ1 has demonstrated that many types of cancer are sensitive to BRD4 inhibition, but that sensitivity is heterogeneous both within and across cancer types. It was shown that tumors with enhanced activity of the Wnt signaling pathway are more resistant to BRD4 inhibition [144]. Initial studies linked BRD4 inhibition to concurrent inhibition of MYC-driven transcriptional programs [136, 138]. However, more recent studies have demonstrated that effects of BRD4 inhibition on MYC activity seem to be secondary to cell's unique dependence on BRD4 for the

transcription of genes associated with SEs [90]. Indeed in some settings, the effect of BRD4 inhibition seems to be MYC-independent [140]. Nevertheless, BRD4 inhibition has shown robust anti-tumor effects in a number of cancer models and is now under investigation in clinical trials (See ClinicaTrials.govBRD4).

These findings further support the utility of studying enhancer function in cancer as a means of identifying novel therapeutic targets. This paradigm has been extended to the development of other small molecule inhibitors of enhancer-mediated transcriptional output in cancer. In order to elicit increased transcription of target genes, enhancers require the activity of a number of enzymatic cofactors including cyclin-dependent kinases (CDKs). Recently, a covalent CDK7 inhibitor, THZ1, has been developed that shows potent activity in T-ALL. The effects of THZ1 on T-ALL were shown to result from suppressed RUNX1 expression, a gene regulated by a highly active SE in these cells [145]. A subsequent study demonstrated that similar to JQ1, THZ1 shows potent anti-tumor activity in MYCN-driven neuroblastoma, resulting from suppression of MYCN-dependent transcriptional programs. This activity seemed to be associated with selective suppression of SE-associated genes including MYCN [146].

Collectively, the JQ1 and THZ1 findings suggest that the malignant phenotype is uniquely dependent on maintained expression of genes regulated by SEs. A recently published study targeting a negative regulator of SE-mediated gene expression suggests that this dependence is not simply due to high levels of expression of SE-associated genes, but precise maintenance of expression. In this study, the Mediator-associated kinases, CDK8 and CDK19, were identified as negative regulators of SE gene expression in AML. The authors identify the natural product cortistatin A (CA) as a selective inhibitor of Mediator kinases that results in disproportionate up-regulation of SE-associated genes. It was shown that CA has robust anti-leukemic activity in vitro and in vivo in certain AML cell lines. Genes up-regulated by CA include tumor suppressors and lineage-determining TFs. Intriguingly, iBET151 down-regulated these genes in CA-sensitive lines, but also showed anti-leukemic activity [95]. Collectively, these findings indicate that tight levels of expression or SE-associated genes are required for maintenance of the malignant phenotype in cancer cells and may open additional therapeutic opportunities in AML and other cancers.

## Future Areas of Study and Clinical Translation

Most studies of enhancer dysregulation in cancer have been focused on understanding the differences between normal cells and cancer cells. While these approaches have yielded unprecedented insight into tumor cell biology, they have been quite broad in their focus. Further studies of enhancer dysregulation as a driving force of specific behaviors of tumor cells are necessary to understand how these master regulators of gene expression contribute to the diverse biology of tumor cell populations. One area of great clinical importance is understanding the molecular etiology of

tumor metastasis. With the exception of CNS tumors, metastasis is the cause of essentially all cancer-caused deaths. Therefore, investigating the contribution of enhancer dysregulation to the metastatic phenotype of cancer cells is an area of utmost clinical interest. To date, no studies investigating the contribution of enhancer dysregulation to tumor metastasis on a genome scale have been published.

Another relatively unexplored area of clinical interest is the use of enhancer alterations as a means of early detection of cancer or pre-malignant lesions, or assessment of tumor burden and progression. A number of studies have demonstrated the utility of profiling circulating tumor cells and cell-free circulating DNA to screen for cancer-specific mutations. This approach is currently being tested as a tool for screening, diagnosis, and prognostication [147–149]. As cancer-specific enhancer mutations are more thoroughly characterized, it is likely that this strategy may be extended to include non-coding mutations, increasing the power of such clinical tools. It is also likely that aberrant epigenetic marks at regulatory regions of tumor cell genomes including enhancers could be detected using similar approaches. Thus, for example, because there is a known inverse correlation between DNA methylation and H3K27ac at enhancers, assays for methylated enhancer DNA may be useful for detecting enhancer dysregulation. Because certain enhancer marks show very high specificity for cancer cell populations, there seems to be a great amount of untapped potential in this space. As our understanding of the contribution of enhancers to cancer formation, maintenance, and progression increases, it is inevitable that this knowledge will transform the clinical landscape of oncology. We and other members of the research and clinical communities are excited about the potential that translating this knowledge into the clinics has for improving the length and quality of life for cancer patients.

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# Chapter 3
# Early Life: Epigenetic Effects on Obesity, Diabetes, and Cancer

**Francine Hughes Einstein**

**Abstract** Abnormal intrauterine growth and other adverse early-life exposures may induce adaptations that in turn predispose the individual to chronic diseases later in life. This type of adaptation may be marked by changes in systems, organs, and tissues. The immediate benefits of early plasticity or adaptability may come at a cost with repercussions, such as increased susceptibility to diabetes, cardiovascular and other age-related diseases, as well as cancer, manifesting in adulthood. Some fetal adaptations may not necessarily be apparent at birth, but may be revealed later in life when invoked by cumulative environmental challenges (e.g., high fat or westernized diet). Early life exposures may represent the advancement of the normal decline of resistance to disease that occurs with aging. Developmental origin of adult disease may be viewed in the same framework of other progressive disorders defined by increasing epigenetic dysregulation, such as cancer and as such may have identifiable biomarkers. Assays that measure epigenetic changes hold great promise as biomarkers for disease states in which risk can be attributable to gene–environment interactions.

**Keywords** Developmental origins of adult disease • Pregnancy • Maternal nutrition • Fetal growth restriction • Obesity • Diabetes • Cancer • Exposome • Epigenome-Wide Associated Studies

## Epigenetics and Development

In 1942, Conrad Waddington coined the term "epigenetics" as the developmental mechanisms that create the phenotype of an individual [1]. Although for all practical purposes every cell in an individual's body has the same DNA sequence, each cell exhibits a unique phenotype. The behavioral response of each cell varies in accordance with internal and external cues. At no other time during the life course

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is the importance of the epigenome more clearly seen than during development. During this critical period of time, rapidly dividing cells with the same DNA sequence replicate, migrate along individualized paths, and differentiate to assume special functional roles to form organ systems. Waddington understood that development includes a complicated and dynamic regulatory process of genotype to produce a range of cellular phenotypes.

Today, epigenetics refers to the study of heritable changes in gene expression that occur without changes in DNA sequence [2]. The epigenome is unique to each cell type and is more dynamic and variable compared to the static nature of DNA sequence [3]. Cellular phenotype and responsiveness to external cues are governed through variations in DNA methylation, histone tail modifications, and chromatin binding and super-coiling. Post-translational regulation of gene and protein expression by microRNAs and other small RNAs also enable fine-tuning of cellular phenotype [4]. At the interface of gene–environment interactions, the epigenome plays a key role in determining phenotype and is shaped by the interaction of DNA sequence variations and exogenous influences on epigenetic regulators. We now have a much clearer view of what these regulators are and intense investigation is underway as to how these gene regulatory marks interact with one another.

The impact of environmental exposures on development is most clearly demonstrated by the relationship between teratogens and birth defects. The tragic events surrounding the use of thalidomide are some of the most well-known examples of disruption of normal human development in recent history. The term disruption refers to a congenital defect that is a result of an extrinsic interruption of the normal developmental process, such that without the presence of the external factor morphological development would proceed normally. In the late 1950s, thalidomide was heavily marketed as a non-addictive sedative that also had antiemetic effects. Thought to be safe, the use of the drug was quickly embraced and given to a large number of women for the treatment of early pregnancy-related nausea and vomiting. Shortly thereafter, an unusually high number of severe birth defects were identified and ultimately the drug was banned in 1961. After just a few years on the market, over 10,000 cases of thalidomide embryopathy were identified. The most commonly related congenital defect is phocomelia or severe shortening of the proximal long bones [5]. Although the underlying mechanisms through which the drug acts are not completely understood, thalidomide is not considered to be a mutagen and therefore involvement of epigenetic mechanisms is heavily implicated. In addition to limb formation, thalidomide can also affect the development of other organs and is associated with increased rates of pregnancy loss and infant mortality in severe cases [5]. Further, in those individuals with no identifiable anomalies, the risk for cardiovascular disease, osteoarthritis, and psychiatric disorders is increased [5, 6], suggesting that the disruption of normal development may be less conspicuous and not necessarily manifest as an observable morphological abnormality at the time of birth.

The thalidomide tragedy not only inspired changes to how we test drugs before clinical use [7], but also demonstrated that specific time-sensitive windows occur during development when adverse exposures may disrupt the normal developmental

process. In the case of thalidomide, exposures occurring between 20 and 36 days after fertilization were most likely to result in limb malformations. Before 20 days, no observable injuries occurred, and after 36 days, disorders related to brain development were the greatest risk [5]. These observations are consistent with findings associated with other known teratogens. For instance, the long-term response to radiation therapy is known to vary depending on the timing of the exposure. Radiation exposure in children who have tissues and organs that are not fully matured is more likely to have changes in growth curves and may have slowing or cessation of maturation. Radiation to the chest, for instance, may lead to a small thoracic circumference in addition to parenchymal lung damage in children. In contrast, adults and in particular elderly people are more likely to have diminished reparative mechanisms leading to fibrotic changes and inflammation, which contribute to diminished functioning [8]. Timing of adverse exposures across the life course is specific to the agent as well as the tissue exposed and the age of the affected individual.

## The Barker Hypothesis and Developmental Origins of Adult Disease and Health

In 1976, Ravelli et al. first reported on increased rates of obesity in individuals exposed in utero to maternal famine based on observations from the Dutch "hunger winter" during World War II [9]. In the late 1980s, the concept of developmental origins of adult disease and health started to attract attention. The late David Barker observed that the highest rates of mortality from cardiovascular disease in the 1980s occurred in regions in England and Wales, which had the highest rates of infant mortality in the early 1900s. At that time, low birth weight was the most common cause of infant mortality [10, 11]. He surmised that for low birth weight infants, the adaptations that enabled survival came at the cost of greater risk for cardiovascular disease in adulthood. From there, a series of epidemiological studies led him to hypothesize that adverse conditions in early life, including those resulting from poor maternal nutrition, lead to a disruption in normal growth and development and contribute to increased susceptibility for chronic disease later in life [12–14].

Other studies from the Dutch "hunger winter" further support the strong association between exposure to poor maternal nutrition and adult disease, specifically obesity, metabolic syndrome, and diabetes in offspring [15, 16]. In response to the Barker Hypothesis, a large number of investigations have demonstrated the link between intrauterine growth restriction and cardiovascular disease risk [17–20] as well as a number of other adult onset diseases, such as, obesity or increased adiposity [21, 22], hypertension [23], stroke [24], osteoporosis [25], and psychopathology [26]. These observations have been recapitulated in many different populations around the world in association with diabetes and metabolic dysfunction later in life [27].

Animal models also demonstrate the importance of maternal diet of offspring phenotype. The Agouti mouse model was one of the first examples showing that maternal diet during pregnancy could have an observable impact on offspring. The yellow agouti mouse ($A^{vy}$) has a mutation that causes yellow fur pigmentation. $A^{vy/a}$ animals manifest a broad range of coat colors from brown to mottled to yellow. Waterland et al. [28] showed that methyl donor supplementation of the diet of pregnant dams leads to methylation of the $A^{vy}$ gene and a shift in the distribution of coat color towards brown in the offspring. Others have shown that diets rich in soy can also lead to changes in DNA methylation, coat color, and decreased obesity of offspring [29].

Animal models of intrauterine growth restriction have been developed, which have enabled greater experimental control of environmental conditions and early life exposures. With these, the association with early growth restriction and a range of phenotypic changes have been demonstrated and are marked by alterations in gene expression in various tissues, including liver [30, 31], muscle [32–35], and hypothalamus [36, 37]. A wide range of experimental protocols and exposures have also been employed in animal models to support the phenomenon of developmental origin of adult disease, including models of malnutrition [38], hypoxia [39], and treatment with antibiotics [40]. Furthermore, some evidence suggests that severe dietary restriction in paternal grandfathers increases the risk for obesity and cardiovascular disease two generations later [41, 42], demonstrating a transgenerational effect that is not limited to the maternal lineage.

Several studies have provided direct evidence of epigenetic modifications as a result of poor maternal nutrition or fetal growth restriction in humans. Using the Dutch Famine Cohort, Heijmans et al. showed a 5 % decrease in methylation levels at the IGF2 differentially methylated region in those exposed to maternal famine in utero compared to unexposed siblings [43]. Others have demonstrated similar findings in global methylation or epigenome-wide changes in a small number of subjects [44–47]. Of note, methylation levels at the H19 and IGF2 differentially methylated regions were more attributable to DNA sequence variation, as opposed to environmental or stochastic events in whole blood of mono- and dizygotic twin pairs [48], reminding us that epigenetic studies should be interpreted with caution as DNA sequence variation has the potential to alter findings, in particular methylation levels at specific loci.

Although interest in the developmental origin of adult disease began with the study of intrauterine undernutrition, overnutrition is now of growing concern and parallels the public health concerns of the obesity epidemic. Paradoxically, infants exposed to excess nutrients in utero commonly as a result of maternal obesity or gestational diabetes are at increased risk for many of the same problems in adulthood as under-nourished infants, including type 2 diabetes, obesity, and cardiovascular disease [22, 49, 50]. Animal models of high fat feeding show that offspring develop a phenotype with features of metabolic syndrome and epigenetic changes in many tissue types (reviewed in [51]). Further, transgenerational effects have been

demonstrated in animal models. For example, maternal high fat feeding results in altered insulin action in two subsequent generations [52] and third generation female offspring have changes in adult body size [53].

Our group examined DNA methylation in hematopoietic stem cells of neonates born with low, normal, and high birth weights, which was used as a surrogate for fetal nutrient availability. Both extremes of fetal growth are associated with global shifts towards hypermethylation of DNA compared to normal weight infants. Although the loci with differential changes in methylation were not identical, the targeted genes had similar functional characteristics and differentially methylated loci were overrepresented in cell type-specific enhancer and promotor regions. Interestingly, male infants with growth restriction and females with overgrowth had more epigenetic dysregulatory events (Fig. 3.1), suggesting sex-specific differences in fetal susceptibility and response to intrauterine exposures [54].

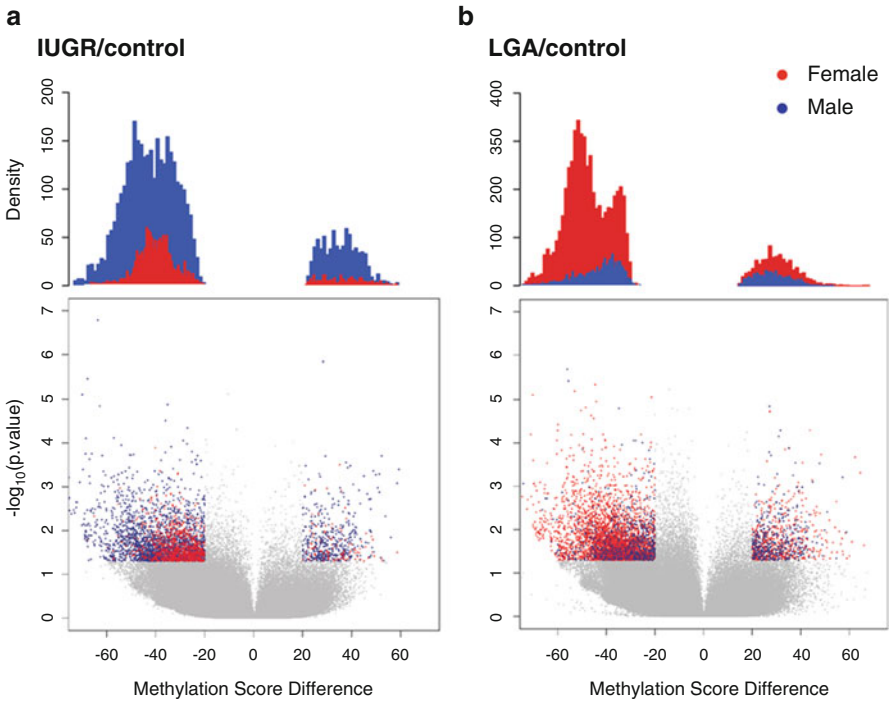


**Fig. 3.1** Sexual dimorphic response associated with Intrauterine Growth Restricted (IUGR) male and over-grown or large for gestational age (LGA) female infants for differentially methylated loci. (**a**) IUGR compared with appropriately grown controls, (**b**) LGA compared with controls. The *lower panels* show volcano plots of DNA methylation score differences and the *top panels* show density plots of differentially methylated loci with $p<0.05$ using analysis of variance with pairwise two-tailed Tukey-tests for methylation difference $>|20|$. From Delahaye et al. [54]

## Epigenetics and Obesity

The alarming rise in rates of obesity across the world is a major public health concern and reproductive aged women are similarly affected. Maternal obesity and diabetes in pregnancy increase the risk of obesity and its complications in exposed offspring [55, 56]. Although the exact mechanisms are not clear, susceptibility for obesity in offspring may stem from disturbances in central regulation of appetite, disruption of metabolic pathways, and the propensity for greater adipogenesis. Changes in DNA methylation and histone modifications may be the underlying mechanisms responsible for the pathophysiological changes observed. In umbilical cords, hypermethylation of RXRα and NOS3 is positively associated with childhood adiposity at 9 years old [57]. Regulation of energy balance may also be disrupted by early life conditions. Decreased methylation levels are found at the glucocorticoid receptor promotor as well as decreased histone lysine 27 trimethylation and increased histone H3 lysine 9 acetylation in hypothalamus of sheep exposed to maternal undernutrition, which is consistent with the increase in glucocorticoid receptor mRNA and protein levels found in the same brain region [38]. Alterations in methylation and expression of dopamine genes in hypothalamus are also seen in another model of maternal high fat feeding [58]. In rodents, maternal high fat feeding induced a metabolic syndrome-like phenotype associated with changes in histone modification and methylation at the adiponectin and leptin genes and the predicted changes in their gene expression in white adipose tissues [59]. Maternal dietary fat intake can also alter expression and epigenetic markings at key genes relevant to fatty acid metabolism [60, 61]. Others have shown enhanced adipocyte differentiation associated with decreased methylation at the promotor of a key transcription factor regulating differentiation in fetal mice exposed to maternal obesity [62].

In addition to maternal diet, our appreciation of the many other environmental exposures that impact health is expanding. A number of endocrine disrupting chemicals have been associated with the rising prevalence of obesity. These agents disrupt regulation of lipid metabolism, metabolic rate, and control of energy balance [63]. In the modernized world, these substances are ubiquitous and can be found in plastic, beauty products, and food packaging, such that exposure may come in the form of ingestion, inhalation, or absorption through the skin. Bisphenol A (BPA) is often a component of plastic products and may be found in the lining of food cans and baby bottles. Human exposure has been documented in urine, serum, breast milk, and both maternal and fetal tissues [63]. A number of other chemical substances have been associated with increased BMI in offspring of exposed pregnant women [64–66].

Polyaromatic hydrocarbons are a class of harmful air pollutants that are formed with incomplete combustion of fossil fuel and are present in grilled meats and tobacco smoke among other sources [67]. Exposure to PAH and tobacco smoke is associated with reduced fetal weight [68, 69]. Chronic exposure to Benzo(a)pyrene (BaP), a member of the polyaromatic hydrocarbon family, induces excessive weight

gain in mice [70]. Individuals exposed to high levels of BaP show evidence of global and gene-specific changes in DNA methylation in peripheral blood lymphocytes [71, 72] as well as increased expression of pro-inflammatory cytokines [73–76]. In pregnancy, BaP crosses the placenta and can cause DNA damage in fetal tissues [77, 78]. Some evidence suggests that in utero BaP exposure may also affect childhood growth and adiposity [68, 79], but others have found reduced weight at 30 months of age [80].

A growing concern for long-term effects of endocrine disruptors on the epigenome stems from the knowledge that certain hormones act in part through epigenetic regulators. As an example, steroid hormones bind to nuclear receptors, enter the nucleus, and form a complex with hormone-responsive elements [81]. In addition, some chemical exposures, which are below the Environmental Protection Agency's (EPA) standard toxicity testing, may have adverse biological effects [82]. A full understanding of how these chemicals affect the health of mothers and their unborn children is still lacking. Greater research is needed to make better assessments of individual exposure and to better understand the mechanisms by which these chemicals exert their influence.

## Epigenetics and Diabetes

Many studies in humans and animal models have demonstrated the link between maternal diet or adverse intrauterine conditions and diabetes in adulthood [83, 84]. Nutrient availability can acutely alter epigenetic regulators of gene expression. For example, in adults hypermethylation of promotor region of peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1-α (PGC-1α) is seen in skeletal muscle of subjects with diabetes and impaired glucose tolerance. These findings coincided with changes in mitochondrial density and plasma lipid concentrations. Further, skeletal muscle explants from non-diabetic subjects showed increases in non-CpG methylation at the PGC-1α promoter region when exposed to palmitate and oleate [85].

Not surprisingly, early life changes in nutrient availability also have an effect on glucose metabolism and insulin action. In rodent models of maternal calorie restriction-induced intrauterine growth restriction, exposed offspring have elevated fasting plasma insulin levels and significant hyperphagia as they age [86]. Preceding evidence of impaired glucose tolerance, a 70% reduction in pancreatic beta cell mass is seen by 3 weeks of age [87]. Growth restricted pups have decreased H3/H4 acetylation and H3K4 trimethylation at the proximal promotor of Pdx1 (pancreatic and duodenal homeobox 1), a transcription factor critical to the regulation of beta cell growth and function [88, 89]. Maternal protein restriction alone can also reduce beta cell proliferation, function and insulin content [90] as well as structural and functional changes in liver [91, 92] and adipose tissue [93], leading to progressive loss of glucose tolerance [94]. Maternal high fat feeding leads to histone modifications in association with transcriptional activation of key regulators of

gluconeogenesis, which may result in increased hepatic glucose production and decreased insulin sensitivity [95]. These findings have led investigators to examine the potential role of DNA methyltransferases and histone modification enzymes in the treatment of metabolic disorders, like diabetes and obesity [96, 97].

## Epigenetics and Cancer

Adverse exposures in early life may increase the risk for other age-related diseases, including cancer [98–100]. The greatest evidence shows an association between hormone-dependent cancers of the reproductive tract and breast. Epidemiological studies show a positive correlation between birth weight and breast cancer risk in women [101–103] and increased risk for aggressive types of prostate cancer in men [104–106]. Evidence of the link between other surrogate markers of fetal nutrition and risk of testicular cancer [107] and lung cancer [108, 109] is beginning to emerge.

Hormone exposure can also have adverse impact on cancer risk later in life. The effect of diethylstilbestrol (DES) exposure in utero is a well-known cause of certain uterine anomalies as well as increased risk for clear cell adenocarcinoma of the vagina [110]. In addition, prenatal DES exposure has been linked to greater risk of breast cancer [111, 112] and may lead to prostate cancer in exposed men [113]. In African American males, the risk of prostate cancer correlated with cord blood testosterone levels [114]. Interestingly, the timing of exposure to excess or exogenous hormones may be critically important to determine risk for later cancer [115] as the effect of estrogen on mammary development differs during the life course [116]. Intrauterine exposure to estrogens may alter architecture of the mammary gland and predispose to cancerous transformation [117], whereas postnatal exposure may promote differentiation to mammary epithelial cells and have a protective effect similar to the effects of pregnancy [115]. Further, exogenous estrogen or high fat diet led to increased mammary cancer in several generations of offspring and was marked by changes in DNA methylation patterns [118].

## Early Life Exposures and Epigenetic Biomarkers

Poor maternal health and adverse intrauterine exposures may lead to a range of changes in gene expression and functional outcomes, ultimately leading to disease susceptibility in adulthood. The phenotypic traits of the offspring include alterations in gene expression, metabolic pathway activity, and changes in structure and function of tissues throughout the body. The sum of the evidence suggests that adverse early life exposures lead to an aging phenotype, marked by increased susceptibility to disease, involvement of multiple organs and tissues, and epigenetic dysregulation. A conceptual model of epigenetic dysregulation characteristic of normal aging and the effect of early life adverse exposures is presented in Fig. 3.2.

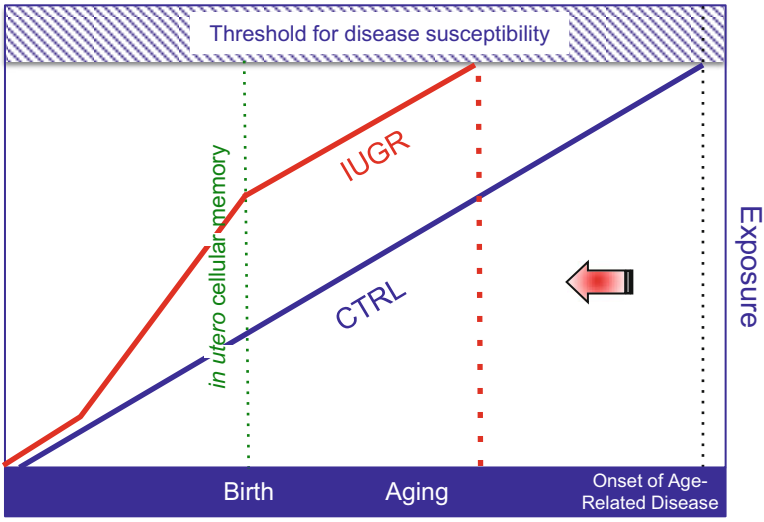


**Fig. 3.2** Paradigm for developmental origin of adult disease. Adverse intrauterine exposures, such as intrauterine growth restriction (IUGR), set a trajectory for reaching threshold for disease earlier in life. *CTRL* control

During development, epigenetic markings are stabilized and are maintained during replication. Assays that measure the epigenome comprehensively provide the opportunity to reflect cellular injury prior to the onset of tissue dysfunction. As a result, epigenetic modifications have the potential to serve as biomarkers predictive of a host of diseases in adulthood. Clinically meaningful biomarkers are specific and sensitive and reliably identify a particular biological point between exposure and disease [119]. Ideally, tools in the future will not only quantify an individual's disease risk, but also provide a measure of their specific biological responsiveness to an exposure and inherent protections from disease as well. As such, epigenetic biomarkers will be more reliable if understood in the context of an individual's genotype. The first step toward developing useful epigenetic biomarkers is to define "normal' or "healthy" on a molecular and cellular level in order to distinguish points along the continuum of disease. Several large consortia have begun to establish genomic maps of all of the different epigenetic marks, such as the NIH Roadmap Epigenomics Mapping Consortium with the major goal of understanding the epigenome in context of DNA sequence variation [120].

Based on some of the work through the Roadmap Epigenomics initiative, a greater appreciation for the importance of confounding sources of variability in epigenetic marks has developed. Defining the extent and nature of epigenomic variability that exists between healthy individuals provides an alternative view of disruption of epigenetic regulation. Our group created methylation variability maps for CD34+ hematopoietic stem and progenitor cells isolated from cord blood [121]. We found that DNA methylation variability is increased at loci of intermediate methylation (Fig. 3.3). DNA methylation variance analysis was developed to create

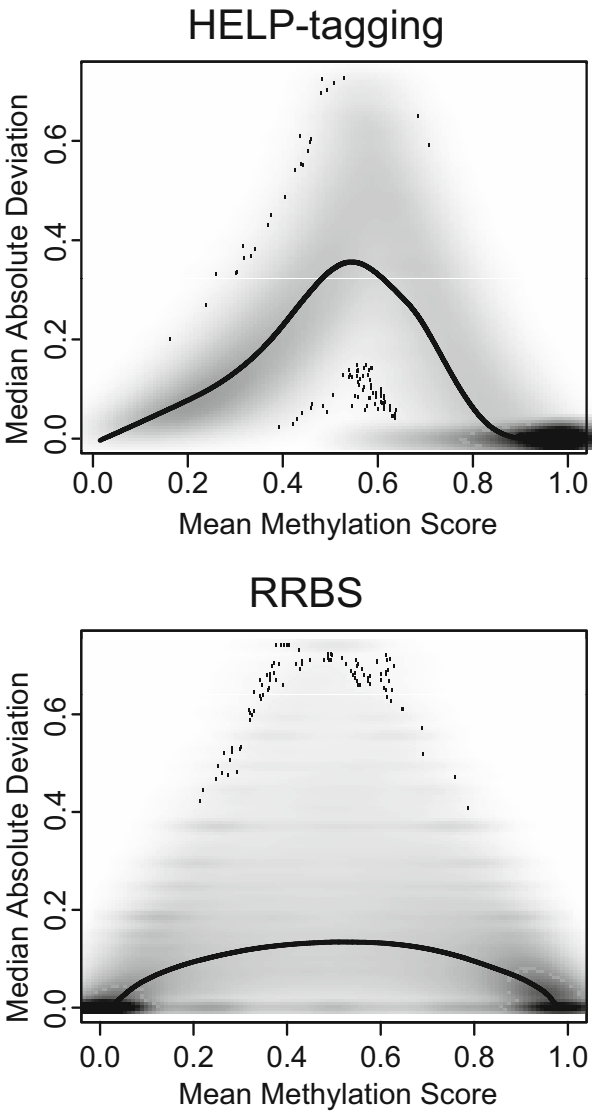


**Fig. 3.3** At loci with intermediate methylation levels, DNA methylation levels are increased. The *top panel* shows the median absolute deviation (MAD) for methylation values in CD34+ hematopoietic stem and progenitor cells from cord blood of 29 healthy, term infants measured by HELP-tagging. The *bottom panel* shows mean DNA methylation across CD34+ cell samples from seven individuals using reduced representation bisulphite sequencing (RRBS). *Gray shading* reflects the number of loci and the *line* indicates the mean MAD value. From Wijetunga et al. [121]

genome-wide maps of inter-individual epigenetic variability in progenitor cells from normal neonates. We made extensive use of Roadmap in Epigenomics resources to identify sources of extreme variability patterns at genomic loci defined by sequence-based properties (like CpG islands, transposable elements, or highly conserved non-coding elements) or by specific epigenetic marks (such as histone modifications or DNAse hypersensitivity peaks), demonstrates chromatin states mapped for CD34+ cells by the Roadmap in Epigenomics to identify methylation changes in cis-regulatory elements specific to this cell type. We found that changes

in methylation at loci that are epigenetically variable may be due to differences in cell subtypes sampled. In contrast, changes in methylation found at invariant loci represent epigenetic changes in a percentage of the cells under studied. Variability maps from normal individual may help to identify regulatory points that are most susceptible to perturbation and those that are more likely to underlie phenotypic changes related to the disease process of interest [121].

The inherent individuality of people also presents challenges for predicting tissue dysfunction or disease progress. Currently, we are heavily reliant on generalized inferences made from the study groups of individuals who share similar clinical characteristics. However, for individual patients, our ability to accurately predict outcomes is often quite limited. The development of molecular classification systems may help to improve upon the clinical risk factor-based assessments that are currently in use. Molecular classifications, such as epigenotyping, exploit the shared molecular features of relevant cells or tissue and are aimed at identifying specific subtypes of disease that are more strongly associated with a particular outcome of interest. The goal of this approach is to use epigenetic or transcriptional profiles to predict clinical outcomes. Similar approaches have been used for classification of cancer subtypes [122, 123] to augment clinical and pathological assessments of cancer risk and disease progression. While changes in DNA methylation patterns have been used to distinguish hepatocellular carcinoma secondary to viral hepatitis versus alcohol intake [124, 125], the applicability of epigenetic profiles as biomarkers of environmental exposures or non-cancerous chronic disease has yet to be determined. Nonetheless, broader application of this approach has the potential to have great clinical impact.

## Challenges to Studying Developmental Origins of Adult Disease

The first major challenge in studying developmental origins of adult disease lies with the difficultly of measuring exposures or the exposome. The "exposome," first coined by Christopher Wild, represents all of the environmental factors influencing internal cellular and chemical reactions that an individual will experience across their lifespan [126]. The exposome includes all environmental conditions, not just acute, high-level toxin exposures, but also low-level exposures that fluctuate over time and may with repeated exposures have cumulative effects. In creating this new term, Wild hoped to create greater balance in the thinking surrounding gene–environment interaction and counterbalance the genome side of the equation. He has argued that the tools available to measure environmental exposures are crude in comparison to the high precision and reproducibility of tools used in genomic assays [126]. Without accurate measures of environmental exposures, the ability to predict how each agent contributes to human disease is limited. In general, genome-wide association studies (GWAS) tend to be more highly regarded due to their

reproducibility, even though the impact of specific environmental exposures may represent a greater contribution to disease risk. Measuring fetal exposures and intrauterine conditions poses even greater challenges given the inaccessibility of the fetus and the additional modifying effect of the placenta on agents in circulating maternal blood.

A second challenge of research involving epigenetics is cell purity of study samples. Unlike mutations in DNA sequence, epigenetic events are thought to be somatic rather than constitutional. Therefore, to test for relevant epigenetic changes, a sample of the cell type mediating a specific phenotype of interest is needed. Conceptually, some very early life exposures may induce more widespread effects, which would justify the use of more accessible samples of surrogate cell types, e.g., peripheral blood as a surrogate of hepatocytes. However, ideally the dysfunctional organ or tissue should be sampled. This is often a limitation of human studies and supports the use of animal models for these purposes. In epigenome-wide association studies (EWAS), a compromise is often needed in choosing the sample type to study. In general, accessibility of cell samples, the specific cell type quantity, and homogeneity of the composite cells in the sample must be balanced with mechanistic relevance of the sample to the disease state or phenotype under investigation. In addition, samples composed of many cell types present another challenge. Histologically, distinct cells have different epigenomes, which dictate their phenotype and behavior. Systematic changes in the proportion of cells or type of cells present in a sample may confound comparisons between groups, particularly because effect sizes are often small in epigenetic studies. Cell purification processes can be helpful as long as sufficient number of cells are available for epigenomic assays.

Another challenge in studying the association between early life events and epigenetic changes is which epigenetic regulator to study and how. Many investigators have focused on cytosine methylation because of the relative stability of cytosine methylation. In addition, sample preparation for chromatin immunoprecipitation-based assays is more complex and prone to technical errors. More recently, there is a push for the use of more comprehensive genome-wide assays as evidence emerges to suggest that epigenetic regulation of gene expression may be occurring at less predictable sites, such as cis-regulatory sequences in the genome that are far from promotors [127]. Microarray-based approaches are cost-efficient and provide genome-wide coverage, but are limited by focus on pre-defined loci such as promotors or CpG islands [128, 129]. Massively parallel sequencing-based approaches are unbiased and can survey the entire genome, but may be cost-prohibitive. Fortunately, the genome-wide assays available continue to improve in comprehensiveness and have become more quantitative, while cost continues to decline.

Similar to GWAS, EWAS aim to discover loci with epigenetic changes that are non-randomly associated with a disease or phenotype. Cost has been a major factor in performing well-designed EWAS in a comprehensive and sensitive manner that can robustly test for epigenetic dysregulation associated with early-life environmental exposures. Michels et al. [130] recently cataloged the major design and analysis issues that are critical to consider when carrying out this type of study. Some of the

issues include careful consideration of a specific research question, choice of study populations, ample sample size, and selection of tissue or cells as mentioned above. In addition, successful EWAS account for sources of biological and technical variability, use various quality control measures, and employ both verification and validation steps in the analytical process [130]. A significant challenge to EWAS studies is the computational resources required for processing and storage of data, particularly with larger cohort sizes and studies that include massively parallel sequencing-based assays. High-performance computing resources and cloud computing are generally necessary to handle the analytical challenges of EWAS.

## Conclusion

Early-life environmental exposures have great biological impact on the health trajectory of an individual over their lifespan. Abnormal fetal growth and exposure to environmental toxins in early life result in significant changes in gene expression in a variety of relevant tissues in animal models. The changes in gene expression are also associated with epigenetic changes and well-characterized tissue dysfunction later in life. While direct evidence demonstrating which agents will cause a specific outcome is limited in humans, this area of research is rapidly advancing. Development of better tools for exposure assessment is critically needed to match the precision of genomic technologies that are currently in use. Comprehensive and quantitative epigenetic assays hold great promise for the development of biomarkers measuring the effects of early-life environmental exposures and prediction of later disease susceptibility if the challenges of study design and cost can be overcome. Investment in well-designed EWAS aimed at understanding the mechanisms underlying early-life exposures that lead to progressive epigenetic dysregulation has the potential to have substantial impact on preventative approaches to most adult chronic diseases.

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# Chapter 4
# Nutritional and Lifestyle Impact on Epigenetics and Cancer

**Eswar Shankar and Sanjay Gupta**

**Abstract** Nutrition and lifestyle factors play an important role in human health as dietary imbalances are major determinants of several diseases including cancer. Emerging studies suggest that diet and nutrition can impact gene expression through epigenetic mechanisms. Epigenetic modifications are heritable and cause potentially reversible changes in gene expression that do not require alteration in DNA sequence. Epigenetic marks include changes in DNA methylation, histone modifications, and small noncoding miRNA. Aberrant epigenetic modifications probably occur at an early stage in neoplastic development and are widely described as essential players in cancer progression. Epigenetic modifications also mediate environmental signals and provide links between susceptibility genes and environmental factors in the etiology of cancer. The present chapter initially highlights the role of various epigenetic mechanisms in the regulation and maintenance of mammalian genome. Focusing on the effect of various endogenous factors that include environmental, lifestyle, nutritional, and social-economic/racial aspects; this chapter discusses their impact on the process of carcinogenesis through various epigenetic modifications. Elucidating the impact of nutrition and lifestyle factors on epigenetic mechanisms may serve as a personalized prediction tool assessing cancer susceptibility and in providing recommendation and guide for prevention and therapeutic options against cancer.

**Keywords** DNA methylation • Histone modification • Noncoding RNA • Dietary agents • Gene–environment interaction • Carcinogens • Chemoprevention

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## Abbreviations

| | |
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| AA | African American |
| BPDE | Benzo(a)pyrene diol epoxide |
| CA | Caucasian American |
| DNA | Deoxyribonucleic acid |
| DNMT | DNA methyltransferases |
| EGCG | Epigallocatechin-3-gallate |
| ER | Estrogen receptor |
| EZH2 | Enhancer of zeste homolog 2 |
| GSTP1 | Glutathione S-transferase pi |
| GTP | Green tea polyphenols |
| HAT | Histone acetyltransferase |
| HDAC | Histone deacetylases |
| HDM | Histone demethylases |
| hMLH1 | Human mutL homolog 1 |
| HMT | Histone methyltransferases |
| HPV | Human papillomavirus |
| IGF | Insulin-like growth factor |
| LINE | Long interspersed nuclear element |
| lncRNA | Long noncoding RNA |
| LSD1 | Lysine specific demethylase 1 |
| MBD | Methyl-binding domain proteins |
| MGMT | *O*(6)-methylguanine methyltransferase |
| miRNA | MicroRNA |
| ncRNA | Noncoding RNA |
| PAH | Polycyclic aromatic hydrocarbons |
| PcG | Polycomb-group proteins |
| piRNA | Piwi-interacting RNA |
| RARbeta | Retinoic acid receptor beta |
| RepA | Short repeat RNA |
| SAH | *S*-adenosyl-L-homocysteine |
| SAM | *S*-adenosyl methionine |
| SFN | Sulforaphane |
| siRNA | Small interfering RNA |
| TIMP-3 | Tissue inhibitor of metalloproteinases-3 |

## Introduction

Cancer is widely recognized as a heterogeneous disease resulting from genetic and epigenetic alterations inherited by a series of transformations in clonally selected cells exhibiting selective growth advantage, sustaining proliferative signaling,

evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Although genetic lesions drive tumor progression, however it is becoming clear that epigenetic perturbations are equally important in cancer development. Epigenetics is referred to as the study of stable inheritance of gene expression that occurs without changes in the DNA sequence. A majority of cancers result from changes that accumulate throughout the lifespan as a result of exposure to various endogenous factors that include environmental, lifestyle, nutritional, and social-economic/racial aspects. Epigenetic disruption of gene expression by these endogenous factors plays a critical role in cancer progression. A number of epigenetic mechanisms have now been identified in mammals. There are three major epigenetic mechanisms which are known to regulate gene expression. These include DNA methylation, modulation of chromatin structure by posttranslational modification of histone or nonhistone proteins, and small noncoding microRNAs (miRNAs) that alter gene expression by either inhibiting translation or causing targeted degradation of specific mRNAs. These mechanisms are critical components in the normal development and growth of cells and their modifications contribute to neoplastic phenotype.

## Mechanisms Underlying Epigenetics

### *DNA Methylation*

Methylation of cytosine residues within the dinucleotide sequence-CpG is one of the most widely studied epigenetic modifications in mammals [1]. Forming an essential component of the cellular epigenetic machinery, DNA methylation in collaboration with histone modification regulates gene expression by modulating DNA packaging and chromatin architecture [2]. DNA methylation is a chemical modification that involves transfer of a methyl ($CH_3$) moiety from the donor *S*-adenosyl methionine (SAM) to the 5′ position of cytosine residue that precedes guanine in the CpG dinucleotide sequence, forming 5-methyl cytosine and *S*-adenosyl-L-homocysteine (SAH) [1, 3–6]. The mammalian genome has been reported to harbor $3 \times 10^7$ methylated cytosine residues mostly within CpG dinucleotide sequences [4]. Although CpG sequences are unevenly distributed throughout the human genome, they are frequently enriched in gene promoters (often referred as CpG islands) and large repetitive sequences such as Long interspersed nuclear element (LINE) and ALU retrotransposon elements [7]. DNA methylation is catalyzed by a group of enzymes known as DNA methyltransferases (DNMTs) [1, 4]. There are three major DNA methyltransferases (Dnmt1, Dnmt3a, and Dnmt3b) identified in mammals. Evidence from phenotypic analyses of mice with mutant DNMT genes have provided useful mechanistic insights into the role and establishment of DNA methylation patterns during development [4, 8]. Dnmt1 enzyme has been demonstrated to have a 5–30-fold more preference for hemimethylated substrates and therefore popularly designated as maintenance methyltransferase. It preserves the existing

methylation patterns in the daughter DNA strands by adding methyl groups to hemimethylated CpG sequences following replication. However Dnmt1 has also been demonstrated to be involved in de novo methylation activity in embryo lysates and its sequence specificity was shown to be confined to 5′-CpG-3′ dinucleotide sequence with little dependence on sequence context or density [9]. Dnmt3a and Dnmt3b enzymes are essential for global de novo methylation as they preferentially target unmethylated CpG sequences [10]. They have been shown to be highly expressed in developing mouse embryos and establish methylation patterns postimplantation [10]. Although Dnmt3L, the fourth family member, lacks intrinsic DNMT activity by itself, it colocalizes with Dnmt3a and Dnmt3b to establish genomic imprints in maternal germ line [11] and facilitate methylation of retroposons. Dnmt2, another member of DNMT family, was found to lack biochemical detectable DNMT activity and its deletion in mice had no obvious phenotypic effects on genomic methylation pattern or methylation of retroviral DNA [10].

Hypermethylation of CpG islands is usually associated with gene silencing. There are multiple routes through which DNA methylation can suppress transcription. A general mechanism is to exclude binding of proteins that modulate transcription through their DNA binding domains [12]. For example, binding of chromatin boundary element binding protein CTCF to DNA is blocked by CpG methylation, which allows the enhancer to activate transcription [13, 14]. This mechanism has been demonstrated to be essential for imprinting of *Igf2* gene [15]. Beside this, CpG methylation has been shown to block the binding of several other transcription factors; however, their biological consequences remain unknown [16]. Another mechanism for DNA methylation mediated gene repression involves binding of specialized DNA binding proteins to the methylated CpG stretches, which form repressor complexes with histone deacetylases (HDACs) and cause chromatin compaction [17–19]. In mammals six methyl-CpG-binding proteins have been characterized to date, which include MeCp2, MBD1-4, and Kaiso. Studies demonstrate that all (except mammalian MBD3) possess a domain that specifically targets them to methylated CpG regions in vitro and in vivo [20, 21].

## *Histone Modifications*

In addition to DNA methylation, posttranslational modification of N-terminal histone tails play a significant role in epigenetic regulation of gene expression [22, 23]. A typical nucleosome unit consists of ~146 bp of DNA wrapped around an octamer of histones (H2A, H2B, H3, and H4) representing the fundamental building unit of eukaryotic chromatin. A diverse array of covalent chemical modification of less structured, protruding N-terminal tails of core histones by methylation, acetylation, ubiquitination, phosphorylation, sumoylation, and ADP-ribosylation dictate the dynamics of chromatin state [24]. Euchromatin is lightly packed form of chromatin where DNA is accessible for transcription, whereas heterochromatin represents

tightly packed chromatin state inaccessible to cellular transcriptional machinery. Most of the chemical modifications occur at Lysine (K), Arginine (R), and Serine (S) residues within the histone tails. These distinct histone modifications on one or more histone tails (often referred to as 'Histone code') which may act sequentially or in combination are recognized by other proteins that signal further downstream events. A number of enzymes have been implicated in catalyzing (addition or removal) various histone modifications. Examples include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone kinases, etc. In brief, HATs catalyze the addition of acetyl group on the ε-amino group of lysine residues in the N-terminal tail of histones, which neutralize the positive charge, relax the chromatin and facilitate the binding of transcriptional machinery to the DNA [25]. Till date 25 HATs have been characterized which are divided into four families. Examples include GNAT (*hGCN5*, *PCAF*), MYST (*MYST*, *Tip60*), p300/CBP (*p300*/*CBP*, SRC (*SRC-1*), and TAFII250 families (TAFII250) [3, 6, 26]. In contrast, HDACs catalyze the removal of acetyl groups from lysine residues resulting in the compaction of chromatin configuration which repress transcription [27]. HDACs are classified into four groups. HDAC-1, -2, -3, and -8 are members of Class I HDAC family while HDAC-4, -5, -6, -7, -9, and -10 belong to class II HDAC family. HDAC-11 belongs to Class IV HDAC group. Sirtuins, which require $NAD^+$ as cofactor for their activity, are structurally unrelated to other HDAC classes, constitute Class III HDAC family [28, 29]. HMTs catalyze the addition of methyl groups to lysine or arginine residues while HDMs act to remove them [30–32]. Examples of histone lysine methyltransferase include EZH2 (Enhancer of zeste homolog 2) and that of histone lysine demethylase include LSD1 (Lysine specific demethylase 1) [33, 34]. Depending on the site of lysine methylation (K4, K9, K27, etc. in Histone H3) and methylation status (mono, di, or tri methylation), histone methylation may have activating or repressive effect on gene expression [26, 34]. H3K4, H3K36, and H3K79 methylation have activating effects on gene transcription, whereas methylation of H3K9, H3K27, and H4K20 is generally associated with gene silencing or transcriptional repression [26, 32, 35]. A plethora of literature is available on each group of histone modifying enzymes, their mechanism of action and various histone modifications, which is beyond the scope of this chapter.

## *Noncoding RNAs*

Recent evidence indicates that noncoding RNA (ncRNA) transcripts play a fundamental role in epigenetic regulation of gene expression and have been implicated in various epigenetic mechanisms such as transposon silencing, X-chromosome inactivation, DNA imprinting, and paramutation [36–38]. In humans, ncRNAs include microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA) which account for majority of transcripts, representing

approximately 98 % of all human transcriptional output [39, 40]. Based on the size, ncRNA can be classified into small ncRNA which are generally less than 200 nucleotides in length and long ncRNA (lncRNA) transcripts that are more than 200 nucleotides in length. They can be divided into further subtypes based on their genomic origin and biogenic processes [37]. Both types of ncRNAs have been shown to be essential '*epigenetic modifiers*' constituting a hidden layer of complex internal signals controlling multiple levels of gene expression associated with development and physiology of an organism [38, 41–43]. lncRNAs have been demonstrated to be involved in gene silencing via mechanisms involving both histone modifications and DNA methylation. For example, the antisense lncRNA located in the *p14*/*p15*/*INK4* locus, *ANRIL*, was reported to cause gene silencing via recruitment of polycomb proteins (PcG) [44, 45]. Another well studied example includes the involvement of a 17 kb lncRNA, *XIST*, in X-chromosome inactivation which ensures X-linked gene dosage compensation in mammalian females [46–49]. This process involves the recruitment of mammalian PRC2 complex containing the histone methyltransferase EZH2 to the locus by a short repeat RNA (RepA) within XIST and deposition and spreading of repressive H3K27me3 marks throughout the X-chromosome. In addition to histone modifications, lncRNAs were also reported to mediate gene silencing through DNA methylation. One such example includes *Kcnq1ot1*, which in addition to interacting with PRC2 complex and G9a, has been implicated in the recruitment of Dnmt1 through a critical 890 bp region to the CpG island of the imprinted genes [50].

Small ncRNAs particularly miRNAs regulate key epigenetic mechanisms. Short RNAs (50–200 nucleotides) were reported to be transcribed from H3K27me3-enriched PRC2 target genes and cause cell-type specific gene silencing in *cis* by stabilizing the PRC2 complex near the transcription site through interactions via formation of stem-loop structures [51]. MiRNAs are known to regulate various components of cellular epigenetic machinery particularly polycomb complexes and thus affect multiple downstream effects [33, 52–54]. One such example include miR-214 which downregulates Ezh2 expression by targeting its 3′-UTR region and accelerates skeletal muscle differentiation and transcription of developmental regulators in embryonic stem cells [55]. There are other miRNAs which have been implicated in the repression of Bmi1, a component of PRC1 complex [56–58]. DNA methylation has also shown to be modulated by miRNAs. Dnmt1 and 3 have been reported to be targeted by the miR-29 family in lung cancer and leukemia cells [59, 60]. In addition to the role of small ncRNAs as regulators of various epigenetic mechanisms, in many instances they are themselves targets of the same epigenetic processes which may lead to further downstream alterations. For example, in human breast tumorigenesis and metastasis decreased expression of a set of miRNAs was attributed to gene hypermethylation [61–63]. In summary, recent evidences suggest that ncRNAs have emerged has key regulators of epigenetic mechanisms and also, that the modulation of these RNA transcripts by the same epigenetic processes may lead to major consequences.

## Factors Affecting the Epigenome

### *Effect of Environmental Factors on the Epigenome*

Environmental factors including chemical carcinogens, environmental pollutants, dietary contaminants, and physical carcinogens play important role in the etiology of human cancer. In general, the degree to which environmental factors influence carcinogenesis depends on the presence of specific hazardous entity and duration of exposure. However, the degree to which hazardous exposures affect cancer largely reflects variation in susceptibility to a given environmental exposure. Generally environmental factors that are capable of initiating tumor development by altering the epigenome include agents which are capable of inducing changes either directly or indirectly in the genomic DNA, and agents that affect critical cellular regulatory processes of gene transcription such as DNA damage and repair, cell cycle control, and cell death process.

Studies demonstrate that the mismatch repair gene *MHL1* is frequently hypermethylated in sporadic tumors exhibiting microsatellite instability [64]. Similarly, silencing of *MGMT*, the DNA repair gene encoding the protein responsible for the removal of carcinogen-induced $O^6$-methylguanine adducts from DNA (which if left unrepaired results in G to A transition mutation), appears to increase the mutation rate in critical cellular regulators, including tumor suppressors and oncogenes [65]. These studies provide cues that environmental exposures alter either the expression or the activity of enzymes involved in de novo DNA methylation (Dnmt3A and Dnmt3B) and/or the maintenance of DNA methylation (Dnmt1) may predispose to mutational events [64, 65]. Additionally, different agents in the environment may also induce mutational events through preferential binding to hypermethylated DNA. Studies on benzo(a)pyrene diol epoxide (BPDE), a carcinogen from tobacco smoke that exhibits preference for methylated CpG sites, resulting in formation of DNA adducts and G to T transfersions, often found in cancers of the aero-digestive tract in tobacco smokers. It has been shown that certain infectious agents such as human papillomavirus (HPV) induce gene silencing via DNA hypermethylation of the promoters of host genes including *CDH1*, *RB1*, *INK4a*/*p16*, *CDNK2A*, *MTHFR*, *PEG3,* and others listed in Table 4.1.

The agents in the second group may alter the pattern of chromatin modifications (histone code) in a transient manner and are likely to induce changes in key cellular processes including gene transcription, DNA damage response, and DNA repair. Primary epigenetic targets for environmental factors in this group may be the proteins and protein complexes responsible for histone modifications such as HATs and HDACs, whose activities are often found deregulated in cancer. Recent studies showed that HATs are involved in the process of DNA repair, suggesting that even moderate and transient inhibition of HAT activity induced by environmental exposures may compromise DNA repair, leading to mutation fixation and genomic instability [110]. Similarly, HDAC was shown to be required for efficient DNA repair, suggesting that the removal of histone acetylation is required for restoration of

**Table 4.1** Targeted genes and epigenetic mechanisms affected by environmental, dietary, and lifestyle factors

| Targeted genes | Epigenetic mechanisms | Agents/exposure | References |
|---|---|---|---|
| Environmental factors | | | |
| *CDKN2A, MGMT* | Binding of PAHs to methylated CpGs | Tobacco smoke | [66–69] |
| *CDKN2A,CDKN2B (p15 gene), MLH1* | Inhibition of histone acetylation; damage of core histone | Nickel | [70–73] |
| *RASSF1A, MGMT CDKN2A, SNSG* | Preferential binding to methylated lysine or modified histone proteins causing DNA damage | AFB1 | [74–76] |
| DNMTs | Inhibition of DNMTs (acute exposure), increased DNMT activity (chronic exposure) | Cadmium | [77] |
| *c-myc* | Decrease of SAM leading to global hypomethylation and activation of oncogenes | Arsenic | [71, 78, 79] |
| *p16/INK4A* | DNA methylation-mediated silencing of key cellular regulators | Ionizing radiation | [69] |
| Genes not identified | Global hypomethylation | UV radiation | [80, 81] |
| Genes not identified | De novo methylation associated with chronic inflammation and cell proliferation | Bacteria (*H. pylori)* | [82–84] |
| Genes not identified | Methylation of viral genome; binding of viral proteins to host gene promoters; hypermethylation of host gene promoter; changes in chromatin modification; patterns and recruitment of HDACs | HPV, EBV, HBV HPV, EB | [85–94] |
| Dietary and lifestyle factors | | | |
| *APC-1A, CDKN2D (p14 gene)* | Alcohol metabolites such as acetaldehyde acts as co-carcinogens with HBV and HCV viruses depleting SAM, aflatoxin, and obesity | Alcohol | [95–98] |
| *CDKN2A, hMLH1, MGMT, RASSFIA, SFRP* | Disruption of heterochromatic silencing; inhibition of SIRT1 | Dihydrocoumarin | [99, 100] |
| Genes not identified | Posttranslational modification of histone and DNA methylation | Folate deficiency | [101] |
| Genes not identified | Modifications in DNA methylation and histone modifications; increased binding of methyl-CpG-binding proteins | Low methionine-intake | [96, 101, 102] |
| Genes not identified | Re-activation of inactive X-chromosome and silent alleles LOI | Unknown | [103–106] |
| *IGF2,* transposable elements | Histone-based germ line inheritance | Transgenerational epigenetic inheritance | [107–109] |

normal (default) chromatin structure following the completion of DNA repair. A tight regulation of HAT and HDAC activity is thus essential for proper regulation of gene transcription and DNA repair. Reduced levels of histone acetylation or enhanced histone deacetylation may result in the compaction of chromatin, blocking access of transcription factors to DNA and/or impeded progression of RNA polymerase. Therefore, different environmental factors may transiently alter chromatin-modifying/remodeling activities and alter patterns of histone modifications impeding DNA repair and other chromatin-based processes.

Another possible epigenetic 'target' of adverse environmental exposure may be general methyl-C-binding proteins, a group of proteins (including MBD1, MBD2, MBD3, MeCP2, and KAISO) that bind to methylated CpG sites [111]. Some members of this family, exemplified by MeCP2, were found to bind and recruit HDAC to chromatin. Changes in MeCP2 protein stability and function elicited by the hazardous agents in diet and environment may thus affect normal gene transcription, leading to aberrant cell proliferation and cancer [112]. Given that histone modifications and DNA methylation appear to work together to establish a permissive or repressive chromatin state, agents in the environment and diet that affect one of these intimately linked and self-reinforcing mechanisms would inevitably affect the other. Although poorly understood, the molecular mechanisms by which epigenetic carcinogens in environment and diet may exhibit adverse effects on histone modifications are beginning to emerge. Several recent studies have examined the effect of specific environmental carcinogens on histone modifications and suggest that these agents may affect the pattern of histone modifications through different mechanisms (Table 4.1).

Another prospective mechanism by which environmental exposure including ingestion affect the epigenome involve transposable elements. Transposons when activated may cause genetic mutations and transcriptional noise [112]. For example, the Alu family alone consists of several hundred thousand elements and is shown to be heavily methylated and transcriptionally silent in somatic cells. It is well documented that the activation of transposable element-derived promoters may be a consequence of perturbed DNA methylation, transposable elements were shown to be activated by various kinds of cellular stress. Therefore, stress induced by environmental agents may activate transposable elements, leading to altered establishment and maintenance of epigenetic states.

## *Epigenetic Modifications by Nutritional Factors*

Studies have demonstrated that maternal nutrition imbalance and metabolic disturbances during embryonic development have a persistent effect on the health of the offspring and may be passed down to the next generation [113]. The potential effect of nutritional factors on phenotype has best demonstrated by studies on the risk of cancer for pregnant women and fetuses. When mother is exposed to adverse conditions, the fetal nutrition may cause alterations in structure, physiology, and metabolism that predispose individuals to several diseases including cancer.

Selected dietary components consumed during early pregnancy may influence postnatal risk of cancer development, although all dietary components are not harmful. In those cases where adverse effects on fetal development were observed, a proposed mechanism includes methylation of genes due to dietary food components in the mother's diet. Both hypermethylation and hypomethylation of selected genes were observed. Genes that were overexpressed included *Klf6*, *Klf9*, *Nid2*, *Ntn4*, *Per1*, and *Txnip*, and genes that were repressed included *Bcar3*, *Cldn12*, *Csf1*, *Jag1*, *Lgals3*, *Lypd3*, *Nme1*, *Ptges2*, *Ptgs1*, and *Smarcb1* [113, 114]. In animal models, deficiencies of macronutrients during placental growth have been shown to affect fetal growth. Most of the genes that contribute to reduced fetal growth are regulated by imprinting, and the maternal allele is affected in these cases. Functionally, the nutrient transport from mother to fetus via the placenta is affected dramatically by the hypomethylation of genes in the embryonic trophectoderm [115].

Direct effects of nutritional factors on epigenetic changes are most studied and among the best understood is the relationship between dietary methionine and DNA methylation [115]. Methionine, an essential amino acid, plays a central role in the epigenetic regulation by serving as methyl donor for methylation reactions. In the process of cytosine methylation, DNMT enzyme converts SAM to *S*-adenosylhomocysteine (SAH); therefore, an optimal supply of SAM or removal of SAH is essential for the normal establishment of genome-wide DNA methylation patterns [116]. CpG methylation patterns are largely erased in the early embryos and then re-established in a tissue-specific manner. Therefore, early embryonic development may represent a sensitive stage, and dietary and environmental factors that affect DNA methylation reaction and the activity of DNMTs may result in permanent fixation of aberrant methylation patterns [110, 116]. In postnatal development and adulthood, established patterns of DNA methylation and histone modifications must be maintained through multiple mitotic divisions; therefore, inappropriate quantities of methionine, other food components, and environmental agents may affect normal patterns of DNA methylation and histone modifications. In this respect, it is interesting to note that in adult men with hyperhomocysteinemia, a disorder occurring in several genetically determined and acquired diseases with uremia, treatment with high doses of folate increases methylation levels at specific genes and restores normal expression [110]. In addition to methylation of DNA, methylation of histones, a distinct epigenetic mechanism dependent on 1-carbon groups, may be affected by consuming excessive levels of specific nutritional factors. Therefore, nutrition factors are likely to directly or indirectly (through changes in DNA methylation) affect histone modifications such as histone methylation.

## *Lifestyle Factors Affecting the Epigenome*

Lifestyle factors including exercise and diet plays an important role in regulating the epigenome and altering gene expression. Exercise can modify the epigenome in order to preserve and prolong life. Exercise has been shown to induce positive changes in DNA methylation within adipose tissue and regulate metabolism in both

healthy and diseased individuals [117]. Increased DNA methylation of genes *Hdac4* and *Ncor2* has also shown to increase lipogenesis following exercise [117]. Exercise also leads to beneficial changes in DNA methylation patterns in skeletal muscle [118]. Not only is obesity an indicator for diseases such as type 2 diabetes and cardiovascular disease, but also puts additional stress on the system which can itself negatively impact health [119]. Acute exercise is associated with DNA hypomethylation of the entire genome in skeletal muscle cells of sedentary individuals and high intensity exercise tends to cause reduction in promoter methylation of certain genes [120]. Exercise is also known to positively influence the expression patterns of miRNAs in leukocyte cells [121]. The health benefits of physical exercise, especially on a long-term and strenuous basis, have a positive effect on epigenetic mechanisms and ultimately may reduce incidence and severity of cancer [122].

Studies in genomic imprinting have revealed how DNA methylation patterns are influenced by diet, and how epigenomic sensitivity to specific diet influences cancer susceptibility. Dietary fat comprises a large part of the Westernized diet, which results in increased adipose tissue via adipocyte hypertrophy and hyperplasia [123]. Dietary fat influences adipokine release through their influence on the epigenome affecting DNA methylation and posttranslation modification of the histone proteins. This represents one of the methods by which dietary fat may influence cancer progression. Overconsumption of well-done meats or saturated fats causes increase in somatic *GSTP1* inactivation by CpG island methylation in the promoter region increasing susceptibility to prostate cancer [124].

Several studies have provided evidence that alcohol consumption is associated with different epigenetic changes in human cancer [125]. In a large epidemiological study (the Netherlands Cohort Study on diet and cancer), analysis of DNA methylation showed that the prevalence of promoter hypermethylation of several genes including *APC-1A*, *CDKN2D*, *CDKN2A*, *hMLH1*, *MGMT,* and *RASSF1A* was higher in colorectal cancer patients with high alcohol (and low folate) intake than among colorectal cancer patients with high folate/low alcohol intake. In addition, the study of human head and neck squamous cell carcinoma showed that the promoter hypermethylation of *MGMT* gene and the genes known to regulate the WNT pathway occurs more frequently in both heavy and light drinkers compared to nondrinkers. The mechanism underlying the epigenetic changes caused by alcohol abuse may also involve SAM. This small metabolite is regenerated from demethylated SAM via the methionine cycle, which involves folate. Therefore, imbalance of this cycle through alcohol consumption may result in depletion of SAM and aberrant epigenetic patterns. In addition, it was shown that the human class I alcohol dehydrogenase (ADH) genes may be regulated by epigenetic mechanism. The class I ADH genes were found to be repressed in human hepatoma through epigenetic modification suggests that changes associated with alcohol-metabolizing genes may also enhance other toxic effects of alcohol on different organs, most notably the liver, including hepatic tumorigenesis [110].

Tobacco smoke is a complex aerosol that contains polycyclic aromatic hydrocarbons (PAHs), mostly benzo[a]pyrene, which is considered the most carcinogenic. Epigenetic targets of the PAHs from tobacco smoke induce DNA damage through

preferential binding to methylated CpG sites, a phenomenon already demonstrated for BPDE, a carcinogen found in tobacco smoke. Several studies have demonstrated hypermethylation and silencing of several genes in lung cancer associated with smoking [126]. The genes frequently altered by promoter hypermethylation in lung cancers of smokers are *p53*, *p16* and *MGMT*. In addition, different components in tobacco smoke induce histone changes and alter histone code. Some potentially novel histone marks, including acetylation, monomethylation, and dimethylation, in specific lysine and arginine residues of histones H3 and H4 in mouse lungs.

Nutrients extracted from the diet enter metabolic pathways and are transformed into useful molecules. These nutrients are known to have epigenetic targets in cells such that they can be used to modify the epigenome in order to correct abnormally activated or silenced genes and can be combined into an "epigenetic diet" useful as a therapeutic and/or chemopreventive measure. During this transitory phase methyl groups are formed from key nutrients including folic acid, B vitamins and s-adenosyl methionine (SAMe), and these methyl groups comprise important epigenetic marks for gene silencing. Diets high in such methyl rich nutrients may significantly alter gene expression and offer protective health benefits [123]. Deficiencies in folate and methionine, both of which are involved in cellular processes that supply methyl groups needed for DNA methylation, can change the expression (imprinting) of growth factor genes such as (IGF1) influencing cancer progression [127]. In addition, several natural nutrients products have interesting biological properties and structural diversity. These include polyphenols present in fruits, vegetables, and other dietary botanicals. Phenolic acids, flavonoids, stilbenes, and lignans are the most abundantly occurring polyphenols that are also an integral part of everyday nutrition in populations worldwide. Certain food components epigenetically increase the levels of DNA repair enzymes such as MGMT and MLH1, others such as blueberry anthocyanins actively decrease DNA damage. Anthocyanin is an effective antioxidant for humans that is found in plants and are easily identified by its potent red or purple pigment. It is found in plants such as eggplant, plums, pomegranate, red onion, cranberries, blueberries, kidney beans, and cherries which all possess anthocyanins. This flavonoid serves as a powerful antioxidant that contributes to scavenging of DNA-damaging free radicals. While the direct fate of anthocyanins in vivo following digestion may be less than 5 % (the majority being rapidly excreted), the potent residual antioxidant property remains in blood following consumption of anthocyanin-rich foods due to metabolic breakdown of the flavonoids and resultant increase in uric acid levels. Some of the common examples of the most studied and promising cancer preventive polyphenols include EGCG (from green tea), curcumin (from curry plant), genistein (from soy), resveratrol (from grapes and berries), and sulforaphane (from broccoli). A large number of dietary agents on DNA methylation, histone modifications, and regulation of expression of noncoding miRNAs in various human cancers are shown in Tables 4.2 and 4.3. Significant gains have been made in understanding the molecular mechanisms underpinning the chemopreventive effects of polyphenols, and consequently, a wide range of mechanisms and gene targets have been identified for individual compounds.

**Table 4.2** Effects of dietary agents on epigenetic modifications

| Source | Dietary agents | Molecular mechanisms | Molecular targets | Cancer type | References |
|---|---|---|---|---|---|
| Soy | Equol | HDAC inhibitor | H2A/H2B/H3/H4 | Breast | [128] |
| | | | Acetylation | | |
| | Genistein | HAT inhibitor | H2A/H2B/H3/H4 | Esophageal; prostate; breast; renal | [128–136] |
| | | HADC inhibitor | Acetylation | | |
| | | | *p21, p16, PTEN,p53* | | |
| | | | *FOXA3, SIRT1, hTERT* | | |
| | Daidzein | HDAC inhibitor | Histone acetylation | Esophageal; prostate | [128, 129, 136] |
| | Biochanin A | DNMT inhibitor | | Esophageal | [129, 136, 137] |
| | Daidzein | DNMT inhibitor | *RARβ,MGMT,CDKN2A,GSTP1,HMGNS,BTG3* | Breast | [129, 136, 137] |
| | Genistein | DNMT inhibitor | *RXRα,CDX2,GSTP1, W1F1* | Prostate | [129, 131, 132, 136, 137] |
| | | | | Esophageal | |
| Poison ivy | Fisetin | Unknown | | Cervix; breast | [138, 139] |
| | | DNMT inhibitor | | Breast, esophageal | [137, 140] |
| Garlic | Allyl mercaptan | SIRT1 inhibitor | H3/H4 acetylation p21/WAF1 | Erythroleukemia liver, colon | [141–144] |
| | S-allylmercapto cysteine | HAT inhibitor | H3/H4 acetylation | Erythroleukemia | [144–146] |
| Cashew nuts | Anacardic acid | HDAC inhibitor | H3K9 and H3K14 deacetylation | Leukemia; cervix breast; prostate | [147–152] |

(continued)

**Table 4.2** (continued)

| Source | Dietary agents | Molecular mechanisms | Molecular targets | Cancer type | References |
|---|---|---|---|---|---|
| Turmeric | Curcumin | HAT and HDAC inhibitor | Histone acetylation | Cervix; HIV; hepatoma; leukemia; prostate brain, lymphoma | [153–164]. |
| | | DNMT inhibitor | *GATA4, EOMES,GZMB* | Leukemia, esophageal | [137, 165, 166] |
| Green tea | Catechin | HAT and DNMT Inhibitor | H3/H4 acetylation | Lymphocytes, breast | [140, 167] |
| | Epicatechin | HAT and DNMT inhibitor | | Lymphocytes, esophageal | [140, 167, 168] |
| | Epicatechin gallate | HAT and DNMT inhibitor | | Colon, prostate, esophageal | [167–169] |
| | Epigalocatechin-3-gallate | HAT inhibitor | H3K27 trimethylation | Colon, prostate | [137, 167–173] |
| | | HMT inhibitor | *EZH2,SUZ12* | Prostate cancer | |
| | | DNMT inhibitor | *RARβ, MGMT,MLH1* | | |
| | | | *CDKN2A, RECK,TERT* | | |
| | | | *RXRα,CDX2,GSTP1, W1F1* | | |
| Green and black tea | Polyphenon B | Increase HDAC1 expression | | Liver cancer | [174] |
| Broccoli | Isothiocyanates | HDAC inhibitor | H3/H4 acetylation | Prostate, leukemia, erythroleukemia | [142, 175–179] |
| | Sulforaphane | HDAC inhibitor | *p21,GSTP1* | Colon, Esophageal | [175, 180] |
| | | Decreases DNMT expression | H3/H4 acetylation | Prostate, breast, colon, kidney, colon, esophageal | [129, 136, 169, 181–183] |
| | | | *RARβ, HBD-2, p21, Bax* | | |

| | | | | | |
|---|---|---|---|---|---|
| Parsley and celery | Luteolin | SIRTI activator and DNMT inhibitor | TNFα, IL-8,RBP | Cervix, esophageal | [137, 138] |
| | Apigenin | DNMT inhibitor | | Esophageal | [137] |
| Grapes, wine and eucalyptus | Resveratrol | SIRTI activator and DNMT inhibitor | | Colon, lungs, breast | [182, 184–186] |
| Indian trumpet | Baicalein | DNMT inhibitor | | Breast cancer | [182] |
| Beat root | Beatnik | DNMT inhibitor | | Breast cancer | [182] |
| Tomatoes | Lycopene | Unknown | | Breast | [187] |
| Citrus | Hesperidin | DNMT inhibitor | | Esophageal | [137] |
| | Quercetin | DNMT inhibitor | | Breast; colon; esophageal | [137, 140, 182, 186, 188] |

**Table 4.3** Dietary agents and changes in mRNA

| Source | Dietary agent | Cancer type | miRNA | Reference |
|---|---|---|---|---|
| Green Tea | EGCG | Neuroblastoma | miR-92, miR-93,miR 106b, miR-34a, miR-99a | [189] |
| | | Hepatocellular carcinoma | miR-467b, miR-487b, miR-197, miR-805, miR-374, let-7f, miR-350, miR-24-1, miR-137, miR-335-3p, let-7a, miR-222, miR-26b, miR-30c-1, let-7d, miR-98, miR-30c, miR-30b, miR-32, miR-674, miR-532-5p, let-7 g,miR-18a, miR-192, miR-302d, miR-30b, miR-802, let-7e, miR-322, miR-720, miR-146b, miR-340-3p, miR-185, miR-425, miR-10a, miR-126-5p, miR-101a, miR-30e, let-7c, miR-141, miR-33, miR-29a,miR-199b,miR-450a-5p,miR-21,miR-23a,miR-101b, miR-148a, miR-193, miR-23b, miR-107, miR-140, miR-551b, miR-466c-5p, miR-106a, miR-590-3p, miR-875-3p, miR-224, miR-292-5p, miR-678, miR-469, let-7b, miR-463, miR-574-3p, miR-201, miR-290-3p, miR-181a, miR-302a, miR-429, miR-133a, miR-190b, miR-710, miR-135b, miR-296-5p, miR-191, miR-188-5p, miR-298, miR-181a-1,miR-466 g, miR-26b,miR-466f-3p,miR-29b, miR-1224,miR-291b-5p, miR-324-5p, miR-486, miR-128, miR-450b-3p, miR-135a, miR-294, miR-671-5p, miR-878-3p, miR-801, miR-370, miR-1, miR-494, miR-133b | [190] |
| | | Lung carcinoma | let-7c, miR-210 | [190] |
| | | Pancreatic cancer | let-7c | [190] |
| | | Colon cancer | miR-27a, miR-20A, miR-17-5P, miR-21 | [191] |
| | | Bladder cancer | miR-203 | [192] |
| Turmeric | Curcumin | Lung cancer | miR-320, miR-26a, let-7i, miR-130a, mir-16, miR-125b,miR-23a, miR-27b, miR-155, miR-625, miR-576-3p, miR-186, miR-9 | [193] |
| | | Leukemia cells | miR-15a, miR-16-1 | [194] |
| | | Pancreatic cancer cells | miR-103, miR-140, miR-146b, miR-148, miR-15b, miR-181a, miR-181b, miR-181d, miR-195, miR-196a, miR-199a, miR-19a, miR-204, miR-20a, miR-21, miR-22 miR-23a,b, miR-24, miR-25, miR-26a, miR-27a, miR-34a, miR-374, miR-510, miR-7, miR-92, miR-93, miR-98 | [195] |
| | | Prostate cancer cells | miR-151a, miR-151-5p, miR-221, miR222 | [196] |

| | | | | |
|---|---|---|---|---|
| Soy | Genistein | Ovarian cancer cells | miR-100, miR-122a, miR-125b, miR-126, miR-135, miR-135b, miR-136, miR-137, miR-141, miR-152, miR-190, miR-196a, miR-196b, miR-204, miR-205, miR-206, miR-217, miR-22, miR-296, miR-30a-3p, miR-30a-5p, miR-331, miR-335, miR-342, miR-362, miR-449b, miR-454, miR-497, miR-500, miR-501, miR-503, miR-515, miR-517c, miR-532, miR-565, miR-578, miR-584, miR-585, miR-590, miR-595, miR-625, miR-647, miR-7, miR-765, miR-766 | [197, 198] |
| | | Pancreatic cancer cells | miR-200 | [130, 135] |
| | | Prostate cancer cells from lymph node | miR-106b, miR-1224-5p, miR-1228, miR-1231, miR-1246, miR-1260, miR-1267, miR-1268, miR-129, miR-1290, miR-1308, miR-1469, miR-149, miR-150, miR-152, miR-15a, miR-17, miR-1825, miR-185, miR-18b, miR-1908, miR-1915, miR-197, miR-1972, miR-1973, miR-1974, miR-1975, miR-1977, miR-1979, miR-20a, miR-20b, miR-24, miR-296-5p, miR-483-5p, miR-513a-5p, miR-548q, miR-572, miR-575, miR-612, miR-638, miR-654-5p, miR-659, miR-671-5p, miR-7, miR-762, miR-764, miR-874, miR-92b, miR-939 | [199] |
| Grapes, wine and eucalyptus | Resveratrol | Prostate cancer cells | miR-101, miR-106a, miR-106b, miR-1274b, miR-136, miR-141, miR-145, miR-17, miR-182, miR-1826, miR-200b, miR-200c, miR-20a, miR-20b, miR-21, miR-214, miR-221, miR-222, miR-302c, miR-375, miR-378, miR-720, miR-768-3, miR-93 | [200] |
| | | Colorectal cancer cells | miR-1, miR-100-1/2, miR-102, miR-103-1, miR-103-2, miR-146a, miR-146b-5p, miR-16-0, miR-17, miR-181a2, miR-194-2, miR-196a1, miR-205, miR-206, miR-21, miR-23a,miR-23b, miR-25, miR-26a, miR-29c, miR-30a-3p, miR-30c-1,miR-30d, miR-30e-5p, miR-323, miR-340, miR-363-5p, miR-424, miR-494, miR-497, miR-560, miR-560, miR-565, miR-565, miR-572, miR-574, miR-594, miR-615, miR-622, miR-629, miR-631, miR-638, miR-639, miR-657, miR-659, miR-663, miR-801, miR-92a-2 | [201] |
| Parsley and celery | Apigenin | Lung cancer cells | miR138, miR-125a-5p | [202] |
| | | Neuroblastoma cells | miR-138 | [203] |
| | Luteolin | Prostate cancer cells | miR-630 | [203] |
| | | Gastric cancer cells | miR-34a | [203] |
| Berries | Ellagitannins | Hepatocellular carcinoma | miR-let-7e, miR-370, let-7a, let-7c, let-7d, let-7a, let-7c, let-7d | [204] |

Several studies have demonstrated that green tea polyphenol (GTP) constituent, EGCG is a potent demethylating agent which inhibits enzymes involved in DNA methylation as well as an effective histone modifying agent [33, 173, 205, 206]. It is well known that CpG island hypermethylation at the promoter region leads to epigenetic repression of several critical tumor suppressor genes during tumorigenesis. A study suggests that EGCG acts as a competitive inhibitor of DNMT ($Ki = 6.89$ μM), which binds to the catalytic pocket and inhibit DNMT activity in a dose-dependent manner [168]. Furthermore, EGCG treatment (5–50 μM for 12–144 h) was found to effectively reactivate methylation-silenced genes—$p16^{INK4a}$, retinoic acid receptor beta *RARbeta*, *O*(6)-methylguanine methyltransferase *MGMT*, and human mutL homolog 1, *hMLH1* in human esophageal cancer KYSE 510 cells. EGCG was also reported to inhibit HDACs and increase permissive or active histone modifications such as histone acetylation at the target gene promoters. Studies from our laboratory showed that exposure of prostate cancer cells to GTP caused re-expression of epigenetically silenced glutathione S-transferase pi, *GSTP1* gene which correlated with the promoter demethylation due to DNMT1 inhibition and histone modifications at the promoter region [173]. However, GTP treatment did not show any global hypomethylation effect which could result in genomic instability as the methylation status of LINE-1 promoter remained unaffected as demonstrated by methylation-specific PCR. GTP treatment decreased mRNA and protein levels of MBD1, MBD4, MeCP2, and HDAC 1-3, whereas acetylated histone H3 (LysH9/18) and H4 were found to be elevated. In another study, we demonstrated that GTP treatment caused cell cycle arrest and apoptosis by inducing proteasomal degradation of class I HDACs in human prostate cancer cells [207]. Studies by Li et al. [206] demonstrated that EGCG in combination with trichostatin A (TSA) could synergistically reactivate ERα expression in ERα negative MDA-MB-231 breast cancer cells by modulating histone methylation and acetylation patter at the gene promoter. In addition, they also reported that treatment with EGCG and/or TSA contributes to transcriptional activation of estrogen receptor (ER)-α by causing a decreased binding of transcription repressor complex, Rb/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 to the regulatory region of the gene.

EGCG has been reported to modulate polycomb proteins such as Bmi-1 and EZH2 [33, 172, 208]. EGCG alone or in combination with DZNep was shown to decrease PcG proteins including EZH2, EED, SUZ12, MEL18, and BMI-1 via a mechanism involving proteasome-associated degradation. The reduction in PcG protein levels correlated with a decrease in repressive chromatin marks—H3K27me3 and H2AK119ub and HDAC-1 levels, whereas accumulation of acetylated H3 levels was found to be elevated. In a recent study, we reported that in breast cancer cells, EGCG or GTP treatment induced expression of epigenetically repressed *TIMP-3* gene is mediated by modulating epigenetic mechanisms involving EZH2 and class I HDACs independent of the promoter DNA methylation [33]. After EGCG or GTP treatment, the protein levels of class I HDACs and EZH2 were significantly reduced. Interestingly, transcriptional activation of TIMP-3 was associated with decreased EZH2 localization and H3K27me3 at the promoter with a concomitant elevation in H3K9/18 acetylation levels.

Numerous epidemiological and experimental studies have demonstrated the chemopreventive effects of genistein and other isoflavones on various cancer types [209]. The role of genistein and other soy isoflavones as epigenetic modulators regulating gene expression has been widely reported by several studies. Genistein has been shown to be more potent DNMT inhibitor as compared to biochanin A or daidzein. A study reported that genistein (2–20 μM/L) could reactivate methylation-silenced genes such as *RARbeta*, *p16INK4a*, and *MGMT* in esophageal squamous carcinoma cells KYSE 510 and prostate cancer LNCaP and PC3 cells [129, 136]. Another study demonstrated that genistein treatment in breast MCF10AT benign cells and MCF-7 cancer cells depletes telomerase (hTERT) activity through epigenetic modulation which involves genistein mediated decrease in Dnmt1, Dnmt3a, and Dnmt3b levels [130, 135]. Furthermore, genistein was shown to repress hTERT promoter by chromatin remodeling which involved increase in trimethyl-H3K9 enrichment with a concomitant decrease in dimetyl-H3K4 chromatin marks. A study by King-Batoon et al. [187] showed that a low, nontoxic dose of genistein (3.125 μM, re-supplemented every 48 h for 1 week) could partially demethylate *GSTP1*, a tumor suppressor gene, in MCF-7, MDA-MB-468, and MCF10A breast cells. Similar in vitro studies in other cancer types provide evidence that genistein is a potent demethylating as well as histone modifying agent, which could reverse the silenced state of critical tumor suppressor genes [131, 132, 210, 211]. A study by Basak et al. [212] demonstrated that AR downregulation in prostate cancer cell line LNCaP by genistein was attributed to the inhibition of HDAC6-Hsp90 co-chaperone function, which is required for AR protein stabilization. Genistein and other soy isoflavones are known to modulate miRNAs as well [135, 197, 198, 213, 214]. Parker et al. [197, 198] performed miRNA profiling of genistein treated and untreated UL-3A and UL-3B cell lines and found 53 miRNAs which were differentially expressed. Upregulation of miR-200 and let-7 by isoflavones was shown to downregulate ZEB1, slug, and vimentin and therefore cause reversal of epithelial to mesenchymal transition (EMT) in gemcitabine resistant pancreatic cancer cells [135]. In human uveal melanoma cells, genistein treatment was demonstrated to cause significant growth inhibition by targeting miR-27a and its target ZBTB10 [214]. However, in vivo clinical studies were inconclusive and did not fall in line with the studies performed in cell line models.

Curcumin has been shown to modulate multiple intracellular pathways associated with proliferation, survival, invasion, apoptosis, and inflammation [215]. In the context of epigenetic pathways, several studies have reported curcumin to be a potent modulator of DNMTs, histone modifying enzymes such as HDACs and HATs as well as miRNAs [216]. In silico molecular docking studies of curcumin with Dnmt1 revealed that it can block or inhibit the catalytic thiol group of C1226 binding site in the enzyme resulting in decreased DNMT activity [216, 217]. This study was further validated by in vitro experimental studies which showed curcumin to be a potent DNA hypomethylating agent [165]. Curcumin was reported to be an effective HDAC inhibitor. Docking studies performed for curcumin binding to HDAC-8 revealed curcumin to be a more potent HDAC inhibitor than known pharmacological inhibitors such as sodium butyrate and valproic acid [164]. Another

study reported that curcumin treatment of B-NHL cell line, Raji cells could reduce HDAC-1,-3. and -8 protein levels in a dose-dependent manner and increase H4 acetylation levels [162]. In agreement with earlier findings, studies by Chen et al. [163] reported significant reduction in p300/CREB binding protein (CBP), HDAC-1, and HDAC-3 levels after exposure of Raji cells to curcumin. Studies revealed curcumin to be a specific inhibitor of p300/CBP HAT, which has emerged a novel target for cancer treatment [149, 154, 218]. Curcumin treatment caused proteasomal degradation of p300 and other closely related CBP proteins with no such effect on HATs such as GCN5 and PCAF [154]. Curcumin has also been closely linked to its ability to modulate miRNAs in cancer cells. A microarray based study of the effect of curcumin (10 μM) on the miRNA profile in pancreatic cancer cells PxBC-3 showed significant changes in the expression of 29 miRNAs (11 upregulated and 18 downregulated) after 72 h treatment [195]. Further studies confirmed that MiRNA-22, which has tumor suppressive function, was upregulated after exposure to curcumin and its downstream target genes SP1 and ESR1 were suppressed in these pancreatic cells. Ali et al. [219] demonstrated that treatment of pancreatic cancer cells with curcumin and its analog CDF could induce gemcitabine sensitivity via induction of miR-200 and inactivation of miR-21 expression.

Epigenetic studies on resveratrol have been previously focused on SIRT1 and acetyl transferase p300 [138, 139, 177, 220]. Resveratrol was identified as a potent dietary activator of SIRT1, which lowers the $K_m$ (Michaelis constant) for both acetylated substrate and $NAD^+$. It was reported to stimulate SIRT1-dependent p53 deacetylation which ultimately contributes to increased cell survival [138]. In another study by Wood et al. [139], resveratrol was shown to activate sirtuins from metazoans—*Caenorhabditis elegans* and *Drosophila melanogaster* and delay aging without any effect on fecundity. The antitumor effect of resveratrol was reported to be mediated partly by SIRT1 [221]. In addition, resveratrol was shown to have a negative effect on *Survivin* gene expression through histone deacetylation at the gene promoter and display a more profound inhibitory effect on BRCA-1 mutant cells both in vitro and in vivo [178]. In prostate cancer cells, resveratrol was reported to cause downregulation of MTA1 (metastasis associated protein) and destabilize the NuRD (Nucleosome remodeling deacetylase) complex thus allowing p53 acetylation. Furthermore, activation of p53 was shown to induce proapoptotic pathways causing apoptosis in prostate cancer cells [222].

Sulforaphane (SFN) at physiological concentrations has been shown to downregulate Dnmt1 gene expression in human colon Caco-2 cells [223]. Studies by Meeran et al. [224] demonstrated that in MCF-7 and MDA-MB-231 breast cancer cells, SFN treatment cause dose and time-dependent inhibition of hTERT (Human telomerase reverse transcriptase) via an epigenetic mechanism involving DNA methylation and histone modifications. SFN treatment was shown to cause downregulation of Dnmt1 and Dnmt3a, which induced site-specific demethylation at hTERT gene first exon facilitating the binding of CTCF associated with hTERT repression. Furthermore, ChIP analysis of hTERT promoter revealed that active histone chromatin marks such as acetyl-H3, acetyl-H3K9, and acetyl-H4 were increased, whereas repressive chromatin marks which include trimethyl-H3K9 and trimethyl-H3K27 were reduced after SFN treatment in a dose-dependent manner.

The SFN-induced hyperacetylation was reported to promote the binding of repressor proteins such as MAD1 and CTCF to the hTERT regulatory region. In another study, Myzak et al. [183] reported that SFN metabolites—SFN–cysteine and SFN-N-acetylcysteine—were more potent HDAC inhibitors in vitro as compared to SFN or its glutathione conjugate. Furthermore, SFN treatment in HCT116 human colorectal cancer cells increased β-catenin-responsive reporter (TOPflash) activity in a dose-dependent manner and inhibited HDAC activity. Consequently, there was an induction in acetylated histone levels bound to p21 (Cip1/Waf1) promoter. In human prostate epithelial cells BPH-1, LNCaP, and PC3, SFN treatment was shown to inhibit HDAC activity, which was accompanied by an increase in acetylated histone levels by 50–100 % and a corresponding induction of p21 and Bax expression which lead to downstream events such as cell cycle arrest and apoptosis [225]. SFN treatment was shown to inhibit HDAC activity in breast cancer cells, but no change in H3 or H4 acetylation was observed [226]. Studies by Myzak et al. [225] provided first evidence for inhibition of in vivo HDAC activity and suppression of tumorigenesis in APC-min mice.

The effect of apigenin on epigenetic related enzymes and their mechanisms was not recognized until recently. Apigenin treatment has been shown to cause a marked decrease in DNMT activity in vitro [137]. Studies from our laboratory demonstrated that apigenin mediated growth arrest and apoptosis in prostate cancer cells was due to the inhibition of class I HDACs [227]. In vivo studies using PC-3 xenografts in athymic nude mice further confirmed that oral intake of apigenin (20 and 50 μg/mouse/d over an 8-week period) reduces tumor burden, HDAC activity, and HDAC -1/-3 protein levels. HDAC-1 and HDAC-3 mRNA and protein levels were found to be significantly decreased in apigenin treated (20–40 μM) PC-3 and 22Rv1 prostate cancer cell lines, which resulted in a global decline in histone H3 and H4 acetylation levels. A corresponding elevation in p21/waf1 and bax levels was observed in both in vitro and in vivo studies, which resulted in the induction of downstream events, that is, apoptosis and cell cycle arrest. In a recent study by Paredes-Gonzalez et al. [228], apigenin was shown to reactive Nrf2 gene which encodes a key transcription factor known for regulating antioxidative defense system and skin homeostasis, in mouse skin epidermal JB6 P+ cells via epigenetic mechanisms. Hypermethylation of 15 CpG sites in Nrf2 promoter was demonstrated to be reversed by apigenin treatment in a dose-dependent manner. Furthermore, apigenin treatment resulted in decreased expression of Dnmt1, Dnmt3A, Dnmt3B, and HDAC (1–8) levels. However, the nuclear localization of Nrf2 was shown to be enhanced and there was increased expression of Nrf2 as well as its target gene NQO1 after apigenin treatment.

## *Social-Economic and Racial Factors Affecting the Epigenome*

Few studies have reported significant epigenetic differences in socio-economic/racial status that account for the differences in cancer and their outcomes [229]. Certain populations are prone to specific types of cancer such as African Americans

(AA) who have 14 % higher incidence and 34 % higher death rates than Caucasian Americans (CA) men. Although access to quality healthcare, socioeconomic status, and genetic make-up is implicated in this disparity, the fundamental causes of such cancer disparity seem to be a complex phenomenon. Many investigators are trying to address various sociocultural determinants as a major cause of cancer disparity and in understanding and underpinning mechanisms for designing better community specific interventions for different populations. For example, AA have been found to have statistically significant lower plasma concentrations of certain antioxidants such as vitamin E, alpha-carotene, beta-carotene, lutein, and zeaxanthin than CA [230]. This report indicates that low levels of antioxidants may affect the epigenome and gene expression leading to higher susceptibility and differential cancer outcomes. More research is needed to fully understand how these epigenetic modifications occur and subsequently affecting cancer outcome in diverse population.

## Summary and Conclusions

From the studies described herein, it is clear that nutritional and lifestyle factors hold great promise in cancer prevention and in therapy by causing epigenetic modifications. As the importance of epigenetic modifications in cancer is well recognized, precise contribution of epigenetic mechanisms and cellular targets of epigenetic alterations by various endogenous factors in human cancer needs further investigation. Although recent advances in the field of cancer epigenetics has enhanced our understanding of epigenetic changes in normal cellular processes and abnormal events leading to tumorigenesis, however deeper understanding of the global patterns of epigenetic modifications by dietary compounds and lifestyle factors in cancer will lead to the design of better strategies to prevent and cure cancer. Moreover, sufficient preclinical and clinical data is required on the epigenetic changes induced by dietary phytochemicals which will lead to better understanding of the epigenetic targets and pathways altered by these agents to elicit their efficacy in cancer. Additional preclinical and clinical studies are required to analyze the safety profile of doses, route of administration, organ bioavailability alone, and in combination in order to obtain maximum beneficial effects. At last, systematic well-designed randomized placebo-controlled trials with adequate power and relevant clinical epigenetic endpoints are needed. Despite these challenges, research on diet and nutrition continues to emerge and will offer new epigenetic targets and promising agents with more opportunities for prevention, and perhaps therapy of cancer in the near future.

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# Chapter 5
# Environmentally Induced Alterations in the Epigenome Affecting Obesity and Cancer in Minority Populations

**David A. Skaar, Randy L. Jirtle, and Cathrine Hoyo**

**Abstract** The obesity epidemic of the last 30–40 years is may be linked to increased environmental chemical exposures with endocrine disrupting potential. The increases in obesity prevalence and severity coincide with increases in several adenocarcinomas at a time when cancer incidence has been generally declining, with disproportionate effects in different ethnic groups. Despite demonstrated associations between such exposures with obesity, and obesity with these cancers, an association between exposure to these environmental chemicals and adenocarcinomas has been difficult to demonstrate in part due to limits in exposure assessment. Exposure to these compounds elicits stable epigenetic responses; thus, if these alterations to the epigenome can be fully characterized, they can be exploited to improve exposure ascertainment. We summarize in this chapter evidence for the influence of environmental exposures on obesity and how epigenetic alterations may contribute to cancers that disproportionately affect minority populations exhibit disparities in incidence and mortality.

**Keywords** Obesity • Obesogen • Prostate cancer • Adenocarcinoma • Disparity • Epigenetic

## Introduction

Due to human industrial activity of the last two centuries, organic compounds such as organophosphates and solvents, as well as heavy metals (e.g., cadmium, lead, chromium, mercury, and inorganic arsenic), are common environmental contaminants,

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particularly in inner city neighborhoods. This urban environmental contamination significantly impacts the health of the poor and underserved residents. Unlike organic compounds that are chemically, biologically, or photo-degradable, heavy metals persist in the environment indefinitely. While dietary intake is a common source of exposure [1], published data [2–5] indicate that these metals also co-contaminate soils in geographic clusters of many urban landscapes. Thus, they are likely tracked indoors to become part of house dust that is subsequently ingested or inhaled [5,6]. With poor mechanisms for their elimination once in the body, heavy metals accumulate in soft tissue and bone and have multiple target organ systems. "Interaction Profiles" from the US Agency for Toxic Substances and Disease Registry suggest that these metals interact synergistically, such that the effects of heavy metal mixtures are greater than would be expected from the known toxicities of the individual metals [7].

Peak elevated blood levels in children occur between 6 and 30 months, coinciding with the developmental window characterized by crawling and oral exploratory behaviors [8]; this age range is also characterized by rapid changes in growth as metabolic set points are being established [9,10]. Fetal exposure via trans-placental transfer from concurrent maternal exposure or heavy metals mobilized from longer-term physiological stores (e.g., soft tissue and bone) has been well-documented [11]. Exposure to these heavy metals leads to well-documented neurodevelopmental disorders including ADHD, which contribute to the achievement gap that disproportionately affects minorities during school years [12] and affects earnings in adulthood. Emerging data also suggest that chronic low-dose exposure to heavy metal *mixtures* increases the risk of cardiometabolic diseases including obesity in adults. These conditions also disproportionately affect minority populations. Although we lack data-linking exposure to these environmental chemicals to cancer directly, what we now know is that rapid acceleration in growth in early postnatal life increases risk of obesity and some cancers in adulthood [13], and obesity is a consistent risk factor for several cancers.

The known effects of Pb and Cd include protein disruption/misfolding, replacement of essential protein cofactors, generation of oxidative stress, and endocrine disruption; however, the mechanisms linking these environmental compounds to cancer, which could be useful in improving exposure ascertainment, are still largely unknown [14–16]. It is becoming clear that the mechanistic framework through which the environment interacts with the genome to alter disease risk has significant epigenetic components. Thus, alterations in DNA methylation, histone marks, and chromatin structure have been proposed to be useful in providing mechanistic insights and past exposure biomarkers to improve risk assessment [17–23]. Cytosine methylation in DNA is the most intensively studied epigenetic mark in human studies, due to the stability of DNA [20] and this covalent modification [18, 20]; however, beyond a handful of epigenomic regions selected for evaluation because of known gene function [8, 16, 24], the epigenomic landscape targeted by these metals is still largely unknown.

Herein, we discuss these environmentally induced epigenetic shifts and how they may play a key role in the development of obesity-related cancers that exhibit health disparities.

## Environmentally Induced Epigenetic Alterations and Obesity

The World Health Organization estimates that approximately 600 million individuals are obese worldwide, and in the U.S., approximately one third of the population is obese. Obesity is a complex condition with an etiology that has only recently begun to be understood. Given the monetary and health costs attributable to obesity, understanding the causes and effects is of critical importance. Obesity is associated with increased risk for a number of cancers and a growing number of cases of metabolic syndrome in early life. Despite progress in genetic research, much of the heritability of obesity is unexplained. The increase in the prevalence of obesity and other complex diseases over the last 30–40 years indicates that the causes for these increases cannot be attributed entirely to genetics; changes in environmental exposures should also be considered as causally related. However, the timing for exposures should not be limited solely to the affected individual, as there is evidence that exposures can have heritable epigenetic alterations that manifest phenotypes in future generations [25–27].

### *Environmental Exposures and Obesity*

That exposure to environmental compounds can induce obesity was first postulated in 2002 [28], based on a correlation between the beginning of the obesity epidemic and the spread of industrial chemicals over the preceding 40 years. Studies from the 1970s had identified a link between low-dose chemical exposures and weight gain in animal models, but there was little follow-up at that time. A number of endocrine-disrupting chemicals, including organophosphates, bisphenol A, heavy metals, and solvents, are now suggested to have the potential to disrupt the normal weight control mechanisms [27, 29–31].

The timing of exposure was a key element of the original "obesogen hypothesis" [32], which posited that exposure to xenobiotic endocrine-disrupting chemicals, particularly during embryonic development, results in reprogramming that ultimately leads to obesity. Potential mechanisms include: (1) increasing the number of adipocytes formed in utero; (2) activating adipogenic pathways during key developmental stages leading to a shift in energy balance towards fat storage; (3) altering basal metabolic rate; (4) promoting fat storage via gut microbiota; and (5) interfering with appetite and satiety feedback mechanisms.

The recognition that endocrine disruption is an important effect of obesogenic chemicals is consistent with the classification of adipose tissue as an endocrine organ [33, 34]. This designation is largely due to the identification of two key signaling molecules primarily produced in adipose cells. The first is the hormone leptin, and the second is the master regulator of fat cell development, peroxisome proliferator-activated receptor gamma (PPARγ). Adipose also has many interactions with steroid hormones, as well as the immune system, through adipokine signaling.

It is through PPARγ signaling that the only currently delineated molecular mechanism for obesogens has been defined for organotin compounds. Organotins, such as tributyltin (TBT), were used as aquatic biocides to coat ship hulls, pipelines, and other underwater equipment starting in the 1960s. Within 10 years, it was found that TBT caused imposex in invertebrates, but a vertebrate effect was not known until 2003, when TBT was shown to masculinize female fish [35]. The pathways affected by TBT turned out to not be through steroid receptors, but rather by activating PPARγ and the retinoid X receptor (RXR) in human, mouse, and frog [36]. The heterodimer of PPARγ and RXR promotes adipose differentiation and storage [37], with TBT exposure increasing production of fat cells at the expense of other tissues during development in frogs and mice [36, 38]. Pubertal exposure to TBT in mice resulted in weight gain, insulin resistance, increased leptin, and fatty liver in males [39], similar to effects seen in humans with metabolic syndrome [40].

Experimental evidence indicates that other chemicals also function as potential obesogens. Some results are correlative, as for cross-sectional studies that found associations between chlorinated organic pollutants and increased body mass and/or type II diabetes, between barium or thallium exposure and increased waist circumference and BMI [41], and between mercury exposure and either increased BMI [30] or metabolic syndrome incidence [42]. Other chemicals have mechanistic evidence in vitro to support their function as an obesogen, such as induction of adipogenesis or activation of PPARγ in cell culture [21, 43]. Maternal cigarette smoking also has obesogenic effects with the children exposed in utero having lower birth weights, but an increased risk of being in the highest decile of BMI after adolescence [44]. Other chemicals with obesogenic effects in animal models include endocrine disrupters such as diethylstilbestrol, bisphenol A, DDT, perflurooctanoates, and phthalates [27, 29, 31].

The difficulty in managing obesity occurring during embryogenesis, early childhood, and adolescence is the increase in the number of fat cells in the individual [45]. One hypothesis is that obesogen exposure at key early developmental points increases fat cell number permanently with a corresponding altered basal metabolism due to the endocrinic activity of the additional adipose [46]. The increased number of fat cells, set during development, cannot be reduced by diet, exercise, or even surgery [45]. Thus, while diet and exercise can shrink fat cells, there are no indications that shrunken cells undergo apoptosis, and lost weight is regained by >80 % of individuals within a few years [47]. The fact that smaller fat cells secrete the least amount of the satiety hormone leptin [48] indicates that the increased number of fat cells, and a correspondingly altered metabolism program, produces a body profile that is difficult to change.

An individual's risk of obesity may not depend solely on their own exposure during key developmental stages, but rather, exposure of their parents, grandparents, or possibly further generations. Trans-generational effects of obesogen exposure have implications for rates of obesity in the population and long-term health outcomes. Animal studies have shown that prenatal exposure to TBT has obesogenic effects persisting into at least the F3 generation [49]. Studies in rats demonstrate that the plastic components BPA, diethylhexyl- and dibutyl- phthalates, jet fuel JP-8, and

DDT each has heritable effects on obesity, although at high doses [27, 29, 31]. Seminal work of multiple generations of humans in Sweden shows that food availability during prepuberty (i.e., 8–12 years) affects longevity and mortality in the individual's grandchildren, demonstrating transgenerational effects, although the mechanisms for these effects are still unclear [26].

## *Environmental Exposures and Epigenetic Shifts*

Transgenerational changes in disease susceptibility are potentially epigenetically inherited. That is, transmitted by mitotically heritable changes that are not based in alterations to DNA sequence. The commonly studied epigenetic marks in humans are cytosine methylation in DNA at CpG dinucleotides, and covalent modifications to histone tails, of which methylation is probably the most epigenetically significant [50]. These epigenetic marks regulate gene expression and are the epigenetic 'signature' of a cell that establishes its identity. Gene promoters in undifferentiated cells tend to have lower amounts of DNA methylation and histone marks indicative of active or poised promoters [51, 52]. During embryonic development as cells differentiate and cell fates are set, promoters for non-cell type genes are epigenetically inactivated by DNA methylation and histone modifications, producing "closed" chromatin [53]. Such epigenetic marks are mitotically heritable, and once established in development and differentiation, all descendant cells carry the established signature.

Mitotic inheritance of epigenetic modifications can explain alterations in cell fate to increase adipose tissue in response to obesogen exposure in the individual directly exposed. However, such somatic modifications are not meiotically heritable and cannot produce phenotypic effects in future generations. The meiotic heritability seen in the animal exposure studies [27, 29, 31], and identified in the Swedish populations [26], would require alterations to the germline of the exposed individual, and these alterations would have to be reproduced in subsequent generations. How such germline modifications are made are still not clear. Whether obesogens act directly on gametes or have effects through systemic alterations, as well as whether specific timing is necessary for gametic alterations, has public health implication for both etiologic understanding and early detection. The blood-follicular-barrier in females [54] and Sertoli cell barrier in males [55] provide protection for the gametes, but are not impermeable. Rosiglitazone, a PPARγ antagonist, is believed to cross both barriers [56] and can alter fatty acid oxidation in mouse oocytes in vitro, which can be fertilized, but develop poorly [57].

The timing for meiotic inheritance has been best demonstrated for food availability and cigarette smoking [58]. For both cases, the critical exposure time in males is in mid-childhood, with food availability during this time affecting mortality relative risk in grandsons, and smokers who began before age 11 having sons with higher obesity risk that increased with age. For females, food availability during fetal/infant development was correlated to mortality relative risk in granddaughters. For food availability, no effects were seen for different exposures during

puberty. The presence of key exposure windows during critical periods of gamete development provides evidence that it is necessary to affect gametogenic cells during differentiation, before the protective barriers have fully formed, or a combination of both. The sex-specific transmission of health outcomes—male exposure to male descendants, female exposure to female descendants—indicates an additional level of complexity that has yet to be clarified. Thus far, none of these studies include minority populations.

Genomically imprinted genes form a group of genes of particular interest for epigenetic studies of environmental exposure. Imprinted genes are expressed in a parent-of-origin specific manner. Expression of these genes is regulated by epigenetic marks originating in the gametes and conserved in all cell types throughout the life of the individual. Many imprinted genes have roles in early development as growth promoters/restrictors [59]. Additionally, imprinted genes function as oncogenes and tumor suppressors. There is also a body of evidence pointing to epigenetic regulation of imprinted genes as a significant target of environmental exposures [60, 61]. Although compelling, the hypothesis that growth and developmental effects of environmental exposures may be disproportionately mediated through altered epigenetic regulation of imprinted genes has yet to be fully tested. Existing data indicate that perturbations occurring at regulatory sequences of genomically imprinted genes as a result of environmental exposure do not vary by race/ethnicity, although the prevalence or dose of exposure itself may vary by race/ethnicity.

Evidence for obesogen action through imprinted genes comes from a number of murine and human studies. High-fat diet-induced obesity in mice is paternally transmitted and correlates with reduced expression in adipocytes of the imprinted and paternally expressed genes, *Igf2* and *Peg3* [62]. In humans, *IGF2* imprint regulatory methylation in newborns was reduced in association with paternal obesity, indicating epigenetic reprogramming in spermatogenesis and a means for heritability of health outcomes [63]. Increased maternal levels of blood Cd and Pb levels is linked to lower birth weight, and decreased offspring methylation at the imprinting control region for maternally expressed *MEG3* [64, 65]. This lower birth weight was associated with rapid growth by age 3 years [64]. These data are consistent with studies showing faster "catch up" childhood growth and increased risk of obesity in lower birth weight children [66]. Possibly, the strongest example of imprinted genes affecting obesity is Prader–Willi syndrome, in which a chromosomal deletion eliminates a region containing paternally expressed genes at chromosome location 15q11–13, resulting in multiple developmental and behavioral symptoms, including significant obesity [67].

## Prostate Cancer Disparities and Environmentally Induced Epigenetic Alterations

There is evidence for environmentally induced epigenetic alterations altering susceptibility to obesity. Furthermore, obesity is associated with several cancers that exhibit ethnic/race disparities, but evidence-linking changes in the epigenome to

cancer directly are limited. One cancer that exhibits prominent disparities in incidence and mortality is prostate cancer (PCa). Globally and in the United States, PCa is the most commonly diagnosed cancer in men. Of 14.9 million diagnosed cancer patients, 1.4 million were cancer of the prostate compared to 1.9 million cases of breast cancer, the most commonly diagnosed cancer in women [68]. PCa is the third most common cancer in the United States in 2015 (~220,800); only breast cancer (~231,800 cases) and lung and bronchus cancer (~221,200) have higher incidences [69]. While prostate cancer diagnoses increase with age—70 % of new cases are in men from age 55–74 [69]—an aging population and increased screening do not fully explain the 3 % worldwide annual increase since the 1970s [70].

The worldwide incidence of PCa is 30.6 per 100,000 [71], with variable incidence rates geographically. The incidence of PCa is 30–100 fold lower in Asian countries than in western Europe, Nordic countries, and the United States [72–74]. The highest incidences are in Australia/New Zealand and North America with age standardized incidence rates of 111.6 and 97.2 per 100,000, respectively. This compares to incidences of 10.5 and 4.5 per 100,000 in Eastern and South-Central Asia, respectively [71]. The current high rates in Oceania, North America, Northern and Western Europe, and the Caribbean are attributed, in part, to a greater number of indolent cases detected by early screening with prostate-specific antigen (PSA) and digital rectal examination [75]. The resulting spike in incidence rates from the late 1980s to the mid-1990s was subsequently followed by a 4.3 % annual decrease from 2003 to 2012 [69], likely due to identification of a large pool of previously undetected, low-grade cancers [75].

Disparities also exist within countries, particularly in the United States. There is a disparity between the incidence in African American (AA) populations and Caucasian American (CA) populations that persisted through the increases in incidence from the 1970s to 2000s (Fig. 5.1) [75]. Incidence rates are also changing in American immigrant populations. Korean immigrants have increased incidences in prostate, breast, colon, and rectal cancers compared to those of people in South Korea with immigrants trending towards a “Western cancer profile”[77]. Similarly, Iranian immigrants to British Columbia show a higher incidence of prostate cancer than Iranian counterparts [78]. These geographical variations in incidence cannot be entirely attributed to the increased PSA screening that began in the 1980s in developed countries, as information gathered prior to PSA screening shows similar geographical differences [79].

## *Prostate Cancer, Obesity, and Metabolism*

Genetic investigations into the role of obesity in prostate cancer stem from the recognition of increased weight as a risk factor for many cancer types, even though the association between obesity and prostate cancer is not consistent and dose-response relationships are not always present. While the percentage of overweight adults has changed little from the early 1960s (32 % in 1962, 33 % in 2010), adult obesity has

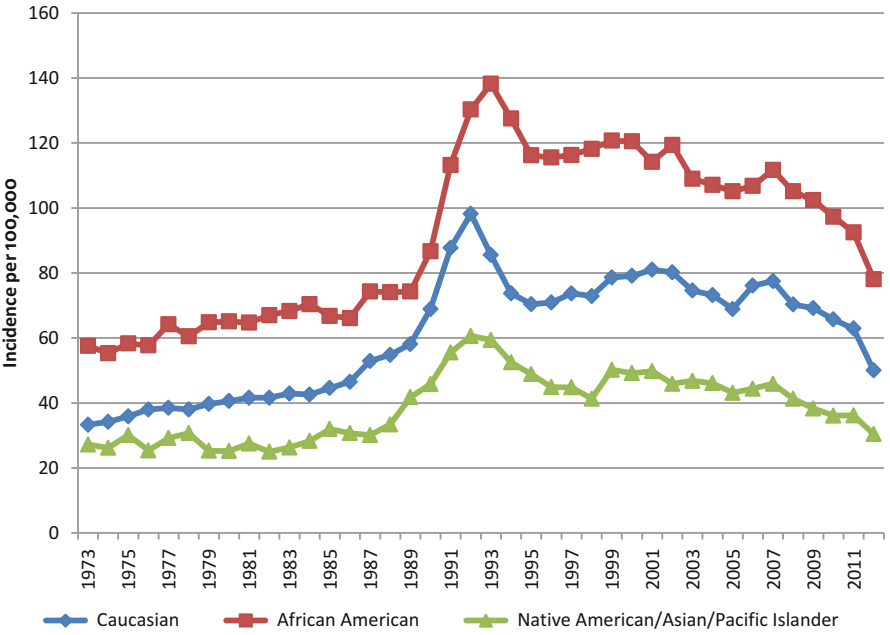


**Fig. 5.1** Incidence of prostate cancer by ethnicity, age-adjusted rates per 100,000, 1973–2012 [76]

drastically increased (13 % in 1962, 36 % in 2010) and extreme obesity has also increased (1 % in 1962, 6 % in 2010) [80–82]. Most of the obesity increase occurred from the late 1970s to 2000, with only a slight increase from 1999 to 2010. This correlates with the spike in prostate cancer from the mid-1980s to early 1990s. Adult obesity rates have racial/ethnic disparities, with 49.5 % of African Americans, 39.1 % of Hispanics, and 34.3 % of Caucasians categorized as obese, and 13.1 % of African Americans, 5 % of Hispanics, and 5.7 % of Caucasians categorized as extremely obese [83].

Obesity as a risk factor for prostate cancer has been reported in cohort studies [84–88] and case-control studies [89–91]. The magnitude of the association is generally modest (<2-fold), and inverse relationships between obesity and prostate cancer have even been reported [92, 93]. Interestingly, the association between obesity and aggressive manifestation (i.e., advanced stage at presentation, progression after primary therapy, or prostate cancer-specific mortality) is stronger [84, 94, 95]. Together, these data provide evidence that prostate cancer origin is a common event, but that progression is variable, and obesity, with a higher prevalence in minority populations, influences progression and tumor aggressiveness.

One of the most compelling hypotheses for a mechanistic link between obesity and prostate cancer is the reduction of androgen levels in obese men. Variable testosterone levels could explain the inconsistent results showing no or inverse relationships between obesity, while the association between body mass index (BMI) and aggressive tumors is decisively positive. Testosterone is required for prostate

tissue proliferation, but it also helps maintain prostatic epithelium differentiation [96]. Thus, lower androgen levels, while possibly being protective against PCa, could also promote the development of poorly differentiated and hormone-insensitive tumors. This is supported by results showing higher recurrence rates after radiotherapy or prostatectomy [97] and higher-grade Gleason scores on initial diagnosis in obese patients [98]. Besides obesity, metabolic syndrome, for which obesity is one of five criteria, is associated with higher prostate cancer grade and is a predictor for shorter survival time in cancer patients treated with the androgen synthesis inhibitor abiraterone [99].

The inflammatory effects of obesity are also hypothesized to contribute to carcinogenesis. White adipose tissue (WAT) comprises most of the body's adipose and can be considered to act as an endocrine organ, regulating homeostasis, immunity, and endocrine functions [84]. Adipocytes, which are the majority cell type in WAT, produce over 50 secreted adipokines, cytokines (e.g. TNF-alpha, TGF-beta, and IL-6), angiogenic factors (e.g. VEGF), and other proteins, such as leptin. The typical function of such inflammatory and angiogenic factors is in wound response and healing; these factors increase in tissue after injury, facilitating cell regeneration and vascularization to restore tissue function, and then decrease following healing. The chronic secretion of adipokines in obesity produces a constant state of inflammatory response, contributing to tumor growth by reducing the anti-inflammatory adipokines, such as adiponectin, or increased insulin secretion [100–102].

Cytokines IL-6 and TNF-α show elevated levels in individuals who are obese [103], insulin-resistant [104], or have one of several cancers [105]. While extensive longitudinal studies have found no association between IL-6 levels and overall prostate cancer risk [106, 107], IL-6 levels are correlated with PSA levels and risk of aggressive prostate cancer [108]. Levels of C-reactive protein (CRP), which is mostly regulated by IL-6, are also associated with obesity and BMI [109–115]. While associations between CRP and cancer mortality, severity, and metastasis have been observed [116–120], other population-based studies using prediagnosis levels do not confirm these associations [96, 107, 121].

Adiponectin and leptin also have significant functions in obesity and inflammation, and associations with prostate cancer have been reported. Adiponectin levels are inversely related to leptin production, and adiponectin inhibits IL-6, IL-10, and NFkB while being down-regulated by increased TNF-α production. Associations between reduced adiponectin levels and high-grade prostate cancer in obese patients have also been found [122, 123], but no association was observed using prediagnostic levels [124]. Leptin is best known as a regulator of body weight homeostasis since the satiety hormone controls appetite, but it is also proinflammatory, with involvement in angiogenesis [125], wound healing [126], and insulin secretion [127].

Additionally, leptin regulates prostate cancer cell growth in cell lines [128–130], and obese men have elevated leptin levels [131]. The first case-control study of leptin and prostate cancer risk found a threefold increase in cancer risk in men with elevated leptin levels [132], but these associations remain unconfirmed [133–135]. It is possible that leptin may have its strongest effect on progression, as leptin was reported to promote proliferation of androgen-resistant cancer cells, supporting a role for leptin as a risk factor for advanced stage cancer [136].

## *Genetic and Epigenetic Contributions to Prostate Cancer Disparities*

The striking differences in prostate cancer incidence across ethnicities led to the investigation of predisposing or protective genetic variants. Functions of particular interest for genetic analysis are based on the known obesity and metabolic functions that have been tied to prostate cancer. Pathways and functions examined include: (1) androgen receptor (AR) activity, which controls normal prostate growth and can promote tumor growth; (2) adipokines and inflammatory cytokines associated with adipose tissue; (3) regulators of preadipocyte recruitment and differentiation, as adipocyte tissue promotes cancer progression; (4) growth factors such as TGF-β, which activates AR and the EGFR family, and can promote malignant transformation and epithelial mesenchymal transition; (5) the insulin growth factor (IGF) axis, a regulator of growth, differentiation, metabolism, and apoptosis; and (6) cell cycle regulators, affecting checkpoint failure and allowing tumor cell progression.

Androgen receptor mutations are uncommon in early-stage prostate cancer, which is typically androgen- and AR-dependent, but mutation frequency increases in advanced cancers that have become androgen-independent [137]. This suggests that the disparities seen in progression and aggressiveness could depend on the degree of AR mutation accumulation in early-stage tumors. Studies of the >70 missense AR mutations known to occur in prostate cancer [138] have found a range of functional effects. One study of 44 mutations found 20 with gain of function [139], while a later study of 45 mutations from metastatic or high-Gleason scored tumors (16 mutations were in common with the first study) found 17 total mutations with some gain of function, 4 of which imparted constitutive activity [140].

Given the typically larger tumor volume, stage, and Gleason score of tumors at presentation in African Americans compared to Caucasian Americans, it has been postulated that differences in AR activity drive this disparity. Immunostaining and expression analysis of AR have identified more frequent positive staining for AR in prostate cancer cells from African Americans than from Caucasian Americans, and even greater AR expression in the prostate cells from benign lesions in African Americans [141]. Examination of AR mutations in prostate cancer patients identified higher rates in African Americans than in Caucasian Americans for both somatic mutations in tumors (8.5 % vs. 2 %) and germline mutations in white blood cells from patients with sporadic prostate cancer (11.3 % vs. 2.7 %).

The AR pathway is also susceptible to epigenetic dysregulation, promoting tumorigenesis and progression in the absence of gene mutations. G-protein-coupled receptor (*GPCR*) can activate AR independently of androgen and is in turn repressed by regulator of G-protein signaling (*RGS2*). The *RGS2* promoter is methylated in prostate cancer, correlating with lower expression, and in vitro expression studies demonstrate that *RGS2* methylation allows for androgen-independent cell growth [142]. Androgen-independent apoptosis is regulated by TGFβ pathway members, with most prostate tumors becoming TGFβ-resistant without any mutations of signaling molecules. This can be partly explained by findings that loss of TGFβ

receptor III, which occurs in most PCa cells, can result from epigenetic silencing [143]. The AR pathway is also affected by mediator of DNA damage checkpoint protein 1 (*MDC1*). *MDC1* expression facilitates an association between AR and histone acetyltransferase *GCN5*, which is associated with less aggressive PCa phenotypes [144]. More general epigenetic effects on PCa progression and aggressiveness are also seen with DNA hypermethylation. Reduction in *TET2* expression by androgens prevents the removal of DNA methylation [145] and histone deacetylase, reducing epithelial-mesenchymal cell plasticity and suppressing metastatic PCa [146].

Given the strong association between obesity and prostate cancer risk and the strong association of decreased adiponectin levels with obesity [147] and aggressive PCa [97], variants in adiponectin (ADIPOQ) and the adiponectin receptor (ADIPOR1) have likewise been investigated as prostate cancer risk factors. While the results have been inconsistent, ADIPOQ SNPs have been associated with obesity, serum adiponectin levels, and prostate cancer risk [148–151], as well as risk of recurrence after radical prostatectomy [152], and ADIPOR1 SNPs are also correlated to prostate cancer risk [151]. Meta-analyses investigating contradictory associations identified one particular marker, 1082G>A, as a protective allele for PCa risk [153,154], particularly in Caucasians. The fact that the 1082G>A variant is also associated with adiponectin expression [155] and that mouse models show adiponectin loss promoting insulin resistance, chronic inflammation (with elevated levels of inflammation factors), and tumor growth [156–158] suggest a mechanism for how obesity promotes tumorigenesis.

Adiponectin is also regulated epigenetically. Thus, alterations in the epigenetic regulation of this gene could affect the genesis of cancer. TGFβ, an inducer of epithelial-mesenchymal transition in cancer cells, increases repressive histone marks at the adiponectin promoter in vitro [159]. Regulation of adiponectic differentiation is also regulated by miRNAs, with miR-369-5 inhibiting and miR-371 promoting differentiation [160]. These results parallel effects that these same miRNAs have on DNA methyltransferases, as DNMT3A and DNMT3B are up-regulated by miR-371 and down-regulated by miR-369-5. This suggests epigenetic mechanisms regulate adipogenesis and that there could be widespread epigenetic effects in association with adipogenesis. Other investigations of obesity-related cytokines and inflammatory factors have identified risk alleles for leptin [161] and IL-10 [162]. The specific allele found for IL-10 controls levels of this anti-inflammatory factor [163], providing another indication of inflammation in PCa risk.

For some time, the insulin growth factor (IGF) axis has been a target for the treatment of many cancers because of its role in cellular growth and differentiation, apoptosis, glucose, and lipid metabolism. Additionally, IGF signaling can activate the androgen receptor by cross-talk [164,165], which is potentially important in late-state androgen-independent prostate cancer growth. Analysis of genetic variants of IGF axis members has identified: (1) variants in the IGF2 antisense RNA and somatotropin receptor 2 associated with increased PCa mortality [166]; (2) IGF1 variants that are associated with incidence and recurrence after radical prostatectomy [167]; and (3) an IGFBP3 genotype that appears protective against cancer development [168]. Examination of IGF1 and IGFBP3 in an African American

cohort identified alleles in both genes (CA19 and 202C, respectively) that are associated with lower serum IGFBP-3 levels. Furthermore, individuals homozygous for the IGFBP3 202C allele have a 2-fold increased risk for prostate cancer [169].

Members of the IGF family of genes and their receptors are known to be epigenetically regulated. For example, *IGF2* is imprinted and expressed only from the paternal allele [170]. *IGF2* upregulation occurs in multiple cancer types, and its overexpression is associated with chemotherapy resistance and poorer prognosis [170–176]. Dysregulation of IGF2 expression due to early developmental exposures is a potential susceptibility factor, with lifetime reduction in regulatory methylation seen in individuals with prenatal exposure to famine [177]; this dysregulation is also associated with an increased risk for obesity [178]. *IGF1R* is also epigenetically dysregulated in tumors with overexpression connected to tumorigenesis and progression [179] resulting from miR-375 downregulation [180].

Cell cycle control is also a factor in PCa, as one of the functions of the androgen receptor is control of cell cycle progression, through cyclin/CDK regulation, with cross-talk between AR and checkpoint pathways affecting androgen response [181]. Examination of check point control genes has identified eleven variants in ten genes that are associated with risk of aggressive PCa in European American and nine variants in seven genes in African American populations. Only two genes were common between the two racial groups, and the most significant associations were in *CCNC* for Caucasians and *CDK2* and *CDK5* for African Americans [182]. These results indicate there are multiple ways of altering one pathway that can increase the risk of developing PCa. The different genes identified provide a possible explanation for ethnic disparities with ethnicity specific variants, or haplotypes, having greater or lesser effects on PCa risk.

*CDK*s also have widespread epigenetic effects on tumor cells by modifying methyltransferase activities. *CDK1*, *CDK2*, and *CDK5* all phosphorylate DNMT1, potentially stimulating its activity [183]. This could then lead to the commonly seen cancer cell DNA hypermethylation in conjunction with tumor-associated cell cycle defects mediated by *CDK* activity. In vitro studies with PCa-derived cells show higher levels and activity of the maintenance methyltransferase, DNMT1, and the de novo methyltransferases, DNMT3a and DNMT3b [184], particularly from tumors with higher Gleason scores. There are also indications that DNMT1 activity is primarily associated with tumorigenesis, while DNMT3a and DNMT3b are mostly involved in progression. Effects on DNMT regulation from initial genetic or epigenetic alterations could start a chain reaction, by targeted dysregulation of DNA methylation resulting in widespread dysregulation of tumor suppressors and oncogenes.

Another targeted examination investigated known PCa-associated biomarkers for ethnic-specific associations. Six biomarkers have significant differential expression between African American and Caucasian patients, with dysregulation of *AMACR*, *ERG*, *FOXP1*, and *GSTP1*, and loss of function of tumor suppressors *NKX3-1* and *RB1* predicting risk of pathologic disease. Additionally, dysregulation of *GOLM1*, *SRD5A2*, and *MKi67* predicts clinical outcomes, including recurrence

and metastasis. Loss of expression of *ERG*, *ETS*, and *SPINK1* in triple-negative PCa is also more common in African American than in Caucasian American men (51 % vs. 35 %) [185].

Integrative genomic screens for regulatory dysfunction have examined miRNAs, identifying regulatory miRNAs with differential expression in PCa. miRNA/mRNA pairs with correlated expression have been found, along with differential expression of miRNAs between ethnic groups. One study identified 22 African American-specific and 18 Caucasian-specific miRNAs with differential expression in tumors compared to matched normal prostate, and 10 miRNAs with differential expression between tumors from African American and European American patients. EGFR signaling was identified as a critical pathway regulated by the African American-specific miRNAs. Loss and gain of function studies in tumor cell lines also identified miRNA/mRNA regulatory pairs driving oncogenesis for *MCL1*, *STAT1*, *FOXO3A*, *ITPR2*, and *PPP2R2A* [186].

Together, these data have identified striking ethnicity-specific associations between obesity and PCa risk. Overall risk assessment shows positive association between BMI and cancer risk in an African American population, but no association in a non-Hispanic Caucasian population. Risk for both low- and high-grade PCa is positively associated with BMI in the African American population. Interestingly, while BMI is positively associated with high-grade disease, it is inversely associated with low-grade disease in the European American population. This is another indicator of likely underlying genetic factors modulating response to environmental and epigenetic influences and contributing to disparities in both overall occurrence and aggressiveness.

## *Esophageal Adenocarcinoma*

Esophageal cancer (EC) is the eighteenth most common cancer in the United States, with incidence of 4.4 per 100,000 population, and it represents only 1.0 % of new cancer cases per year with a 0.5 % lifetime risk (2015 estimates). Nevertheless, it is one of the most lethal cancers, the tenth most common type of cancer death in the U.S., and had 5-year survival of 17.9 % from 2005 to 2011 [69]. Incidence rates are higher in other countries with about 81 % of cases occurring in less developed countries, making EC the eighth most common cancer worldwide. Estimated incidence and deaths from EC in the U.S. for 2015 are 16,980 and 15,590, respectively. Overall, EC is a disease of aging with an average age at diagnosis of 67, and an average age at death of 69. Individuals less than 55 years old comprise only ~14 % of new cases and ~12 % of deaths. EC incidence spikes in the 55–64 years age group, with 27 %, 28 %, and 22 % of diagnoses coming in the three age decades from 55 to 84 years, respectively.

EC is also much more common in men than women and shows significant ethnic variability. Age-adjusted EC incidence per 100,000 is 7.6 in men and 1.7 in women

with comparable death rates (7.5 and 1.5). Incidence and death rates in Caucasian and African-American populations were similar. Incidence rates per 100,000 were 8.0 and 7.6 in Caucasian and African-American men, respectively, and 1.7 and 2.5 in Caucasian and African-American women, respectively. Death rates per 100,000 were 7.7 and 7.0 in Caucasian and African-American men, respectively, and 1.5 and 2.0 in Caucasian and African-American women, respectively. Other ethnic groups in the U.S. had significantly lower incidence rates, with respective incidences per 100,000 for men and women in Asian/Pacific Islander, Native American/Alaska Native, and Hispanic groups of 3.6 and 1.0, 4.9 and 1.6, and 5.2 and 1.0, respectively.

These data make it clear that there are sex-specific risk factors for the initiation of EC, but that progression is comparable between sexes. There also appear to be ethnicity-specific risk factors for EC, with Asian and Native American populations having lower rates of incidence and death than Caucasian Americans and African Americans. Although the incidence and death rates of EC in Caucasian Americans and African Americans are similar, separation of ECs into subtypes has identified significant disparities in cancer risk and outcome in these two populations.

While overall esophageal cancer rates in the United States did not change appreciably from 1992 to 2012 [187], subdivision by ethnicity and cancer type highlights reveals several changing trends. Firstly, overall incidence and mortality for African American men has consistently and dramatically dropped, from an incidence of 17–18 per 100,000 in 1991, to parity with Caucasian American men by 2004–2005, to the slightly lower incidence and mortality rates measured for 2008–2012. Decreased incidence/mortality has not been seen for any other ethnic group. Secondly, simultaneously with this deceasing trend in African American men, there has been a less dramatic but consistent increase in incidence/mortality in Caucasian American men—from ~6 per 100,000 in 1991 to ~8 per 100,000 for 2008–2012.

The increased incidence and mortality for esophageal cancer in Caucasian American men can be better understood by separation of esophageal cancer into the primary subtypes of squamous cell carcinoma (ESCC) and adenocarcinoma (EAC). Adenocarcinoma rates began to rise in Caucasian American men around 1975, surpassing the rate of squamous cell carcinoma by 1995 [188] (1). Simultaneously, there was a slight increase in adenocarcinoma for African American men during this period while still remaining <1 per 100,000. The observed decrease in squamous cell carcinoma began in the 1990s (Fig. 5.2b), providing the primary contribution to the overall drop in African American esophageal cancer rates.

Nevertheless, the overall incidence of esophageal adenocarcinoma has increased 500 % in the United States over the last three decades, the greatest increase of any cancer. While overall esophageal cancer rates are higher in less developed regions, with Africa and Asia having the highest incidence rates (EC is the sixth most common cancer in Asia) [71], the majority of these cases are squamous cell carcinoma [189]. These striking differences in EC rates and types between developed and developing countries, African Americans and Caucasian Americans, and men and women make it important to determine the genetic, epigenetic, and environmental factors that affect the risk of developing this deadly cancer.

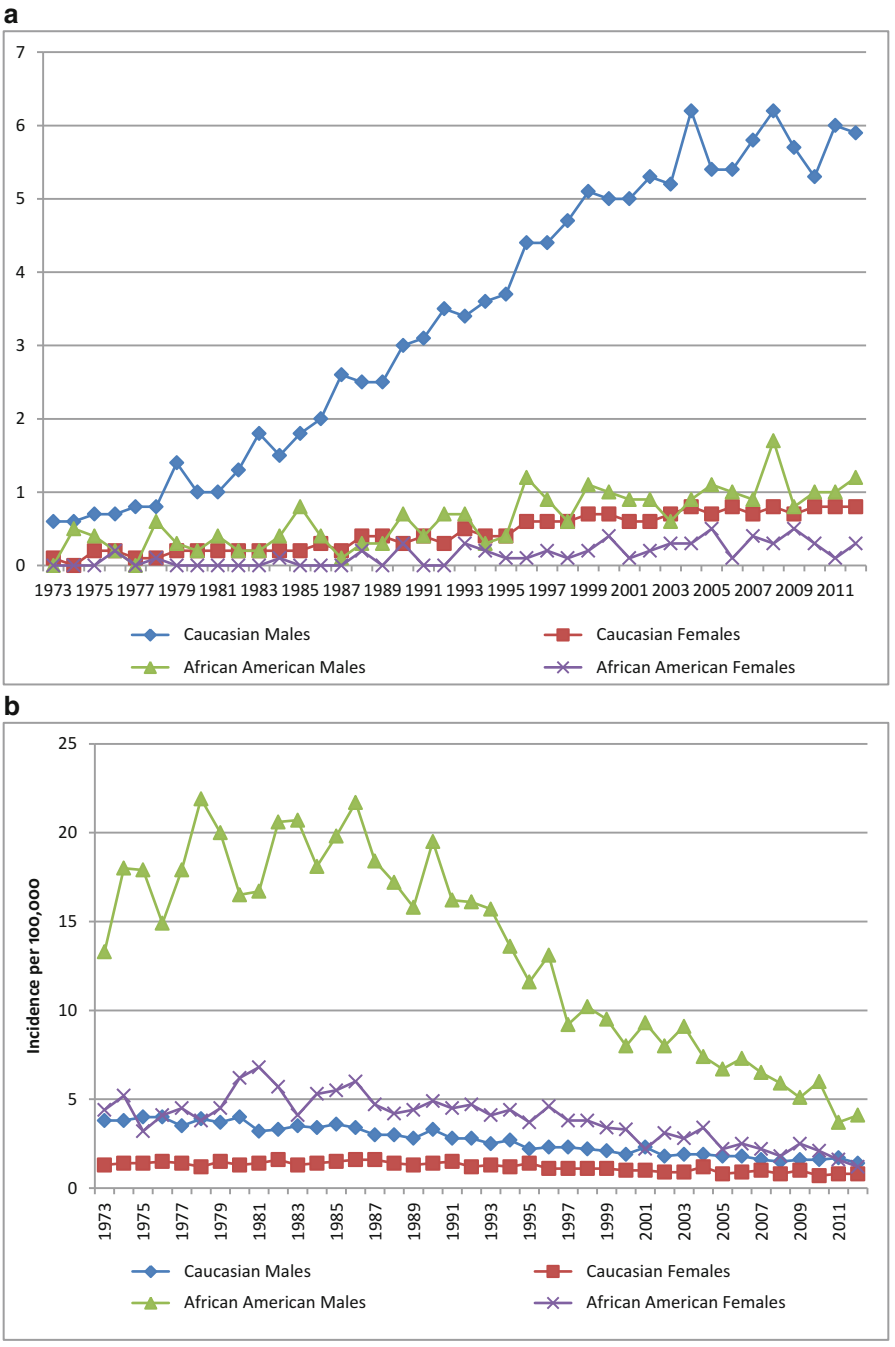


**Fig. 5.2** Incidence of esophageal cancers by ethnicity and sex, age-adjusted rates per 100,000, 1973–2012. (**a**) Adenocarcinoma incidence, (**b**) squamous cell carcinoma incidence [76]

## *Gastroesophageal Reflux Disease and Obesity*

Esophageal cancer has several known environmental and lifestyle factors. They include: smoking, betel nut and/or tobacco chewing, human papilloma virus, consumption of alcoholic drinks, and overweight/obese status. There are also results suggesting that diet can affect EC risk, particularly fruits and vegetables (protective effect) and red meat (risk factor, particularly processed meats [190]), and vitamin deficiencies. By far, the most consistently associated risk factors for EC are gastroesophageal reflux (GERD), Barrett's esophagus (BE), male sex, and obesity.

### Gastroesophageal Reflux

GERD is considered a primary risk for developing BE through erosion of the esophageal lining by a combination of gastric acids, bile salts, pancreatic enzymes, and partially digested food triggering acute and chronic inflammation [191]. In patients with reflux and esophagitis, inflammatory cytokine profiles are also found with increased levels of IL-1β and IL-10 [192]. Expression analysis has identified genes differentially expressed in BE cells, as compared to normal esophageal cells, including: (1) downregulation of transcription factors involved in esophageal squamous epithelium development (i.e., P63, *SOX2*, *PAX9*); (2) overexpression of transcription factors involved in intestinal development (i.e., CDX homebox genes, HNF genes, *GATA4*, and *GATA6*); and 3) activation of the TGFβ/BMP signaling pathway [193].

There are large race/ethnic and regional variations in GERD frequency with reflux symptoms being traditionally uncommon in Asian populations. There are both biological and environmental theories to explain the low incidence of GERD in Asians. They have an inherently smaller gastric parietal cell mass and lower acid production, and the typical Asian low fat diet also reduces reflux issues [194]. However, multiple studies have reported variations among Asian ethnic groups, with Indians having higher GERD incidence rates than Chinese or Malays (7.5 %, 0.8 %, and 3.0 %, respectively) [195]. For the ethnicities in Malaysia, Indians have a higher odds ratio than Malays, 3.25 versus 1.67, respectively [196]. For the Indians, Chinese, and Malays in Southeast Asian, Indians had the highest rates of BE metaplasia and esophagitis [197].

While GERD/esophagitis rates dramatically increased in the U.S. and Europe from 1982 to 2005, there was no significant increase in Asia during this time. It was not until the late 1990s that an increasing trend became apparent in Asia [198]. Japan has the greatest increase in GERD/esophagitis from ~3 % in the 1970s to 14–16 % by 2000. Other Asian countries have also shown increased GERD/esophagitis rates; however, as previously noted, the incidence rates are significantly different among ethnicities. Current rate estimates range from 3 % in China and 6 % in the Philippines, to 7–10 % in Korea and 12–15 % in Taiwan. While there are caveats to these estimates, since different diagnostic criteria are used, and some diagnoses are based on frequency of reflux and others on endoscopic examination, the rising trend is clear.

Whether this increase in GERD will translate into increases in BE and EAC remains to be seen. BE is still typically uncommon across most of Asia (incidence of 0.06–6 %), except in Japan and India. Incidences as high as 19.9 % have been reported in Japan [199], and rates in India range from 6 %[200] to nearly 25 % [201]. The full extent of GERD and BE incidence in Asia will be more precisely determined as longitudinal studies with larger samples sizes and consistent diagnostic criteria are used to better determine the long-term trends.

One environmental factor that may be involved in regional variation in GERD incidence is *Helicobacter pylori* infection. While treatment for *H. pylori* has reduced infection rates and associated gastric symptoms in the United States and Europe, it is still more common in other parts of the world. Although *H. pylori* causes peptic ulcers and distal gastric cancer, it is inversely correlated to GERD, BE, and EC [202]. This suggests that decreasing *H. pylori* infection rates in the western world may contribute to the increase in the incidence of GERD. The more recent increase in GERD in Japan has also been linked to a reduction of *H. pylori* through a national eradication effort [203]. In a study with African Americans, *H. pylori* infection was inversely associated to GERD incidence [204], providing a possible reason for the observed lower incidence of GERD, BE, and EAC in African Americans.

**Barrett's Esophagus**

Barrett's esophagus is characterized by the presence of intestinal metaplasia in the columnar-lined esophagus, with secretory cells, including goblet cells, replacing the stratified squamous epithelial cells that normally line the esophagus. Biopsy data indicates that BE and the presence of intestinal metaplasia is a necessary precursor to EAC [205, 206]. This is not surprising given that EAC is a cancer of glandular cells, rather than typical epithelial squamous cells, which become malignant in squamous cell carcinoma.

Despite the apparent requirement for BE as an EAC precursor, BE is not sufficient for progression to a cancerous state. In one study tracking for progression of BE, people diagnosed with gastric BE and only the presence of cardiac or fundic metaplasia showed no disease progression over 4 years of monitoring. In contrast, 13.4 % of people with BE with intestinal metaplasia demonstrated progression either to cell abnormality/dysplasia or adenocarcinoma [205]. Meta-analysis of studies that followed 11,434 BE patients found an annual incidence for EAC of 0.33 % in patients who first presented with BE without dysplasia [207]. A single study that followed 11,000 BE patients for 5.2 years, after excluding EAC diagnosis within the first year of tracking to eliminate possible misdiagnoses from the original biopsies, found an annual incidence of 0.12 %[208]. It is unlikely that ethnic disparity in EAC is due to a difference in progression from BE to EAC. BE occurrence in African Americans and Hispanics is proportional to EAC incidence in these groups, indicating that it is the development of BE that is the key step, and that progression from BE is relatively consistent [209].

## Obesity

Risk factors for BE are essentially the same factors for EAC risk—male sex, Caucasian-American, GERD, and obesity. Independent predictors of progression from BE to EAC were increased age, male gender, and being overweight (i.e., BMI 25–29.9) [210]. As obesity is a significant risk factor within the sex and ethnicity risk pools, as well as a potential contributor to GERD, it is a study target of great interest.

It has been hypothesized that obesity contributes to BE development by influencing GERD, with male-specific obesity patterns contributing to the gender disparity. Central obesity, which is more common in men [211], increases intra-abdominal pressure, relaxing the lower esophageal sphincter [212], exposing the lower esophagus to gastric acid, increasing the risk of BE metaplasia, and EAC [212–217]. Analyses comparing BMI and abdominal obesity to GERD have associated central obesity with symptomatic reflux in a dose-dependent manner that is independent of BMI [218]. However, contradicting the pressure hypothesis are results showing that while a high BMI increases gastric and gastroesophageal pressure, increased pressure does not correlate to elevated esophageal acid exposure [215]. Furthermore, enhanced risk of EA is similar in patients with and without GERD [219].

Therefore, it is unlikely that GERD is influenced by obesity purely mechanically, suggesting that other means of translating obesity/adipose deposition to GERD are functioning. The possibility that increased BE and EAC in males is due to different patterns of adipose tissue is consistent with data demonstrating that fat type and distribution are the risk factors rather than simply BMI. One meta-analysis reported that it is waist circumference rather than BMI that increases BE risk [220]. Other studies show that it is the visceral component of obesity, not the subcutaneous fat, that is the greatest risk factor for BE [221, 222]. Similarly, abdominal obesity has been reported to be a risk factor for EAC that is independent of BMI [223]. Moreover, it persists despite inclusion of cofounders, such as reflux, exercise, smoking, overall calorie intake, and consumption of red meat, fruits, and vegetables [224]. Attenuation of the significance of BMI linkage with GERD occurs when adjusting for waist circumference indicating that the effect seen for BMI is due to increased abdominal obesity that occurs with increased BMI [225]. Additionally, visceral adipose assessed by CT scan is associated with the duration of GERD symptoms [222]. Taken together, these findings indicate that centrally deposited adipose has an additive metabolic and/or signaling effect on reflux symptoms and risk for developing BE and EAC.

Multiple studies have found BMI or abdominal obesity associations with BE or EAC that are independent of acid reflux [219, 223, 226–228]. This indicates that obesity association with EAC has comparable magnitude and patterns in individuals with and without GERD. Several studies have demonstrated an association between obesity, GERD, and BE in Japanese patients. This is particularly intriguing given the previously described increase in GERD and BE diagnoses in Japan. One study identified a positive association between visceral fat and reflux esophagitis in both men and women, as well as an association between smoking and serum triglyceride levels with reflux in men only [229]; however, there was no association between amount of visceral fat and either reflux severity or the presence of BE. A second

Japanese study of patients with BE determined that being overweight or obese significantly increased BE risk, independent of GERD parameters [230]. This analysis also found that being slightly overweight (BMI: 23.0–24.9), which is not a risk factor for BE in Caucasian populations, is a risk factor in the Japanese. While these studies are inconsistent with respect to risk of increased BMI for BE incidence in Japanese populations, they are consistent with the finding that BE incidence is independent of GERD.

To take a wider view of the possible role of GERD in developing EAC, it is important to note that GERD symptoms are distributed globally, but it is Caucasian males who have the highest incidence of BE and EAC. It is also this group that has the greatest increase in visceral obesity, and it is from this group that the majority of epidemiological studies have been performed. It is postulated that the smaller amount of data from other populations has precluded the revelation of other factors affecting on BE and EAC incidence in other ethnicities or geographic regions [231].

This point is illustrated by comparing the incidences of BE and EAC for ethnicities within the United States. While obesity rates among Hispanics and African Americans have increased along with Caucasian Americans, the associated increase in EAC for these groups is not as dramatic. Likewise, GERD symptoms in the United States show different ethnic trends than do BE and EAC incidences. A survey among Caucasian Americans, African Americans, Asian, and Hispanics in the United States showed that Hispanics have the highest rate of GERD symptoms (i.e., occurring at least monthly in 50 %). The rates are lower in Caucasian Americans (37 %), African Americans (31 %), and Asians (20 %) [232]. Interestingly, African Americans have lower visceral adipose than Caucasians or Hispanics, even with comparable BMI and waist circumference [233]. This provides a plausible explanation for the low incidence of EAC in African Americans, if it is visceral adipose tissue that is key. However, the amount of visceral adipose in Hispanics is comparable to that in Caucasians, whereas the BE and EAC rates in Hispanics are significantly lower. Therefore, there may be underlying ethnicity-specific factors mitigating the effects of visceral adipose and GERD on risk of developing BE and EAC.

The inconsistent association between GERD and EAC risk, as well as between obesity severity and GERD severity, indicates that obesity has biological effects on the risk for developing BE and EAC biologically (as was discussed for prostate cancer), rather than just mechanical. Adipose type, location, and amount could be the significant factors affecting cancer formation, possibly by affecting the inflammatory response. Additionally, the high incidence of BE and EAC in Caucasian Americans, that is independent of obesity and GERD, suggests potential ethnicity-specific susceptibility.

### Obesity and Inflammation

Obesity is considered a state of chronic meta-inflammation, metabolically triggered inflammation [234], with wide metabolic effects. Adipose tissue secretes a variety of proinflammatory cytokines that are thought to contribute to metabolic syndrome, insulin resistance, and increased risk for a number of cancers [235]. Visceral obesity

in particular is associated with increased levels of these factors, including TNF-α, leptin, and resistin, as well as reduced adiponectin. These alterations are at least partly responsible for increased circulating insulin and insulin growth factor-1 and can stimulate the insulin growth factor receptor complex, promoting cellular proliferation. Such insulin resistance is associated with progression to EAC in a cohort with BE [236], but other conflicting data [237].

Other factors significant for associating adipose to BE and EAC are leptin and adiponectin. In the majority of obese individuals, serum leptin levels are elevated, and this increase is considered a risk factor for cancers, including breast, colorectal, prostate, ovarian, lung, and endometrial [238]. Leptin levels have been associated with an increased risk for BE progression, and leptin receptor expression has been detected in BE and EAC cells [231]. Cell culture models have shown that a combined leptin-acid exposure (mimicking obesity and GERD) produced synergistic proliferation and apoptosis resistance in EAC cell lines [239]. Adiponectin levels have an inverse relationship to visceral fat levels [240] and an inverse association to the formation of BE and erosive esophagitis. Adiponectin affects pathways that inhibit leptin-induced proliferation, invasion and migration, and the antiapoptotic effects of leptin [231].

Leptin and adiponectin have opposite relationships to the amount of visceral fat and are antagonists in BE progression. These facts provide a compelling model for how visceral adiposity is the form of obesity with the greatest risk for EAC development.

## Genetic and Epigenetic Factors

Genetic studies have the potential to determine both general and ethnicity-specific factors that affect EAC risk. One study specifically examined genetic markers for obesity, using a study population with BE and EAC patients. A set of 29 genetic variants associated to BMI were used as an instrument for lifetime BMI and showed significant associations between BMI and both BE and EAC. This indicates that genetic propensity to obesity is a risk factor for esophageal metaplasia and neoplasia [241].

Two untargeted whole genome association studies identified a number of SNPs associated with BE and/or EAC risk: (1) SNPs in the HLA region on chromosome 6p21 and near *FOXF1* [242]; and (2) SNPs near *CRTC1*, *BARX1*, and *FOXP1* (BEACON) [243]. Follow-up studies in separate datasets supported the identification of the SNPs near *CRTC1*, *BARX1*, and *FOXP1* [244], as well as identifying other associations near *GDF7* and *TBX5*, and within *ALDH1A2* [244], *XRCC2,* and *GATA6* [245]. Analysis of the *FOXP1* polymorphism determined that individuals homozygous for the major allele have a stronger association between reflux symptoms and BE risk than those carrying the minor allele [246].

The genes in proximity to these SNPs have functions relevant to BE and EAC development. *FOXP1*, *BARX1*, and *TBX5* encode transcription factors with roles in esophageal development or specification, and *GATA6* produces a zinc finger

transcription factor involved in cellular differentiation and organogenesis. *ALDH1A2* is involved in retinoic acid/retinol synthesis and is important for signaling in developing and adult tissues. *FOXP1* is a putative tumor suppressor gene, *CRTC1* produces a CREB-regulated transcription coactivator with oncogenic potential when inappropriately activated, and *XRCC2* produces a RecA/Rad51-related factor involved in DNA repair. Finally, the HLA/HMC cluster controls antigen display on immune cells, regulating self/nonself identification and autoimmune response. The protein functions, both in early development and tumorigenesis, suggest a model of esophageal cancer formation that is susceptible to environmental influence (e.g., obesity-mediated inflammation).

MicroRNAs have also been the focus of much study in determining BE and EAC mechanisms and biomarker profiles. This is due to the recognized significance of miRNAs in gene expression, particularly with the determination of miRNA dysregulation profiles in multiple cancers associated with development, pathogenesis, metastasis, prognosis, and response to treatment [247–251]. Comparison of 11 studies identified miRNAs with replicating results in: (1) BE versus normal epithelium, 8 were overexpressed and 5 underexpressed; (2) EAC versus normal epithelium, 9 were overexpressed and 8 underexpressed; and (3) EAC versus BE, 4 were overexpressed and 5 underexpressed [252]. Another meta-analysis of multiple studies identified increased miR-192, miR -194, and miR -215 and decreased miR-203 and miR-205 expression in both BE and EAC compared to controls. These findings indicate the utility of these miRNAs as biomarkers or treatment targets [253]. Nearly 50 miRNAs have been identified as aberrantly expressed at some stage of progression with over 50 known gene targets [254].

MiR-192 has potential oncogenic properties, with overexpression having proliferative effects in lung cancer [255], as well as overexpression with effects on cell cycle progression in colon cancer [256]. MiR-205 is also downregulated in prostate cancer [257] and breast cancer where it targets *HER3* and *VEGF-A* [258, 259]; its downregulation is significantly associated with poor survival in head and neck cancer [260]. MiR-196a has been reported as upregulated during EAC progression by four studies [252]. One group identified miR-196a downregulation of *ANXA1* has an antiapoptotic effect in EAC cells, improving their survival and providing a good biomarker for neoplastic progression from BE to EAC [261, 262].

Epigenetic studies have begun to identify regulatory modifications that are changed during the progression from BE to EAC. Global DNA hypomethylation is seen in BE and EAC cells when compared to normal squamous cells [254, 263, 264]; such widespread hypomethylation is common in cancerous cells. Information on hypomethylation of specific genes is still limited, but there is the potential for the activation of a number of oncogenes normally involved in normal tissue development. Potential oncogene candidates include *CDX1* and *CDX2*, skeletal and intestinal developmental regulators normally expressed only in intestine, but detected in EAC cells [265]. *B3GAT2* and *ZNF793* are also identified as being significantly hypermethylated in BE cells. They may be useful diagnostic markers, but no functional role in BE development or progression has been defined [266].

## Summary

Data relating common environmental chemical exposures to adenocarcinomas that exhibit disparities are limited; however, evidence is accumulating that (1) environmental contaminants are found more frequently in higher concentrations in minority populations; (2) exposure to environmental toxicants increases the risk of epigenetic shifts at regulatory regions that have been associated with the more proximal disease of obesity, and (3) obesity is a risk factor for progression in these cancers. Studies to test the hypothesis that environmentally induced epigenetic alterations contribute to disparate cancer risk are critically required.

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# Chapter 6
# Stress, Exercise, and Epigenetic Modulation of Cancer

**Giuseppe Lippi, Elisa Danese, and Fabian Sanchis-Gomar**

**Abstract** The term epigenetics is generally referred to phenotype modifications occurring in the DNA or the chromatin's structure, which may influence the transcription of many genes independently of their primary nucleotide sequences. Although epigenetics is still in its infancy in the field of physical exercise, some studies convincingly suggest that epigenetic regulations may play an important role in modulating the favorable effects of exercise on development and progression of cancer. Several lines of evidence demonstrated that regular physical activity decreased the risk of several types of malignancies, and some of these beneficial effects are seemingly mediated by epigenetic modifications. More specifically, it has been clearly demonstrated that physical exercise is effective to induce histone modifications, methylation and acetylation of DNA, modulatory expression of microRNAs (miRNAs), as well as additional influences on proteins and biological pathways implicated in cancer biology such as tumor suppressor p53, lipoprotein(a), and hypoxia-inducible factor-1 (HIF-1). Although the available evidence does not support the notion that exercise-induced epigenetic changes always follow a unidirectional path in terms of cancer risk, the favorable effects of reduced cancer development and progression probably overwhelm cancer-promoting activities. If preliminary findings are confirmed in larger studies, physical exercise may hence be regarded as an appealing perspective for reducing the risk of cancer in different populations.

**Keywords** Epigenetics • Cancer • Physical activity • Physical exercise • Sport

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## Cancer and Exercise: Review of Epidemiological Evidence

The association between physical activity (PA) and health is an old theory, having been first speculated more than 2000 years ago by the Greek physician Hippocrates, who advised: "eating alone will not keep a man well; he must also take exercise" [1]. With the decline of the Hellenic civilization, the interest in the relationship between PA, fitness, and health faded for centuries and then rose again with the advent of the Industrial Revolution. During that period scientists began to measure the benefits of exercise more objectively, thus inspiring a new era in which the association between exercise and human health could be analyzed more scientifically, by using numerical quantification. Since then, evidence accumulated from a number of epidemiological studies unequivocally supports the existence of an inverse, independent, and graded association between PA health and overall mortality, especially in the field of cardiovascular medicine [2, 3].

The earliest pioneer studies postulating the inverse relationship between PA and cancer risk were concomitantly published in 1922 by two independent groups [4, 5]. The authors assessed cancer mortality rates in men with different occupations and concluded that those employed in physically demanding jobs experienced lower cancer mortality rates than those engaged in less strenuous activities.

Thereafter, modest progress was made until the mid-1980s, when the interest on this topic re-emerged, thus leading to a rapid increase of scientific literature on this topic. Since then, more than 600 epidemiological studies were carried out in both genders, in different ethnic groups, in broad age classes, in a variety of social and occupational groups, and in most continents around the world. Although individual studies have demonstrated that PA might be virtually protective against all types of cancer, the evidence emerged was judged as mostly inconsistent for some cancer types because of the impossibility to perform high-quality meta-analyses from existing data. In particular, the use of different definition of PA, the lack of consistency in the methods used to quantify it, the difference in the study design, the lack of uniformity in adjusting for confounding factors, as well as the intrinsic bias associated with self-reported questionnaires, represent some of the main drawbacks which hampered cumulative data analysis due to the large differences in risk estimates across the different studies [6]. Following a rigorous evaluation of the available literature, the report published in 2007 by the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) judged that the evidence supporting a protective role of PA against cancer was "convincing" for colon cancer, "probable" for postmenopausal breast and endometrial cancer and "limited-suggestive" for lung, pancreas, and premenopausal breast cancer [6, 7].

The best evidence for a protective influence of PA against cancer currently emerges from epidemiologic studies on colon cancer. Wolin and colleagues conducted the first formal estimation of the magnitude of association between PA and reduction of colon cancer risk in 2009. This meta-analysis of 24 cohort studies and 28 case–control studies reported a significant 24 % reduced risk when comparing the most vs. the least active individuals across all studies (relative risk [RR],

0.76; 95 % CI, 0.72–0.81). The effect was similar in men and women. The findings from case–control studies were stronger (RR, 0.69; 95 % CI, 0.65–0.74) than from cohort studies (RR, 0.83; 95 % CI, 0.78–0.88) [8]. More recent results of an ensuing meta-analysis provide convincing evidence that the association between PA and the risk of colon cancer does not differ by anatomical subsite. By calculating the summary risk estimates from 21 studies, the authors observed that the risks of both proximal colon cancer and distal colon cancer were, respectively, 27 and 26 % lower among the most physically active people compared with the least active people [9]. Results from the two meta-analyses were substantially similar, although evidence emerged from the recent one appears more consistent due to the low grade of statistical heterogeneity in the estimates across studies ($I^2$, 31.3 %; $p = 0.057$ and $I^2$, 0.0 %; $P = 0.473$ for proximal and distal colon cancer, respectively). The lower risk of colon cancer associated with PA appears not consistently modified by other well-known risk factors, including body mass index (BMI), cigarette smoking, energy intake, and high-risk diet [10, 11]. In contrast to colon cancer, most studies on rectal cancer reported no significant relation between increased PA and cancer risk.

There is substantial evidence that breast cancer risk is statistically significant decreased among physically active individuals. The best confirmation for this association comes from studies of postmenopausal breast cancer for which the evidence has been judged as 'probable' by both the WCRF/AICR [12], and the IARC [13].

Studies on premenopausal women reached fewer certain results, thus making the association "limited-suggestive" according to experts' conclusions. However, a recent meta-analysis pooling results of 31 studies reported that premenopausal active women had a stronger reduction of breast cancer risk [RR, 0.72; 95 % CI, 0.65–0.81] than those in the postmenopausal state (RR; 0.87; 95 % CI, 0.87–0.92 [14]. At variance with these findings, another meta-analysis published in 2014 reconfirmed that PA seemingly prevents breast cancer, especially in postmenopausal women [15]. Notably, the homogeneity with which the menopausal status has been defined and applied to stratify study populations in these investigations is probably the main cause of the different conclusions reached in the two meta-analyses. Therefore, further investigations using a clear and unique definition of menopausal status to cluster study population will be needed to clarify this important aspect. Regardless the influence of menopausal status, an agreement in literature exists for assuming that women who were most active in their occupational and/or recreational activities may have a lower incidence of breast cancer than their sedentary counterparts. In addition, the housework seems to reduce the risk of breast cancer by itself as well as in combination with spare time activity such as walking, cycling, or playing sport [16, 17]. The reported reduction in the risk ranges from 10 to 80 %, is on average 25 %, and appears to be stronger for subjects with BMI < 30 kg/m$^2$, parous women, women without a family history of breast cancer, and estrogen and progesterone receptor-negative women [18, 19]. Finally, most prospective studies found evidence for a dose–response relationship showing a lower risk of breast cancer with higher levels of PA [20, 21].

To date, five publications have summarized the available epidemiologic evidence regarding the association between PA and risk of endometrial cancer [22–26]. Concertedly, findings from these studies suggest that PA is associated with an

18–30 % reduction in endometrial cancer risk with high versus low PA levels. The association was observed for broad range of activity domains, including both recreational and occupational PA, and for different intensities, including light, moderate, and vigorous activities. Particular protection from endometrial cancer through PA participation was found for women who were overweight or obese [26].

Recent epidemiologic investigations support the hypothesis that PA might reduce the risk of many other cancers other than those previously discussed, thus including malignancies of lung, pancreas, prostate, and stomach. Nevertheless, the current evidence remains limited and additional data are needed [27].

Observational studies evaluating the association between PA and risk of death among survivors of cancer suggest that PA prolongs overall and cancer-specific survival [28–30]. Even in such cases, most convincing data come from studies on colon and breast cancer. In particular, a meta-analysis of prospective studies published through June 2013 showed that engaging in the approximate equivalent of 150 min of at least moderate intensity PA per week after cancer diagnosis was associated with a 24 % reduced risk of total mortality among breast cancer survivors and 28 % decreased total mortality risk among colorectal cancer survivors [31]. The apparent protection afforded by PA was observed even after adjustments for tumor stage, cancer treatment, smoking, and adiposity and was confirmed in different geographical settings, in both large and small studies, as well as in surveys using self-reported and interview-based PA assessments. The authors also confirmed previous findings suggestive of a beneficial effect of PA performed before cancer diagnosis on both total and cancer mortality. They found that high versus low prediagnosis PA was associated with decreased risk of total mortality. More specifically, higher prediagnosis PA was associated with a 13 % decreased risk of total mortality among breast cancer survivors and a 14 % decreased total mortality risk among colorectal cancer survivors. Finally, they showed that an increase in PA from pre- to postdiagnosis further reduces total mortality risk.

## Recommended Dose of Physical Activity for Health Benefits

According to a real biological perspective, physical exercise should indeed be regarded as a form of probably “favorable” stress. The large experience accumulated during the past suggests in fact that PA induces a large number of metabolic adaptations that are magnified in the process of transition from a sedentary to a physically active state [32]. What has become rather clear after decades of research in this field is that a linear dose–response relationship seemingly exists between physical exercise and metabolic changes. Interestingly, it was recently shown that former participants in the Tour de France (i.e., the most famous worldwide professional 3-week cycling race) have a considerable increase in average longevity (+17 %) compared to the general population [33], thus underpinning that the burden of the most frequent causes of death (especially cancer and cardiovascular disease) may be consistently reduced by PA [34].

The recent guidelines of the American College of Sports Medicine and the American Heart Association recommend that health promotion and maintenance in healthy adults aged 18–65 years can be achieved by engagement in moderate-intensity aerobic (endurance) PA for not less than 30 min on 5 days each week or vigorous-intensity aerobic PA for not less than 20 min on 3 days each week [35]. In older subjects, these indications should also consider the aerobic fitness and risk of fall and should include exercise to promote or enhance flexibility [36]. Additional recommendations have been published by the World Health Organization (WHO) [37]. Specifically, children and youth aged 5–17 years should be engaged in at least 60 min of moderate- to vigorous-intensity PA (preferably aerobic) every day; amounts greater than 60 min provide additional health benefits. Adults aged 18–64 years should be engaged in not less than 150 min of moderate-intensity aerobic PA per week or not less than 75 min of vigorous-intensity aerobic PA per week or an equivalent combination of moderate- and vigorous-intensity activity. Moderate-intensity aerobic PA and vigorous-intensity aerobic PA may be increased to 300 min and 150 min per week, respectively, for gaining additional health benefits. These recommendations also apply to subjects aged 65 years or older, although it is clearly stated that older adults who are unable to perform the minimum amount of PA due to health conditions should be as physically active as their abilities and conditions allow. As specifically regards cancer prevention, the American Cancer Society (ACS) endorsed that adults should be engaged in not less than 150 min of moderate intensity or 75 min of vigorous intensity activity per week (or a combination of these), preferably spread throughout the week, whereas children and teens should be engaged in not less than 1 h of moderate or vigorous intensity activity per day, with vigorous activity on at least 3 days each week [38]. Overall, the time spent on sedentary behavior such as sitting, lying down, watching television, and other forms of screen-based entertainment should be very limited. Interestingly, the ACS has also released PA guidelines for cancer survivors [39], indicating that these individuals should avoid inactivity (i.e., aiming to exercise not less than 150 min per week, including strength training exercises not less than 2 days per week), and return to normal daily activities as soon as possible after diagnosis.

Therefore, despite universal recommendations do exist, a generalization seems inappropriate wherein the number and type of congenital and acquired risk factors for both cancer and cardiovascular disease varies widely across the population. As especially regards cancer, a more personalized approach seems advisable, which should take into account the family history (i.e., the genetic predisposition to develop some forms of cancer), demographic determinants (i.e., age, gender, and racial origin), and the exposure to environmental risk factors (e.g., diet and ambient pollutants). In particular, although the relationship between exercise, health and fitness seems now virtually unquestionable, a major dilemma remains, that is to definitively establish which is the adequate amount of physical exercise needed for the single patient (regardless of the age) for improving health without reaching the so-called wrecking point, after which the potential benefits may be outweighed by the adverse consequence of excess stress (Fig. 6.1).

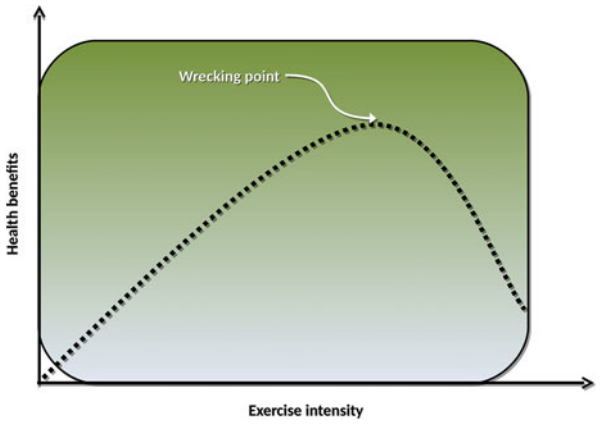


**Fig. 6.1** Relationship between physical activity and health

## *Physical Activity and Epigenetic Modulation of Cancer*

PA is thought to act in a variety of ways to affect cancer risk [40]. Indeed, one of the most important mechanisms mediating the association between PA and cancer prevention is the direct and indirect effects that exercise has on weight control. Regular PA, in fact, helps maintain a healthy body weight by balancing caloric intake with energy expenditure and by regulating the circulating levels of sex hormones, adipokines, insulin, and pro-inflammatory cytokines. PA has also been hypothesized to reduce carcinogenic prostaglandin production. In addition, PA increases colon motility, leading to decreased transit time and, perhaps, reduced mucosal exposure time to carcinogens. Another potential mechanism is through the beneficial effects of chronic PA on DNA oxidative damage or repair and on immune system [27, 41].

Interestingly, it has been recently postulated that PA may regulate molecular pathways related to inflammatory processes, metabolism, and energy consumption through the induction of epigenetic modifications. Changes in the concentrations of metabolites, such as oxygen, tricarboxylic acid (TCA) intermediates, 2-oxoglutarate, 2-hydroxyglutarate, and β-hydroxybutyrate, are potentially dependent upon epigenetic modifications and many epigenetic enzymes [42]. PA induces fluctuations in these enzymes in a tissue-dependent manner. Many of these changes are regulated by epigenetic modifiers such as DNA methyl transferases, histone acetyltransferases, histone deacetylases, and histone demethylases, between others [42]. Therefore, these substrates and signaling molecules, regulated by PA, affect important epigenetic mechanisms which ultimately control the gene expression involved in metabolism [42].

The term epigenetics is generally referred to phenotype modifications occurring in the DNA or the chromatin's structure that can influence the transcription of several genes independently of their primary nucleotide sequences [43]. The most common epigenetic changes induced by exercise include histone modifications, such as methylation and acetylation, DNA methylation, and synthesis of noncoding mRNA [44]. The role of such exercise-induced modifications in cancer is discussed below.

## *Physical Activity and DNA Methylation*

DNA methylation is a process in which methyl groups are added to the DNA, thus modifying its function, most frequently by suppressing gene transcription [45]. The process is usually catalyzed by DNA methyltransferases and entails the covalent linkage of methyl groups in cytosine within CpG dinucleotides concentrated in large clusters conventionally known as "CpG islands." Interestingly, although hypermethylation within the promoter region may lead to inactivation of tumor-suppressor genes, generalized hypomethylation is also associated with genomic instability and may hence contribute to make the cell more vulnerable to transformation. Therefore, both aspects are deeply involved in the pathogenesis of human cancer, wherein hypomethylation may be associated with hyperactivation of oncogenes whereas hypermethylation may be linked to transcriptional silencing of critical growth regulators such as tumor suppressor genes, [46].

Brown recently performed a meta-analysis of 16 studies, totaling 387 genes and 1580 subjects, to comprehensively summarize exercise-associated DNA methylation changes [47]. Overall, DNA methylation was found to be significantly reduced with exercise (as many as 478 genetic elements showed exercise-associated DNA methylation patterns), especially with ageing (i.e., after the age of 40). Five exercise-associated imprinted loci could also be identified, including *KCNQ1* (potassium channel, voltage-gated KQT-like subfamily Q, member), *MEG3* (maternally expressed 3), *GRB10* (growth factor receptor-bound protein 10), *L3MBTL1* (l(3) mbt-like 1), and *PLAGL1* (pleomorphic adenoma gene-like 1). DNA methylation decreased with exercise (60 % of loci). Importantly, the genes displaying reduction of DNA methylation were part of a microRNA-regulated gene network functioning to suppress cancer. More specifically, hypermethylation was found for the genes *CXCL10* (C-X-C motif chemokine 10 also known as chemokine interferon-γ inducible protein 10), *DCC* (deleted in colorectal carcinoma), *PPP2R3A* (protein phosphatase 2, regulatory subunit B, alpha), *RASA1* (RAS p21 protein activator (GTPase activating protein) 1), *SULF1* (Sulfatase 1), *TMEM100* (transmembrane protein 100), and *WNT7A* (Protein Wnt-7a), whereas hypomethylation was found for the genes *GAB1* (the GRB2-associated binding protein 1), *L3MBTL1* (Lethal(3)malignant brain tumor-like protein 1), *PLAGL1* (pleomorphic adenoma gene-like 1), *WNK3* (With-No-Lysine Kinase 3), *BCL2L11* (Bcl-2-like protein 11), and *CACNA2D3* (calcium channel, voltage-dependent, alpha 2/delta subunit 3). Although the real significance of most of these changes remains to be elucidated, some of these genes are particularly interesting for cancer biology.

CXCL10 belongs to the large CXC chemokine family, composed by elements that bind to the chemokine (C-X-C motif) receptor 3 (CXCR3) and exert a wide spectrum of either carcinogenic or anticancer activities. In particular, it has been shown that CXCL10 is capable to bind to G-protein-coupled receptors, thus inducing a wide spectrum of biological and physiological activities which include an enhancement of cell growth and proliferation, as well as overresponsiveness to cytokines synthesized by malignant and inflammatory cells [48].

WNT7A is a glycoprotein which not only plays an important role in modulating cellular proliferation and differentiation, but also influences carcinogenesis and tumor progression. Overexpression of this protein has recently been observed in patients with endometrial cancer, and its expression strongly correlates with disease progression [49]. Lethal(3)malignant brain tumor-like protein 1, the transcription product of the *L3MBTL1* gene, has been shown to bind histones, thus increasing the order of chromatin structure and generating transcriptional repression. Recent evidence shows that depletion of this gene is associated with replicative stress, enhancement of DNA injury, development of DNA damage response, and overall genomic instability [50]. *L3MBTL1* can hence be considered a powerful tumor suppressor gene, and it is conceivable that hypomethylation of this gene during exercise should be regarded as protective mechanism against cancer machinery. The *PLAGL1* is another (putative) tumor suppressor gene. More specifically, this gene is frequently silenced in both breast and ovarian malignancies, whereas its overexpression (as may result from hypomethylation during exercise) may be associated with antiproliferative effects [51].

It is also noteworthy, however, that the relationship between sport, DNA methylation and cancer may be complex and not unidirectional. In fact, although the hypermethylated genes are indeed involved in cancer biology, most of them exert tumor suppressor functions rather than tumor promoting activities. *DCC* is a putative tumor suppressor gene and has been found to be frequently mutated or downregulated in colorectal cancer and esophageal carcinoma [52]. *PPP2R3A* encodes one of the regulatory subunits of the protein phosphatase 2 (PP2), which is involved in negative modulation of cell growth and division [53]. Interestingly, silencing of *RASA1* is also associated with unregulated cell proliferation and carcinogenesis [54], whereas enhanced expression of *SULF1* seemingly reduces cell proliferation, migration, and invasion [55]. *TMEM100*, an activin receptor-like kinase-1 (*ALK1*) signaling-dependent gene essential for arterial endothelium differentiation and vascular morphogenesis, inhibits metastasis and cancer cell proliferation [56]. The *GAB1* gene encodes the GRB2-associated binding protein 1, which is a pivotal mediator of cellular growth, transformation and apoptosis. Recent evidence suggests that overexpression of *GAB1* enhances cell growth and strongly promotes tumorigenesis [57], so that hypomethylation of this gene during exercise may influence vulnerability to developing cancer. WNK3 belongs to the "with no lysine" family of serine–threonine protein kinases. Its expression has been found consistently increased in several human cancers [58]. Importantly, overtranscription of this gene (e.g., as a result of hypomethylation) may increase cell survival by delaying apoptosis.

According to this evidence, it seems hence more reasonable to conclude that PA-induced DNA methylation does not follow a unidirectional path in terms of cancer risk, and additional studies may be needed to clearly define this issue.

## *Physical Activity and Histone Modification*

Histone modifications are posttranslational alterations on the lysine-rich tail region of histones. They mainly include not only acetylation and methylation, but also some less-studied modifications such as phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination, and proline isomerization. Each of these histone modifications directly or indirectly affects chromatin structure, thereby leading to alterations in DNA repair, replication, and gene transcription.

Histone acetylation is regulated by a balance between histone acetyl-transferase (HAT) and histone deacetylase (HDAC) activities, with the former involved in a process of addiction of an acetyl group to the α-amino groups on the N-terminal tails of histones, whereas the activity of the latter enzyme entails removing acetyl groups from DNA. In chromatin, DNA is tightly wrapped around histones. Lysine acetylation by HATs is believed to neutralize the positive charge of histone tails, weakening histone-DNA or nucleosome-nucleosome bindings, and inducing an open (euchromatin-like) conformational change. As a consequence, the access of transcription factors to DNA is facilitated and gene expression concomitantly enhanced. On the other hand, deacetylation of histones by HDACs reduces the space between nucleosome and DNA, thus leading to a closed (heterochromatin-like) chromatin conformation that lower the accessibility for transcription factors, ultimately decreasing gene expression [59, 60].

Histone methylation is a reversible process that occurs through histone methyl-transferases (HMTs), which are enzymes that add methyl groups to lysine and arginine tail regions of histones. The most heavily methylated histone is H3, followed by H4. Both arginine and lysine methylation can occur in mono-, di-, and trimethylated forms. Although, the current knowledge on the biological role of this modification is still rather limited, it has been suggested that it may reflect transcriptionally active euchromatin or transcriptionally repressed heterochromatin [61].

Current data on the effect of PA on histone modifications are limited and mainly come from studies exploring molecular pathways implicated in metabolic processes. For example, it has been concluded that histone modifications do regulate glucose transporter type 4 (*GLUT4*) expression in response to exercise [62–64]. In human skeletal muscle, the highly expressed class IIa HDACs is known to interact with the myocyte-specific enhancer factor 2 (MEF2) by creating a complex which removes acetyl groups from MEF2 and represses the expression of GLUT4, a MEF2-dependent transcription.

Following acute exercise, the HDACs activity is reduced as a consequence of a ubiquitin-mediated proteasomal degradation, and of its phosphorylation by $Ca^{2+}$/Calmodulin-dependent protein kinase (CaMK), AMP-dependent protein kinase (AMPK), or protein kinase D (PKD), which both cause a dissociation between HDACs and MET2 and the exit of HDACs from the nucleus [65]. Upon removal of the transcriptional repressive function of HDACs, the expression of *GLUT4* increases. Such enhancement of skeletal muscle GLUT4 seems to occur as rapidly in response to an exercise stimulus as it declines with cessation of training [66]. The

epigenetic modifications of GLUT4 expression by exercise may have remarkable clinical implications. The GLUT4 glucose transporter is the major mediator of glucose removal from the circulation, and a key regulator of whole-body glucose homeostasis. Therefore, the ability of PA to interfere with its trafficking pathways may have therapeutic potential in obesity, type II diabetes, metabolic syndrome as well as in associated comorbidities including obesity-related cancers.

Another intriguing effect of exercise-induced histone modification is that involved in the activation of the hypothalamus–adipocyte axis. In 2010 Cao and colleagues demonstrated that physical as well as social cognitive stimulations may trigger brain-derived neurotrophic factor (BDNF) expression in the hypothalamus leading to preferential sympathoneural activation of white adipose tissue. The elevated sympathetic drive is then effective to activate adipocyte β-adrenergic receptors, inhibiting leptin expression and release, and ultimately suppressing cancer growth and preventing metastasis [67]. More specifically, exercise is able to increase the expression of BDNF through at least two pathways: by inducing the acetylation of histone H3 in the *BDNF* promoter IV, which result in the transcription of *BDNF* gene, as well as by increasing the phosphorylation levels of cAMP response element binding protein (*CREB*) and *CaMKII*, that once activated, acquire a strong histone acetylation transferase-promoting activity and, in turn, activate BDNF transcription [68].

## *Physical Activity and microRNA*

A growing body of evidence suggests that PA may affect the production of microRNAs (miRNA), small noncoding single-stranded RNA of approximately 20 nucleotide in length that play critical roles in many biological processes including cell development, differentiation, proliferation, and apoptosis. At variance with protein-coding RNA, miRNAs represent a large portion of eukaryotic transcript and do not result in protein production. Instead, they are known to regulate about 30 % of human gene expression. The miRNAs act at a post-transcriptional level by targeting the 3′ untranslated region of mRNAs, thus regulating translation of mRNA to amino acids. MiRNA regulation is dynamic. Their effects can be temporary, when the miRNA temporarily binds an mRNA to suppress translation, or permanent, causing degradation of the mRNA strand.

What is currently known about the association between exercise and microRNAs has been summarized in a systematic review published in the early 2015 by Flowers et al., and including as many as 14 studies [69]. With the exception of few miRNAs which have been found repeatedly modulated across studies, most articles reported different findings. The reasons for this discrepancy mainly reside in the different type of exercise that has been evaluated (i.e., cardiorespiratory fitness vs. resistance training) and in the timing of miRNAs evaluation (i.e., acute-phase vs. long-term responses). For example, miR-146a was increased immediately following acute exercise, but consistently declined after resistance

training. Similarity, miR-20a and miR-20b both decreased following cycle ergometry, but their concentration was enhanced after completion of a 90-day exercise training program. Among the over 100 miRNAs which have been found to be up- or downregulated in response to exercise, some may play an important role in tumorigenesis. For example, it has been reported that miR-15a and miR-16-1 that target *BCL2*, an antiapoptotic gene, were downregulated in B-cell chronic lymphocytic leukemia [70], whereas miR-145 was downregulated in colorectal cancer [71, 72]. Amplification or overexpression of the miR-17-92 cluster has been reported in patients with lymphomas [73].

According with a recent hypothesis, miRNAs may also represent a plausible mechanistic link between PA, telomeres, and improved health [74]. Telomeres are specialized nucleoprotein structures that protect the ends of linear chromosomes and progressively shorten with each round of cellular division. Telomere length, shorter than the average length for a specific age group, has been associated with increased incidence of age-related diseases and decreased lifespan in humans. Moreover, excessive or accelerated telomere shortening can induce genomic instability by mediating interchromosomal fusion and may contribute to telomere stabilization and development of cancer [75]. Several studies indicate that individuals with shorter telomeres have a greater risk for development of lung, bladder, renal cell, gastrointestinal, head, and neck cancers [76–78].

By investigating the acute effects of 30 min of intense cardiorespiratory exercise on the expression of genes involved in telomere regulation in white blood cells (WBCs), Chilton and colleagues were able to identify four miRNAs (miR-186, miR-181, miR-15a, and miR-96) that potentially targeted telomeric gene mRNA [79]. In particular, telomeric repeat binding factor 2 interacting protein (TERF2IP), a protein that is part of a complex involved in telomere length shortening, was identified as a potential binding target for miR-186 and miR-96 [80, 81]. The expression of both miR-186 and miR-96 was found to be increased from immediately after to 60 min postexercise and was accompanied by a parallel and simultaneous downregulation of *TERF2IP* mRNA expression. Such findings suggest that intense cardiorespiratory exercise may be sufficient to block the oncogenic insult of *TERF2IP*, and that the effect may be mediated by microRNAs.

Using a similar exercise protocol, Tonevitsky and coauthors [82] identified four miRNA–mRNA networks dynamically regulated by 30 min of exercise. The target mRNAs were involved in immune function, transcription regulation, and membrane traffic of proteins. Most importantly, some of these miRNA–mRNA networks including hsa-miR-24-2-5p-MYC and hsa-miR-21-5p-TGFBR3 have a role in cancer development and progression, since they were found to be involved in cell proliferation, apoptosis, transformation, migration invasion, angiogenesis, and metastasis.

## *Physical Activity and Modulation of the Tumor Suppressor p53 Pathway*

The p53 tumor suppressor protein is a transcription factor that regulates the expression of stress response genes and exerts multiple, antiproliferative functions [83]. Tumor protein p53 is one of the most important proteins that protect against cancer and has also been identified as the most important guardian of the human genome. Therefore, it is not a surprise that the *p53* gene is mutated or dysfunctional in the majority of human tumors. The disruption of normal p53 function represents one of the main prerequisite for the initiation and/or progression of tumors.

Recently, p53 has been described as an important regulator of miRNAs [84]. In 2007, several independent groups identified different miRNAs as direct transcriptional targets of p53 [85]. Among all miRNAs, the members of the miR-34 family displayed the highest induction by p53 [86]. Some years later, evidence suggests that miRNAs not only mediate the downstream effect of p53, but are also involved in the upstream regulation of p53, thus further highlighting the importance of miRNAs in human tumors. An overview of the role of miRNAs in the p53 network is shown in Fig. 6.2. Interestingly, some of these regulators of p53 may in turn be modulated by stress and exercise [87]. In particular, depending on the intensity of training, these miRNAs may cause upregulation or downregulation of the tumor suppressor p53.

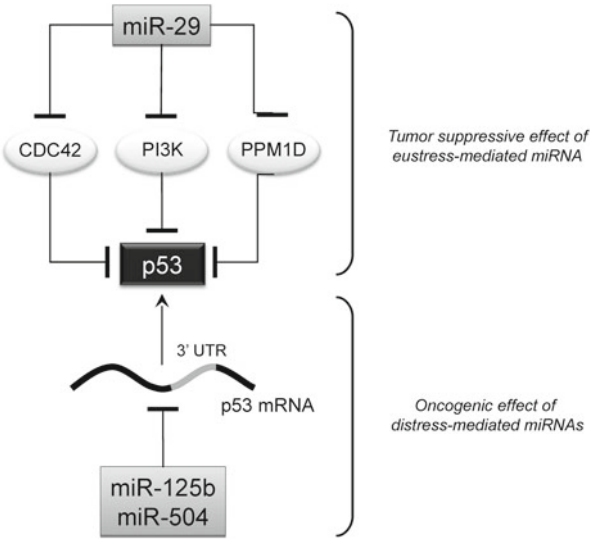


**Fig. 6.2** Exercise-induced regulation of p53 protein by miRNAs. miR-29 upregulates p53 by inhibiting the expression of negative regulators of p53 (*CDC42, PI3K, PPM1D*). miR-125b and miR-504 downregulate p53 by binding the 3′ untraslated region of p53 mRNA. *CDC42* cell division control protein 42 homolog, *PI3K* phosphatidylinositol-4,5-bisphosphate 3-kinase, *PPM1D* protein phosphatase $Mg^{2+}/Mn^{2+}$-dependent 1D; *UTR* 3′ untraslated region

The miR29a, whose changes in expression levels are associated with immediate response to cardiorespiratory fitness [88], is effective to increase p53 activity by targeting two negative regulators of p53, namely p85α, the regulatory subunit of phosphatidylinositol-3 kinase (P13K), and CDC42 (cell division control protein 42 homolog), a Rho GTPase [89]. Furthermore, miR-29 was shown to target the protein phosphatase $Mg^{2+}/Mn^{2+}$-dependent 1D (PPM1D) during ageing, which is another negative regulator of p53. Thus, miR-29 and p53 form a positive feedback loop that reinforces p53 functions, such as apoptosis and senescence.

The first report suggesting a negative regulation of p53 by miRNAs was published in 2009. Le and colleagues, by performing an in silico search for putative miRNA binding site in the p53 3′ untranslated region, demonstrated that miR-125b can bind to human and zebrafish p53 mRNA, thus reducing p53 expression. Overexpression of miR-125b is capable to repress the endogenous level of p53 protein, thus suppressing apoptosis in human neuroblastoma cells and human lung fibroblast cells. In contrast, knockdown of miR-125b is effective to enhance the concentration of p53 protein, thus inducing apoptosis in human lung fibroblasts and in the zebrafish brain [90]. Elevated expression levels of miR-125b were associated with increased tumor size and invasion in 89 colorectal cancer samples, and also correlated with poor prognosis and decreased survival [91]. It is also noteworthy that miR125b belongs to a class of inflammatory microRNAs whose expression levels are modulated by acute exercise [92–94]. One year later, Hu et al. demonstrated that miR-504, a stress-induced miRNA [95], can regulate p53 expression through its binding to two binding sites in human p53 3′-untranslated region [96]. Experiments presented by the authors demonstrated that overexpression of miR-504 reduces p53 protein levels and impairs p53 functions, including apoptosis and cell cycle arrest. Furthermore, miR-504 promotes tumorigenicity of cells in vivo.

Taken together, these findings suggested that PA and stress may positively or negatively regulate the activity and function of the p53 signaling pathway by modulating the effect of tumor suppressor or oncogenic miRNAs.

## *Physical Activity and Hypoxia-Inducible Factor-1 Pathway*

The hypoxia-inducible factor-1 (HIF-1) pathway plays a pivotal role in cancer biology. More specifically, hyperactivation of this pathway has been associated with increased angiogenesis, enhanced cell survival and local or distant cancer spread, so that inhibitors of HIF-1 are increasingly developed and used as anticancer therapeutics [97]. In brief, the HIF pathway is composed by two different proteins (HIF-1α and HIF-1β). After synthesis, HIF-1α can only exert its transcription activities at the DNA level by stabilization by HIF-1β, so that the HIF-1α/HIF-1β complex can cross the nuclear membrane, bind to intranuclear proteins and trigger gene transcription. The activity of HIF-1 pathway is hence modulated at multiple levels, which entail protein stabilization, transactivation, and target gene availability. More specifically, prolyl-hydroxylase (PHD) and

asparaginyl hydroxylase both promote HIF-1α degradation, whereas histone deacetylase sirtuin-6 (SIRT6) and factor-inhibiting HIF (FIH) substantially inhibit its transcriptional activity.

Recent evidence suggests that training may promote a negative regulation of the HIF-1 pathway. Lindholm et al. studied skeletal muscle tissue in matched populations of moderately active individuals and elite athletes [98]. When compared with moderately active individuals, elite athletes displayed a significantly higher expression of all negative HIF-1 modulators, including PHD (73.5 ± 9.5 vs. 98.0 ± 6.6), FIH (4.3 ± 0.2 vs. 31.0 ± 8.0), and SIRT6 (0.2 ± 0.1 vs. 11.4 ± 2.2). Similar evidence was previously published in an animal model, wherein Koltai et al. showed that exercise training was effective to reduce the expression of carbonylated proteins, including HIF-1-alpha, in rats [99].

## The Intriguing Relationship Between Lipoprotein[a], Physical Exercise, and Cancer

An interesting aspect in the intriguing relationship between sports, epigenetics, and cancer recently emerged from studies on lipoprotein[a] (Lp[a]) metabolism. Lp[a] is a highly atherogenic lipoprotein which strictly resembles a low-density lipoprotein (LDL) particle since it is composed by apolipoprotein B100 (i.e., the main protein moiety of LDL) covalently linked to a single copy of the unique and enigmatic apolipoprotein[a] (Apo[a]). The latter protein is unique to humans, Old World monkeys and apes, although an Apo[a]-like protein also exists in the blood of the hedgehog, in which it probably appeared independently, as result of a process of convergent evolution. The appearance of Apo[a] in the hedgehog genome, its preservation throughout the evolution of this small animal and primates, combined with the evidence that high levels of Lp[a] are compatible with longevity, do suggest that Lp[a] may confer some kinds of evolutionary advantage to those species who are capable to produce it. Although the enigma remains still inexplicable, it seems reasonable to conceive that the negative impact of Lp[a] on the cardiovascular system may be somehow offset by some favorable biological effects. Indeed, the large cholesterol content of this lipoprotein has been identified as beneficial for cell regeneration and organism recovery after trauma. Nevertheless, a more intriguing biological pathway has recently been elucidated, according to which Lp[a] may enhance survivor by decreasing mortality for cancer. The biochemical structure of Apo[a] is homologous to that of plasminogen, since this protein contains a protease domain, a single copy of plasminogen kringle V and multiple repeats of domains similar to plasminogen kringle IV (Fig. 6.3). Angiostatin is a natural modulator of angiogenesis, which is prevalently produced by catabolism of kringle-containing precursor proteins, which also include Apo[a]. Angiostatin exerts a kaleidoscope of anticancer effects such as upregulation of p53 protein, stimulation of FasL-mediated signaling pathways, and inhibition of Akt. All these activities ultimately

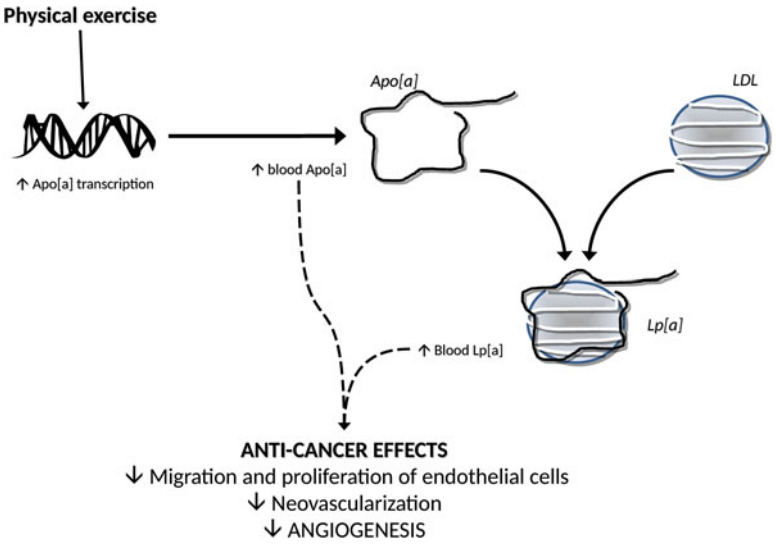


**Fig. 6.3** Putative anti-cancer effects of apolipoprotein[a] (Apo[a]) and lipoprotein[a] (Lp[a])

converge to promote apoptosis of endothelial cell and inhibition of angiogenic signaling pathway activated by a number of angiogenic factors including fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF). Several lines of evidence now attest that Lp[a] is an active phase protein, wherein the transcription of the Apo[a] gene can be substantially amplified by various types of stress, including physical exercise [100]. The main effector regulating Apo[a] synthesis is probably interleukin-6 (IL-6), which activates the promoter region of the gene and ultimately increases the blood concentration of Apo[a], thus magnifying its putative anticancer potential [101] (Fig. 6.3). This hypothesis has recently been supported by epidemiological evidence attesting that low Lp[a] levels are associated with both all-cause and cancer death [102], and that elderly patients display equivalent or even higher values of Lp[a] than those of the general population aged 75 years or younger.

## Future Perspectives

Although epigenetics is still in its infancy in the field of exercise, studies have already suggested that epigenetic regulation may play an important role in modulating the effect of exercise on cancer development and progression. Research findings demonstrate that the benefits from PA occur when activity is at least of moderate intensity and performed regularly and is sustained over lifetime or at least for a long term. However, the effects of various modalities of exercise in modulating epigenetic modifications in different cancer sites remain largely unknown. Future studies

will focus on the effect of cardiorespiratory versus resistant training and of acute versus prolonged exercise on epigenetics changes measured in acute phase and at long term. Findings derived from such studies might have two clinically relevant implications. First they may prompt the development of cancer-specific recommendations and guidelines establishing the exact type, intensity, and duration of exercise required for improved health outcomes in different group populations. Second, they will provide a number of epigenetic markers which could be used to monitor patients' response to exercise interventions and predict health benefits.

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# Chapter 7
# Epigenetic Effects of Gut Microbiota on Obesity and Gastrointestinal Cancers

**Joice Kuroiwa-Trzmielina and Luke B. Hesson**

**Abstract** The microbial census within our gastrointestinal tract outnumbers our own cells by roughly tenfold. Our gastrointestinal microbiota performs many important roles including contributing to the energy harvest from our diet, protecting us from colonization by pathogenic species of bacteria, eliminating harmful metabolites and carcinogens, and shaping our immune system to prevent chronic mucosal inflammation. Given these important functions, as well as the shear abundance of bacteria within our gastrointestinal tract, it seems intuitive that our microbiota must play some role in disease. Many studies have described changes in the gastrointestinal microbiota in various disease states including cancer, inflammatory bowel disease, diabetes, and obesity. However, important questions remain; are these changes in the microbiota a cause or consequence of disease, or are they merely correlative? If changes in the gastrointestinal microbiota are a potential cause of disease, then what's the mechanism? Here we consider the evidence that the gastrointestinal microbiota can induce epigenetic changes in host cells and whether this is a potential contributing factor to obesity and gastrointestinal cancers.

**Keywords** Microbiota • Microbiome • Epigenetic • Methylation • Histone • Metabolites • Cancer • Gastrointestinal • Dysbiosis

## Introduction

The term gastrointestinal microbiota refers to the bacteria, archaea, viruses, protozoa, helminths, and fungi that inhabit the gastrointestinal tract, whereas the term microbiome refers to the collective genomes of these microorganisms. The gastrointestinal tract of a healthy human contains around 100 trillion microorganisms, which exceeds the

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total number of human cells in the body by a factor of ~10. Between 1000 and 1150 bacterial species have been identified in human feces, with around 160 species shared between most individuals [1]. Our gastrointestinal microbiota consists of 99.1 % bacteria and is comprised of four major microbial phyla: *Firmicutes*, 30.6–83 % (*Clostridium*, *Ruminococcus*, *Eubacterium*, *Dorea*, *Peptostreptococcus*, *Peptococcus*, *Lactobacillus*); *Bacteroidetes*, 8–48 % (*Bacteroides*); *Proteobacteria*, 0.1–26.6 % (*Enterobacteriaceae*); and *Actinobacteria*, 0.7–16.7 % (*Bifidobacterium*) [1–3]. The composition of the gut microbiota is shaped by multiple factors including diet, medications (such as antibiotics), sanitization, animal exposure, host factors (genetics, pH, transit time, bile acids, digestive enzymes, and mucus production), and bacterial factors (adhesion capacity, the production of specific enzymes, and metabolic activity) [4–6].

The wider recognition of the importance of the human microbiota is reflected in recent international efforts to better characterize its composition. The Human Microbiome Project (HMP) was launched in 2008 with the aim of identifying and characterizing the human microbiomes associated with healthy and diseased humans [7]. Data were derived from a cohort of 242 healthy adults sampled at 15 (male) to 18 (female) body sites (skin, nose, mouth, throat, vagina, and feces) up to three times over 22 months. The result of this multicenter, multisite collective project is the catalogue of 5177 microbial taxonomic profiles from 16S ribosomal RNA genes and over 3.5 terabytes of metagenomic sequence. The HMP complements the Metagenomics of the Human Intestinal Tract (MetaHIT) consortium, which aims to use metagenomic sequencing of fecal DNA from European individuals to characterize and quantify microbial communities specifically within the human gut [8]. Ultimately, these databases will help to construct a reference gene catalogue of gastrointestinal microbes that will be invaluable for understanding the role of the microbiota in health and disease.

There is increasing evidence that the gastrointestinal microbiome influences our health [9]. But how compelling is the evidence that alterations in the microbiota (termed dysbiosis) can cause disease? In this chapter, we will consider whether the gastrointestinal microbiota can induce epigenetic changes in host cells that lead to disease such as obesity and cancer. We will firstly describe the basic mechanisms involved in the epigenetic regulation of gene expression. Following this, the role of the gastrointestinal microbiota in maintaining our health will be discussed. We will then objectively describe the direct and indirect evidence that bacterially derived molecules and bacteria themselves contribute to disease with an emphasis on the mechanisms by which bacteria are associated with signaling, transcriptional, and epigenetic changes. Finally, therapeutic opportunities of modulating our microbiota to treat a range of diseases will be discussed.

## Epigenetic Control of Gene Expression

Epigenetic regulation of gene expression involves a dynamic multilayered network of chromatin modifications. The definition of the term epigenetics has been intensely debated for many years and remains controversial. For the purposes of this chapter,

the term epigenetics refers to the structural adaptation of the genome, without a change in the DNA sequence. By this definition, several chemical modifications can be considered to be epigenetic in nature. These include DNA methylation, histone modifications, the presence of specific histone variants, and the positioning of nucleosomes. These chemical and structural modifications to chromatin can regulate DNA function by affecting DNA accessibility or by serving as docking sites for the recruitment of additional proteins.

The most widely studied chromatin modification is DNA methylation. DNA methylation describes the addition of a methyl ($CH_3$) group to the fifth carbon of a cytosine residue to create 5-methylcytosine (5mC). This usually occurs in the context of cytosines immediately preceding (5′) guanine (CpG methylation). However, it should be noted that methylation of cytosines in other contexts (i.e., CpA, CpT, and CpC methylation) has been described in some cell and tissue types, though this is considered much rarer and its function remains unclear [10]. About 70–80 % of CpG dinucleotides in the mammalian genome are methylated [11]. Regions with a high content of CpG sites, denominated CpG islands, are found at the promoter region of approximately 70 % of genes in the human genome [12]. The function of DNA methylation is context dependent [13]. For example, methylation of CpG island promoter regions correlates with transcriptional silencing of the associated gene [14, 15]. However, high levels of methylation within gene bodies are associated with high gene expression levels [16]. CpG methylation can also regulate interactions between enhancers and promoters over long distances. For example, hypermethylation of the distal enhancers of the *NANOG*/*OCT4* and glucocorticoid receptor promoters prevents them from activating the promoters of these genes [17, 18]. The expression of the imprinted *IGF2* gene is regulated by methylation at a nearby region, which prevents binding of the CTCF insulator protein and allows interaction of the *IGF2* gene promoter with its enhancer [19]. CpG methylation is also important in maintaining genomic stability by suppressing the expression of transposable elements [20, 21]. DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosyl-L-methionine (SAM) to cytosine. Intracellular stores of SAM, also a substrate of various histone methyltransferases, are dependent on the continued supply of vitamin B6, vitamin B12, and folic acid. DNMT1 has a key role in maintenance of methylation throughout the genome and in ensuring that methylation is a long-term and heritable DNA modification. DNMT3A and B are primarily responsible for new (de novo) methylation. In recent years, ten-eleven translocation methylcytosine dioxygenase 1 and 2 (TET1/2) have been identified as active DNA demethylation enzymes [11]. This family of enzymes catalyzes the conversion of 5mC to 5-hydroxymethylcytosine (5hmC), which following oxidation produces 5-formylcytosine (5fC) and 5-carboxyl-cytosine (5caC). The function of these chemical modifications remains unclear, though they are generally rare in adult human cells.

The functional unit of chromatin is the nucleosome, which consists of a histone octamer containing one histone $(H3–H4)_2$ tetramer and two histone H2A–H2B dimers, around which approximately 146 bp of DNA is wrapped [22]. More than 60 different histone modifications have been identified to date. These modifications

combine with other layers of epigenetic modifications to create a variety of functional contexts capable of orchestrating complex processes such as gene transcription, DNA repair, DNA replication, and splicing [23]. Histone acetylation, particularly the acetylation of lysine 9 on histone H3 (H3K9ac), as well as the trimethylation of lysine 4 on histone H3 (H3K4me3) at promoter regions, is associated with gene expression. However, modifications such as H3K9me3 and H3K27me3 are associated with gene silencing. Finally, the precise positioning of nucleosomes is also important in the regulation of gene expression, and nucleosome occlusion of gene promoters or enhancers is associated with the transcriptional silencing of genes [18, 24].

The promoter regions of expressed genes are usually unmethylated and associated with active histone marks such as H3K9ac, H3K14ac, and H3K4me3 (Fig. 7.1a). Transcriptionally silent genes may or may not show promoter hypermethylation, but other markers of transcriptional inactivity include low levels of active histone marks, the presence of the repressive histone marks H3K9me3 or H3K27me3, and nucleosome occlusion of the transcription start site (Fig. 7.1b).

## The Role of the Gastrointestinal Microbiota in Maintaining Health

The human gastrointestinal microbiota has many beneficial activities related to host defense and immunity. These include the displacement of pathogenic bacteria through competition for limited nutrients or cell surface receptors and the production of factors that inhibit the growth of other bacterial species. The gastrointestinal microbiota also helps to shape the host immune system and contributes to epithelial barrier fortification through maintenance of tight junctions (molecular gaskets that seal the cells of the gastrointestinal tract together). Other beneficial activities include the production of essential vitamins, including vitamin K, biotin, and folate, and the metabolism of nondigestible carbohydrates to produce short-chain fatty acids (SCFAs) such as butyrate, which is an important source of energy for colonocytes [25, 26].

Arguably, the shaping of mucosal immunity is one of the most vital roles of the gastrointestinal microbiota. The multitude of bacteria within the gut provides a variety of molecular cues that our immune system relies on to maintain an intact epithelial barrier. These molecular cues are known as microbe-associated molecular patterns (MAMPs). Examples of MAMPs include lipopolysaccharide (LPS), bacterial flagellin, lipoteichoic acid, peptidoglycan, and nucleic acids. To recognize MAMPs, host cells use pattern recognition receptors (PRRs), toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) proteins, and NOD-like receptors (NLRs). These receptors activate signaling cascades leading to transcriptional activation of immunity genes such as cytokine genes [27, 28]. For example, the outer membrane of Gram-negative bacteria contains LPS, which is recognized by Toll-like receptor 4 (TLR4) molecules on the surface of host cells. This induces a signaling cascade through the nuclear factor kappa B (NFkB) and mitogen-activated protein kinase (MAPK) cascades leading to increased transcription of the genes encoding the pro-inflammatory

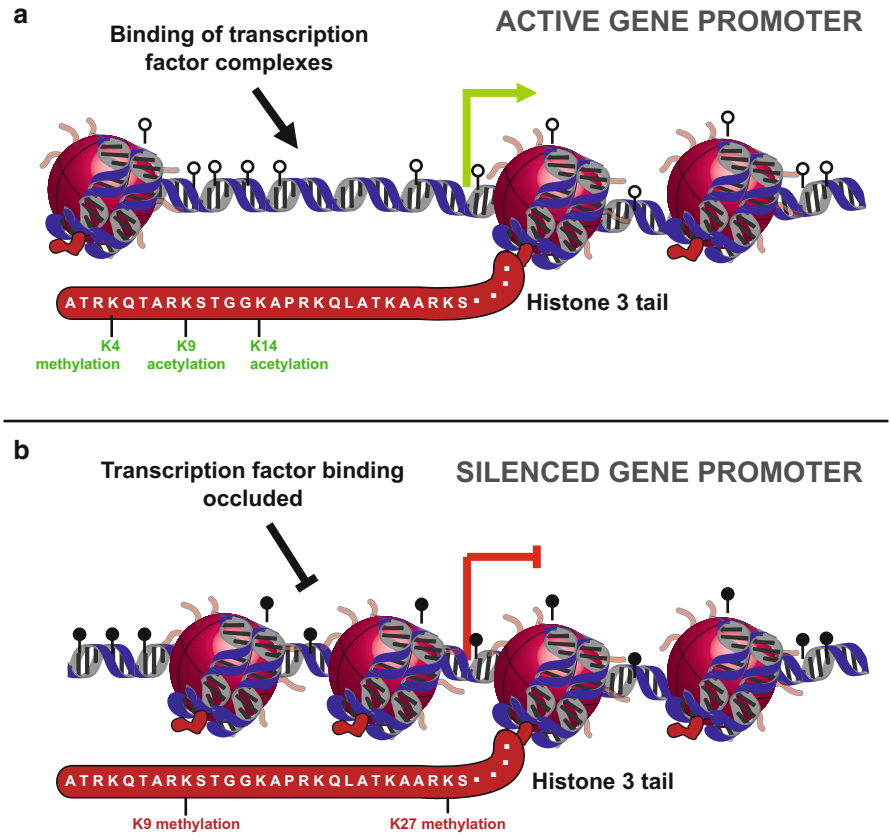


**Fig. 7.1** A simplified schematic of the epigenetic inactivation of CpG island gene promoters. (**a**) The promoters of expressed genes contain unmethylated CpG dinucleotides (*white circles*) and nucleosomes (*red spheres*) that are modified on histone tails (*red lines*) by active histone modifications (*green text*). Active histone modifications include trimethylation of lysine 4 on histone H3 (H3K4me3) and acetylation of lysines 9 and 14 on histone H3 (H3K9/14ac). CpG island promoters are also characterized by a nucleosome-depleted region upstream and encompassing the transcription start site (indicated by the *green arrow*). This nucleosome-depleted region enables access to the promoter region by the transcriptional machinery. (**b**) Epigenetic silencing of genes is often associated with methylated CpG dinucleotides (*black circles*) and nucleosomes that are modified on histone tails by repressive histone modifications (*red text*). Repressive histone modifications include H3K9me3 and H3K27me3. Transcriptional silencing (*blunt-ended red arrow*) is also associated with nucleosome occlusion of the promoter, which prevents access to the transcriptional machinery

cytokines interleukin (IL)-6, IL-12, and tumor necrosis factor (TNF) [27]. Commensal bacteria also induce the production of immunoglobulin A (IgA) from intestinal dendritic cells within mesenteric lymph nodes, which ensure that the intestinal mucosa is primed to protect against local breach of the epithelial layer [29]. Dysregulation of the complex interplay between the immune system and the gastrointestinal microbiota can lead to chronic inflammation, tissue damage, loss of epithelial barrier function, and hyperproliferation through chronic exposure of epithelial cells to pro-tumorigenic inflammatory cytokines.

## Obesity and Cancer Risk

Obesity (defined as a body mass index (BMI) > 30 kg/m$^2$) is a major and potentially preventable cause of cancer [30]. Excess body weight contributes to approximately 3.2 and 8.6 % of all cancers in men and women [31] and is a risk factor for the development of cancers throughout the gastrointestinal and hepatobiliary tract [30]. Obesity is also associated with a worse prognosis following diagnosis of gastrointestinal cancers [32].

The mechanisms by which obesity contributes to cancer risk are likely to be multifactorial; however chronic inflammation and increased systemic levels of pro-inflammatory cytokines, such as insulin and insulin-like growth factor 1 (IGF1), are likely to play a major role [33]. Chronic inflammation is a hallmark of cancer [34], and the risk of cancer accumulates with increasing duration of inflammation [35]. The long-term consequences of chronic inflammation are seen in patients with inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis, which both confer an increased risk of developing colorectal cancer (CRC) [36, 37]. In obese individuals the source of chronic inflammation is partly due to the secretion of various cytokines from increased stores of adipose tissues. These include IL-6, IL-8, C-reactive protein (CRP), TNF, and macrophage migration inhibitory factor (MIF) [30, 38, 39]. In the intestinal epithelium, fecal calprotectin also acts locally to promote intestinal inflammation in obese individuals [40]. Inflammation promotes tumorigenesis by increasing cell proliferation, invasion, migration, and angiogenesis. Inflammation also activates the NFkB and STAT3 transcription factors resulting in the transactivation of genes that suppress apoptosis and promote cell cycle progression [41].

Data concerning DNA methylation changes in obesity remain inconsistent and at most suggest subtle or site-specific differences. Most studies have investigated DNA from peripheral blood lymphocytes (PBLs), with some examining the muscle, placenta, and colon [42]. In obese individuals, total levels of methylcytosine have been reported to be increased, decreased, or not significantly different from controls [43–48]. These studies are compromised by the fact that methylation levels can be altered by many factors including deficiency of the methyl donor folate, gender, ethnic background, age, exposure to chemicals, tobacco smoke, alcohol, and diet [43–46, 48–52]. Given these difficulties in assessing differences in total methylation levels between individuals, many studies have instead focused on specific genes, and several have been reported to be differentially methylated in various tissues from obese individuals [42]. The PPARγ coactivator 1 alpha (*PGC1A*) and pyruvate dehydrogenase kinase (*PDK4*) genes, which are involved in mitochondrial function and fuel utilization, are two particularly interesting examples of genes that are epigenetically regulated in obesity and other metabolic disorders including type 2 diabetes. Methylation within the *PGC1A* promoter negatively correlates with *PGC1A* expression and is acutely inducible by exposure to systemic factors associated with insulin resistance such as TNF or the free fatty acids palmitate and oleate [53]. In the skeletal muscle of obese individuals, methylation within the *PGC1A* promoter

positively correlated with markers of obesity including BMI, CRP, and leptin levels, whereas *PDK4* promoter methylation negatively correlated with these markers [54]. A direct relationship between methylation at these promoters and obesity was shown by the observation that gastric bypass surgery and weight loss restored methylation to nonobese levels [54]. Acute bouts of exercise can also cause transient reductions in *PGC1A* promoter methylation and increased *PGC1A* expression [55]. These studies demonstrate that epigenetic regulation of specific genes is likely to be important in obesity.

Several studies have shown that gastrointestinal dysbiosis is also associated with obesity. For example, a high-fat diet can lead to increased gut *Firmicutes* and decreased *Bacteroidetes* [56]. These alterations may play an important role in obesity-related inflammation [57]. In a recent study, mice fed on a high-fat diet showed alterations in the composition of the gastrointestinal microbiota that resulted in the production of a microbial metabolite known as deoxycholic acid (DCA). Enterohepatic circulation of DCA stimulated the production of a signature profile of inflammatory cytokines, chemokines, and proteases from hepatic stellate cells following exposure to chemical carcinogens resulting in the development of hepatocellular carcinoma (HCC) [57]. Finally, alterations in gut permeability, also often associated with gastrointestinal dysbiosis, can lead to raised systemic levels of LPS, which have been linked to chronic inflammation [58].

## Dysbiosis and Gastrointestinal Cancers

### *Barrett's Esophagus and Esophageal Adenocarcinoma*

Barrett's esophagus is a premalignant lesion of the lower esophagus that is associated with histological changes to the esophageal mucosa and an increased risk of esophageal adenocarcinoma [59]. Differences in the esophageal microbiome have been linked to reflux, esophagitis, and Barrett's esophagus [60]. For example, healthy controls show a predominance of Gram-positive *Firmicutes*, whereas in those affected by reflux, esophagitis, and Barrett's esophagus, the predominant species are Gram-negative *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Spirochaetes* [60]. These differences have been linked to the production of LPS, which triggers TLR4 and NFkB activation and the production of inflammatory cytokines [60].

### *Gastric Cancer*

The most abundant human gastric bacterium phyla are *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* [61]. However, in individuals infected with *Helicobacter pylori*, gastric microbial diversity is reduced, and *Helicobacter pylori* becomes the predominant bacterium [62]. *Helicobacter pylori* is a Gram-negative bacterium that colonizes

the stomach of approximately half of the world's population and represents the most significant risk factor for the development of gastric cancer [63, 64]. Infection occurs via oral-oral or fecal-oral route and may persist throughout the host's life. The World Health Organization (WHO) has classified *Helicobacter pylori* as a class I carcinogen. Cancer risk in *Helicobacter pylori*-infected individuals is influenced by factors such as bacterial strain, host genetics, and specific interactions between the host, microbiota, and environment. Strains containing the cytotoxin-associated gene A (*CagA*) are known as C*agA*+ and are associated with an increased risk of gastric cancer compared to C*agA* strains [65, 66]. The CagA protein becomes tethered to the membrane of host cells and activates several signaling pathways including the MAPK pathway, via recruitment and phosphorylation of SHP2, and the Wnt signaling pathway, via disruption of the interaction between E-cadherin and β-catenin [67]. CagA interacts with several other proteins to disrupt other pathways, including the PI3K, AKT, GSK3β, and STAT3 signaling pathways [68–71]. Some *Cag*+ strains of *Helicobacter pylori* produce a variant of the CagA protein that has been linked to the activation of NFkB and the expression of IL-8 [72]. *Helicobacter pylori* also produces the multifunctional VacA toxin that has been linked to alterations in the gastric epithelium including altered plasma and membrane permeability autophagy, apoptosis, and reduced proliferation of immune T cells, which are important for attenuating inflammatory responses [73].

## *Colorectal Cancer*

Numerous reports have suggested that dysbiosis occurs in the colon of CRC patients including an overall decrease in the diversity of species present [74–76]. Specific bacterial species that have been implicated are *Bacteroides*, *Streptococcus gallolyticus*, *Helicobacter pylori*, *Escherichia coli*, *Clostridium leptum*, *Coriobacteridae*, *Clostridium coccoides*, and *Fusobacterium* [74, 76–82] which are all elevated relative to healthy individuals. High levels of *Fusobacterium* in the colon of colorectal cancer patients are the most consistently reported finding. Decreases in butyrate-producing bacteria have also been described in the colon of CRC patients [1, 83, 84]. The wide range of bacterial species linked to the development of CRC most likely reflects the likelihood that multiple species play a role [77] but also the difficulties in defining the composition of a "normal" microbiome. Changes in colonic microbiome diversity in patients with adenomas compared to those without adenomas [85] possibly indicate that dysbiosis occurs early in the development of neoplasia.

Numerous theories have been proposed to explain how dysbiosis can lead to CRC. These include the increased production of harmful by-products such as amines, phenols, reactive oxygen species (ROS), and reactive nitrogen species (RNS) that lead to increased DNA damage in colonocytes [77, 86]. Other possibilities include

the production of bacterial toxins that also cause DNA damage or alterations in MAMPs leading to chronic inflammation [77]. Recently, it was shown that inflammatory bowel disease (IBD), a known risk factor for colorectal cancer [36, 37], is associated with the accumulation of invariant natural killer T immune (iNKT) cells in the colonic mucosa in germfree mice and hypermethylation of a region upstream of the *Cxcl16* gene [87]. The importance of Cxcl16 in this process was demonstrated using a Cxcl16 neutralizing antibody, which attenuated iNKT cell accumulation [87]. However, neonatal colonization with commensal microbiota provides protection against this accumulation, which is associated with reduced inflammation and hypomethylation of the *Cxcl16* gene [87]. Interestingly, in this study, hypermethylation of the region upstream of the *Cxcl16* gene was associated with upregulation of *Cxcl16* expression, possibly suggesting the inactivation of a repressor or chromatin insulator domain. Another example of the link between the inflammation, the gastrointestinal microbiota, and the development of cancer is described by Arthur et al. [78]. In this study, *IL-10* knockout mice (*IL-10*$^{-/-}$) developed colitis, showed increased abundance of the commensal *Escherichia coli*, and were susceptible to CRC following exposure to the chemical carcinogen azoxymethane in 80 % of cases. However, the same mice colonized with the commensal *Enterococcus faecalis* rarely develop tumors [78]. This shows that inflammation is crucial to the development of some CRCs and that commensal bacteria can modify the risk of developing cancer.

## A Striking Example of Bacteria-Associated Epigenetic Reprogramming

What's the evidence that dysbiosis can induce epigenetic changes? A striking example of bacteria-associated epigenetic changes is the reprogramming of adult human Schwann cells following infection with *Mycobacterium leprae*, which causes human leprosy. Infection with the leprosy bacterium transcriptionally silences Schwann cell differentiation genes, such as *Sox10*, and upregulates mesodermal-specific genes and master regulators of epithelial-mesenchymal transition, such as *Twist1*, *Prrx1*, *Tbx18*, and *Bmp6* [88]. These changes in gene expression were accompanied by hypomethylation of the *Twist1*, *Prrx1*, *Tbx18*, and *Bmp6* and by hypermethylation of the S*ox10* gene promoters in reprogrammed cells [88]. Furthermore, Schwann cell reprogramming actually helps promote bacterial dissemination between host cells by promoting the local migration of cells and through the formation of bacteria-laden granulomas within recruited macrophages [88]. Though these changes in gene expression and DNA methylation are driven by an intracellular pathogen, the reprogramming of Schwann cells by *Mycobacterium leprae* is a stark illustration that bacteria can induce epigenetic alterations or indeed entirely reprogram host cells.

## Dysbiosis-Associated Epigenetic Changes in Gastrointestinal Cancers

Epigenetic changes have been investigated extensively in the context of *Helicobacter pylori*-associated gastric cancer. A study of gastric biopsy samples collected from patients infected with *Helicobacter pylori* showed that chronic gastritis was associated with promoter hypermethylation of the DNA repair gene *MGMT* [89]. Others have reported hypermethylation at several other gene promoters in gastric mucosa biopsies from *Helicobacter pylori*-infected patients including the *CDH1* [90] and *MLH1* [91] genes. Interestingly, the *CagA*-positive strain of *Helicobacter pylori*, which induces a more pronounced inflammatory response in the gastric mucosa [92], is associated with the highest frequency of *MGMT* hypermethylation, and eradication of *Helicobacter pylori* infection in these patients results in reduced *MGMT* methylation [89], indicating that hypermethylation of specific genes may, at the very least, be a marker of disease severity. Chronic *Helicobacter pylori*-associated gastritis is also associated with the secretion of a protein known as HP0175 that induces the expression and secretion of IL-6 from macrophages [93]. This increase in IL-6 expression was induced through the activation of the NFkB and MAPK pathways via the TLR4 receptor and was associated with the phosphorylation of serine 10 on histone H3 within the IL-6 gene promoter [93]. Increases in cyclooxygenase 2 (COX-2) expression also accompany *Helicobacter pylori* infection [94]. This increase in expression is associated with epigenetic alterations around the COX-2 gene transcriptional start site including H3 acetylation and H3K4 dimethylation, decreased H3K9 dimethylation and H3K27 trimethylation, and rapid cyclical DNA methylation/demethylation at eight CpG sites [94].

Numerous genes become epigenetically inactivated by promoter hypermethylation in the progression of Barrett's esophagus to esophageal adenocarcinoma [95], including genes involved in the regulation of the cell cycle (*CDKN2A*) [96, 97], DNA repair (*MGMT*) [98], Wnt signaling (*SFRP1* and *WIF1*) [96], and apoptosis (*DAPK1* and *RUNX3*) [99, 100]. It's unclear whether these epigenetic changes are a cause or consequence of the neoplastic process, and to date, no studies have investigated whether specific changes in the microbiota are linked with specific epigenetic alterations.

In the colon, the production of the SCFA butyrate from dietary polysaccharides is performed by *Firmicutes* such as *Lachnospiraceae* and *Ruminococcaceae*. The production of butyrate in the colon contributes approximately 5–15 % of the total caloric requirements in humans [101]. Colonocytes obtain about 70 % of their energy from the metabolism of butyrate [102]. Butyrate has differential effects on the survival of normal colonocytes and colorectal cancer cells (Fig. 7.2), inhibiting growth and inducing apoptosis in the latter [102–104]. These differential effects are explained by the Warburg effect in cancer cells, which metabolize relatively little butyrate and instead use glucose as their primary energy source [105]. Consequently, butyrate accumulates within cells and acts as an inhibitor of histone deacetylases (HDACs). It has been known for some time that the inhibition of HDACs induces multiacetylated

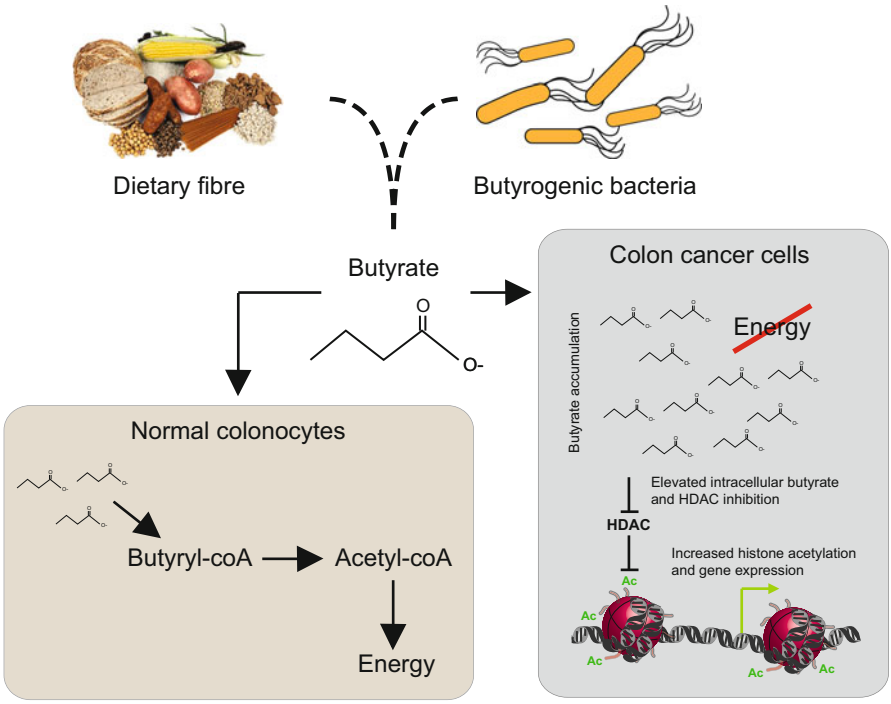


**Fig. 7.2** The different effects of microbially derived butyrate on normal colonocytes and cancer cells. Butyrogenic bacteria such as *Firmicutes* produce butyrate from dietary polysaccharides. Colonocytes derive the majority of their energy from butyrate. However, cancer cells derive most of their energy from glycolysis and lactic acid fermentation, leading to an accumulation of butyrate. At higher concentrations butyrate acts as an inhibitor of histone deacetylases (HDACs). The reduced activity of HDACs leads to the accumulation of hyperacetylated histones (Ac), which may increase the expression of specific genes

forms of histones H3 and H4 [106] leading to the transcriptional upregulation of specific genes (Fig. 7.2). A study that administered butyrate daily for two weeks by enema (at concentrations that can be achieved by consumption of a high-fiber diet) found alterations in the expression of over 500 genes in human colonic mucosa [107]. Several components of the Wnt signaling pathway have been shown to become hyperactivated in response to butyrate as well as synthetic inhibitors of HDACs [103, 108]. Recently, however, the suppressive effects of butyrate on cancer cell growth have been challenged by a study showing that it can also act as an oncometabolite that drives proliferation of tumor cells [109]. This disparity may be explained by the degree of dependence of cells on aerobic glycolysis, host genetic background, or the presence of other bacterial metabolites such as the omega-3 fatty acid docosahexaenoic acid, which synergizes with butyrate to induce apoptosis [110].

Commensal microbe-derived butyrate has been reported to ameliorate colitis (an inflammatory bowel condition) by inducing the differentiation of colonic regulatory T cells [111]. Regulatory T cells are crucial in the suppression of inflammatory or

allergic responses in the colon. The mechanism by which butyrate does this is likely due to increases in acetylation at the forkhead box p3 (*Foxp3*) gene, which is a critical marker of regulatory T cell differentiation [111]. In another study, Donohoe et al. [105] demonstrated that mice colonized with *Butyrivibrio fibrisolvens* (a butyrate-producing bacteria) that are fed a high-fiber diet were protected against azoxymethane/dextran sodium sulfate (AOM/DSS)-induced CRC. Similarly, in this study, protection against CRC was also observed in mice fed a tributyrin-fortified diet. However, protection was lost in mice colonized with a mutant form of *Butyrivibrio fibrisolvens*, which produced sevenfold less butyrate [105], thereby providing strong evidence that high levels of butyrate protect against CRC.

Elevated levels of folic acid have previously been linked to the development of colorectal neoplasia, and folic acid supplementation has been reported to cause increases in DNA methylation in leukocytes and colonic mucosa in humans [112]. However, the accuracy of the in vitro methyl acceptance assay [113] used to assess global methylation levels has since been questioned after the development of more quantitative mass spectrometry-based methods [114, 115]. Daily diet is the primary source of folate in humans; however, folate-synthesizing bacteria such as *Bifidobacterium* spp. in the human gut also contribute to adequate folate supply [116]. High doses of folinic acid are associated with mild increases in methylation at the *Cxcl16* gene in mouse models of IBD [87]. However, simply correlating the levels of folate with DNA methylation levels is unlikely to be informative. This is because folate exists as functionally distinct coenzyme species that are required for DNA methylation (5-methyltetrahydrofolate) or DNA synthesis (5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate) [114]. A recent study of global methylation in normal colorectal mucosa from colorectal cancer patients and healthy individuals demonstrated that the relative abundance of these different folate species determines methylation levels rather than total folate levels [114]. Consequently, future studies should investigate whether dysbiosis is associated with changes to the relative levels of the different folate species in the colorectal epithelium.

In intestinal macrophages, bacterially derived LPS can induce the expression of inflammatory mediators such as IL-12. This induction of IL-12 expression involves the activation of p38α MAPK and TLR4 signaling, phosphorylation and acetylation of histone H3 serine 10 and lysine 14, and nucleosome eviction from the IL-12 gene promoter [117, 118]. This chromatin remodeling is essential for the binding of NFkB to the IL-12 gene promoter [119]. However, prolonged expression of inflammatory genes can cause a phenomenon named LPS or endotoxin tolerance, characterized by epigenetic changes such as methylation of H3K9 and reduced phosphorylation of H3S10 at inflammatory gene promoters [120].

## Clinical Implications of Microbiota Intervention

*Dietary intervention.* Though host genetics plays an important role in shaping the microbial census of mice [121] and humans [122], evidence now suggests that dietary changes overrule genotype-related differences [123]. For example, a study of

the effects of two distinct diets (a high-fat, high-sugar diet and a low-fat, high-plant polysaccharide diet) on the gastrointestinal microbiota of five genetically distinct inbred mouse strains showed that a high-fat, high-sugar diet led to reproducible shifts in fecal microbiota composition [124]. Importantly, mice used in this study included strains deficient for genes with an established role in shaping the gut microbiome (*Myd88–/–*, *Nod2–/–*, ob/ob, and *Rag1–/–*), which provides strong evidence that genetic influence was secondary to that of diet [124]. These findings are not wholly unsurprising given that monozygotic twins still exhibit a substantial degree of microbiome individuality [122] and that isogenic mouse strains exhibit substantial intergenerational variation in their gastrointestinal microbiomes [125]. Studies such as these suggest our diet can be used to manipulate the composition of our gastrointestinal microbiota. However, dietary manipulation must be carefully considered. An illustration of this is the use of *Lactobacilli*-containing food supplements, which may have beneficial effects in patients with type 2 diabetes [126] but can result in weight gain [127].

When compared with a normal diet, a high-fat diet leads to increases in the abundance of *Enterobacteriaceae*, *Desulfovibrionaceae*, *Porphyromonadaceae*, *Rikenellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Coriobacteriaceae*, and *Deferribacteraceae* and significant decreases in the abundance of *Bifidobacteriaceae*, *Peptostreptococcaceae*, *Roseburia*, and *Butyricicoccus* in the colon [128]. These alterations occur independently of obesity and can induce tumor progression in the small intestine of mice susceptible to cancer [128]. The mechanism by which this high-fat diet promotes cancer is unclear, but one theory is that dysbiosis associated with a high-fat diet alters the intestinal mucosal barrier resulting in intestinal inflammation [128].

## *Prebiotics and Probiotics*

Gibson and Roberfroid defined prebiotics as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon that have the potential to improve host health" [129]. This definition includes nondigestible oligosaccharides (e.g., inulin, fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS)), poorly digested carbohydrates, certain fibers (e.g., wheat bran), and resistant starches [130, 131]. One benefit of dietary supplementation with prebiotic fiber is a potential shift in the gut microbiota composition toward butyrogenic strains. However, evidence that prebiotics help to maintain health and prevent chronic diseases in humans is limited, and neither the US Food and Drug Administration (FDA) nor the European Food Safety Authority (EFSA) have established definitions for prebiotics.

More recently, using deep metagenomic sequencing, we found that prebiotic feeding not only affects gut microbiota at the taxonomic level but also profoundly changes metabolic functions of the gut microbiota during both normal diet and high-fat diet (HFD) feeding [35]. A total of 20 genera were significantly affected by

the HFD compared to the control diet, whereas prebiotic treatment mitigated the impact of HFD on gut microbiota composition and metabolic functions, along with host metabolic parameters, such as obesity, diabetes, and inflammation [35].

The term probiotic is currently defined as a living microorganism, which upon ingestion in certain numbers, exerts health benefits beyond that of general nutrition [130, 132]. Most strains of probiotics are lactic acid bacteria (LAB), *Lactobacillus*, and *Bifidobacterium*, but *Enterococcus*, *Streptococcus thermophilus*, *Escherichia coli*, and *Saccharomyces boulardii* may also be present [130, 133, 134]. Probiotics have been reported to modulate inflammation, the immune system, and intestinal permeability [135]. Probiotics may show benefits in alleviating the symptoms of lactose intolerance, ulcerative colitis, atopic eczema, constipation, diarrhea, hypercholesterolemia, and drug-induced colitis [130, 134]. Importantly however, probiotics are transient members of the microbiota for the period during which they are administered or for a short period afterward. They do not become part of the established normal microbiota [136] meaning that they must be continually ingested. The possibility of treating CRC patients with probiotics has been investigated in large-scale clinical trial studies. For example, evaluation of 2–4 years administration of *Lactobacillus casei* in 380 individuals [137] showed that the incidence of tumors with a grade of moderate atypia or higher was significantly lower in the probiotic-treated group compared to controls. More recently, a 12-year follow-up study with 45,241 volunteers reported that intake of yogurt containing more than 107 organisms per gram of viable *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* was associated with a decrease in the incidence of CRC [138].

Synbiotics are a mixture of prebiotics and probiotics that improve the survival and colonization of a probiotic and that beneficially affect the host [129]. A recent systematic review has suggested that synbiotics may also help to prevent CRC [139].

## *Genetically Modified Bacteria*

Tumors often contain regions of hypoxia that may provide a niche for the growth of anaerobic bacteria [140]. Anaerobic bacteria produce high levels of butyrate suggesting that the presence of certain bacterial species may provide a mechanism to deliver localized butyrate to tumor sites [141]. Taking this concept of targeted delivery a step further, several studies have shown that genetically engineered *Bifidobacterium* can serve as a delivery system for the treatment of breast cancer [142, 143].

Bacterial strains including *Lactobacillus casei* and *Lactococcus lactis* that have been engineered to produce a protein called elafin may help to decrease inflammation in mouse models of colitis. Ex vivo culture of cells from the inflamed mucosa of human colitis patients shows that cytokine production and cell permeability can be attenuated by the presence of these engineered bacteria [144]. An engineered strain of *Lactobacillus gasseri* that overexpresses the antioxidant superoxide dismutase has also been shown to diminish colitis in an *IL-10* knockout mouse model [145].

## The Potential Role of the Gastrointestinal Microbiota in Modifying Response to Cancer Therapies

Two recent studies have suggested that the composition of the gastrointestinal microbiota may determine how effective specific cancer therapies are. For example, oral administration of *Bifidobacterium* suppressed tumor growth to the same extent as an antibody cancer therapy known as programmed cell death protein 1 ligand 1 (PD-L1), and the two together almost abolished tumor growth [146]. Response to another cancer immunotherapy, this time targeting the cytotoxic T-lymphocyte-associated protein **4** (CTLA-4), is also mediated by certain bacteria in the *Bacteroides* and *Burkholderia* genera [147]. Though these studies were performed in mice, they raise the possibility that modifying the microbiota in humans may enhance cancer therapies and produce responses in patients who might otherwise not respond.

*Antibiotics.* Several studies have shown that gastrointestinal polyps can be driven by gut microbiota-induced inflammation and that treatment with antibiotics can reduce the number of polyps that develop [109, 148, 149]. However, manipulating the gut microbiome should be considered with caution and may have unforeseen consequences. For example, prolonged antibiotic treatment cannot only lead to antibiotic-resistant strains of *Enterobacteriaceae* [150] and *Staphylococcus epidermidis* [151] but will undoubtedly reshape commensal microbiome profiles and potentially ablate protective bacterial species that prevent pathogenic infection [152]. The progressive eradication of *Helicobacter pylori* has reduced the risk of gastric cancer as intended, but this has been associated with an increased risk of developing gastroesophageal reflux disease, Barrett's esophagus, and esophageal adenocarcinoma [153]. A potential solution to the off-target effects of antibiotic treatment is to use bacterium-targeting viruses known as bacteriophages to target specific cancer-associated strains of bacteria [154].

*Fecal microbiota transplantation (FMT).* One approach to modifying the gastrointestinal microbiota is to inoculate the gastrointestinal tract of an individual with the microbiota from a healthy person. This therapeutic approach, known as FMT, is an effective treatment for recurrent *Clostridium difficile* infection and has shown promise in the treatment of IBD and obesity [155]. The inoculation of lean germfree mice with gastrointestinal microbiota from obese mice results in fat gain in recipients [156], whereas in humans, FMT from lean to obese volunteers can result in improved insulin sensitivity concomitant with changes in the composition of the gastrointestinal microbiota [157].

*Screening and early diagnosis.* Some studies have investigated the potential of gastrointestinal microbiota profiling as a screening tool for early-stage disease, including colorectal cancer [158, 159]. These studies have shown that different microbial profiles can be detected in stool samples from patients with colorectal adenomas or cancers when compared with healthy individuals. When combined with known clinical risk factors for colorectal cancer, such as body mass index, age, and race, gut microbiota profiles significantly improved the ability to distinguish healthy, adenoma, and cancer groups when compared with each risk factor alone [158].

However, a recent study showed that bacterial populations within the colonic lumen (i.e., within the feces) are not representative of the bacterial within the mucosal compartment [160]; therefore, caution must be exercised when interpreting results.

## Conclusions

Undoubtedly the gastrointestinal microbiota is a significant factor in maintaining our health, and dysbiosis is associated with several diseases. In this chapter we have focused on some specific examples of disease-associated microbial changes and, where possible, the molecular mechanisms involved. In doing this, we have shown that dysbiosis can be associated with epigenetic changes; however, the mechanisms differ between host site, the bacterial species involved, and the pathological condition being studied. Surprisingly few studies have attempted to identify specific epigenetically altered genes or to attempt to determine whether dysbiosis and the associated epigenetic alterations are a cause or consequence of disease. In the vast majority of cases, these remain open questions. The potential therapeutic implications of modifying our gastrointestinal microbiota are staggering and could have broad implications for cancer therapy, stemming the obesity epidemic and diagnostic screening to name a few.

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# Chapter 8
# Epigenetics in Obesity and Esophageal Cancer

**Andrew M. Kaz and William M. Grady**

**Abstract** Barrett's esophagus (BE) is a metaplastic condition that is believed to develop in the esophagus due to chronic acid reflux and can progress to esophageal adenocarcinoma (EAC). Obesity, a well-known risk factor for BE and EAC, may increase the risk for BE/EAC indirectly by increasing the frequency of gastro-esophageal reflux and directly through obesity-mediated inflammation and the secretion of cancer-promoting molecules by adipocytes. Epigenetic alterations, which are commonly seen in BE and EAC, have been associated with chronic inflammation in the esophagus and also with obesity in other tissues. There is emerging evidence that elevated BMI is associated with the altered DNA methylation observed in BE, dysplastic BE, and EAC tissues. There is also some suggestion that genes involved in cancer-related pathways and pathways implicated in obesity-related cancers and adipose-mediated inflammation (insulin, IGF-1) demonstrate altered methylation in obese individuals. Thus, obesity appears to influence the formation and progression of BE to EAC via epigenetic mechanisms.

**Keywords** Barrett's esophagus • Esophageal adenocarcinoma • DNA methylation • Obesity • Gastro esophageal reflux disease

## Abbreviations

| | |
|---|---|
| BE | Barrett's esophagus |
| EAC | Esophageal adenocarcinoma |
| LGD | Low-grade dysplasia |
| HGD | High-grade dysplasia |

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|---|---|
| SQ | Squamous esophagus |
| FFPE | Formalin-fixed paraffin-embedded |
| HM450 | HumanMethylation450 |
| UTR | Untranslated region |
| DML | Differentially methylated loci/locus |
| DMR | Differentially methylated region |
| BMI | Body mass index |
| NCI-PID | National Cancer Institute Pathway Interaction Database |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| GO | Gene ontology |

## Introduction

Esophageal adenocarcinoma (EAC) is thought to develop from a pre-cancerous condition of the esophagus called Barrett's esophagus (BE). BE is a metaplastic condition where specialized intestinal columnar epithelium replaces the normal squamous epithelium in the esophagus [76]. BE is suspected when a salmon pink-colored mucosal lining is visualized in the tubular esophagus during an endoscopic examination and is confirmed by histopathological evaluation demonstrating intestinal-type epithelium in biopsies obtained proximal to the gastro-esophageal junction (GEJ) [78]. Clinically, BE is important because it is recognized as the precursor to esophageal adenocarcinoma (EAC); although the absolute risk of BE progression is low (approximately 0.2 %/year), individuals with BE have an approximately 25× increased risk of developing EAC compared to the general population [76].

The incidence of EAC has been rapidly increasing in the US for reasons that are not entirely clear [6], but might be related to an increase in the prevalence of known risk factors for BE and/or EAC. Risk factors for EAC include smoking, overweight and obesity, central adiposity, and chronic gastroesophageal reflux disease, which is thought to trigger BE [73].

EAC appears to arise via a metaplasia-dysplasia-carcinoma sequence where Barrett's metaplasia progresses through low- and high-grade dysplasia to invasive carcinoma [76]. Although the molecular and genetic events involved in initiation and progression of BE/EAC are still being investigated, certain histologic and associated genetic alterations have been described for EAC [21, 28, 58, 70, 71, 79, 88, 90]. Epigenetic alterations, mainly aberrant DNA methylation, have also been demonstrated to frequently occur in BE and EAC [11, 43, 50, 81]. Some epigenetically altered genes are known tumor suppressors, and in some cases, aberrant methylation is predicted to play a causative role in the pathogenesis of these EACs. Additionally, some of these aberrantly methylated genes (e.g., *p16, RUNX3, HPP1, NELL1, TAC1, SST, AKAP12*, and *CDH13*) might be useful prognostic markers to predict the progression of BE to EAC [11, 43].

Although elevated body mass index (BMI) has been associated with altered DNA methylation in several prior studies [17, 33, 37, 60], there is currently little information on the relationship between obesity, epigenetic alterations, and BE/EAC. In this chapter, we will: (1) briefly review epigenetic alterations in BE and EAC, (2) describe the relationship between obesity, gastro-esophageal reflux, and esophageal inflammation, and (3) further explore the relationship between obesity and epigenetic alterations in these conditions.

## Epigenetic Alterations in DNA methylation in BE and EAC

Previous studies have evaluated global patterns of DNA methylation in BE and EAC and found that epigenetic alterations occur in BE tissue before the development of dysplasia or cancer [93]. Xu et al. noted that both BE ($N=77$) and EAC ($N=117$) samples were highly methylated compared to normal squamous esophagus ($N=94$), providing evidence that DNA hypermethylation occurs early in the BE to EAC progression sequence. Another study used microarrays to compare DNA methylation between BE and EAC samples and found that methylation alterations in certain genes could distinguish between BE, BE with dysplasia, or EAC [3]. The authors found a panel of genes (*SLC22A18*, *PIGR*, *GJA12*, and *RIN2*) could accurately discriminate BE from dysplasia/EAC and could stratify patients into low-, intermediate-, or high-risk groups based on DNA methylation patterns.

Distinct genome-wide methylation patterns can be found when comparing normal squamous esophagus (SQ), BE, dysplastic BE, and EAC samples [50]. Additionally, there appear to be subsets of both BE and EAC that demonstrate relatively high methylation levels compared to other BE or EAC cases. Previously, Kaz et al. examined BE and EAC tissue samples using methylation microarrays and found there were subgroups with distinct methylation signatures (high and low methylation epigenotypes), suggesting that there may be a CpG Island Methylator Phenotype (CIMP) molecular class of BE and EAC [50]. This observation needs to be verified with additional studies.

Altered methylation of promoter CpG islands, which is associated with gene silencing in some cases, has been shown to occur frequently in BE, dysplastic BE, and EAC. Studies evaluating the methylation status of several dozen candidate genes that are epigenetically altered in other cancers have been evaluated in BE and EAC. One of the first tumor suppressor genes shown to be aberrantly methylated in BE was *CDKN2A/p16*, which normally inhibits CDK-mediated phosphorylation of the Rb protein and inhibits cell cycle progression. *CDKN2A/p16* promoter hypermethylation combined with 9p21 chromosomal loss leads to inactivation of this gene in some cases of EAC or BE with dysplasia [52, 91]. CpG island hypermethylation of the *CDKN2A/p16* promoter ranging from 3 to 77 % of BE cases has been reported in several publications, suggesting that *CDKN2A/p16* methylation is in early event in BE pathogenesis [5, 23, 85, 92].

Other candidate tumor suppressor genes, such as *APC*, *ESR1*, and *CDH1,* also show aberrant promoter CpG island methylation in esophageal samples. In a study evaluating 107 distinct spatial locations in six esophagectomy specimens, which contained both BE and EAC, spatial methylation maps were created to define methylation patterns in the BE and adjacent EAC [22]. Eads et al. found a high incidence of methylated *ESR1*, *APC,* and *CDKN2A* in different sites of individual cases of BE, BE with dysplasia, and EAC in a pattern suggesting simultaneous methylation in large contiguous fields or clonal expansion of cells that acquired methylation early in the BE → EAC progression sequence [22]. Similar patterns of widely distributed genetic alterations consistent with clonal expansion in BE have been reported in studies that focused on LOH events or mutations of *APC*, *TP53*, and *CDKN2A/p16* [4, 67, 92].

Other groups have focused on altered *APC* and *CDH1* methylation in BE and EAC as well [7, 49]. In a study of 52 patients with BE and EAC, Kawakami et al. found hypermethylated *APC* in 39.5 % of cases of BE and 92 % of EAC cases, but not in matched normal esophagus. When they looked at plasma samples from these patients, they could detect methylated *APC* in 25 % of EAC patients; this was associated with reduced survival [49]. Meanwhile, Smith et al. found high levels of methylated *APC* in >95 % of BE and EAC tissues studied, supporting the concept that aberrant methylation of putative tumor suppressor genes occurs early in the BE → EAC sequence [74].

Numerous other genes implicated in carcinogenesis have been found to be aberrantly methylated in their CpG island promoter regions in BE/EAC, including the STAT-induced STAT inhibitors (SSIs), suppressors of cytokine signaling (*SOCS-1* and *-3*) and Reprimo (*RPRM*), and members of the glutathione S-transferase (GST) and glutathione peroxidase (GPX) family [34, 65, 81]. Jin et al. identified aberrant methylation of somatostatin (*SST*), tachykinin-1 (*TAC1*), *NELL1*, and *CDH13* and noted that the incidence of methylation of these genes was higher in BE, BE with dysplasia, and EAC vs. normal esophageal samples [44–48]. These investigators further showed that treatment of esophageal cell cultures with the demethylating agent 5-aza-deoxycytidine caused increased mRNA expression levels of these hypermethylated genes, supporting the association between methylation and transcriptional repression. It is clear from these and other studies that, in general, aberrant methylation of genes is detectable in cases of BE without dysplasia, suggesting that many of the epigenetic alterations that occur in EAC are already present in BE.

## The Role of Obesity in Chronic Gastroesophageal Reflux Disease (GERD) and Esophageal Inflammation

Gastro-esophageal reflux is a strong risk factor for both esophageal inflammation and BE [66, 87]. Reflux of gastric acid and enzymes as well as bile from the duodenum are likely responsible for inducing an inflammatory response in the esophagus that is associated with Barrett's metaplasia [29, 41]. GERD and reflux esophagitis have been associated with the presence of pro-inflammatory and pro-tumorigenic cytokines, including IL-8 and IL-1 beta, which are discussed in more detail below [27].

Obesity, in particular central adiposity, is also a well-documented risk factor for developing both BE and EAC [13, 14, 53, 87]. The current guidelines for selecting individuals to screen for BE reflect this risk; those with multiple risk factors for EAC, including elevated BMI and intra-abdominal distribution of body fat, are advised to undergo screening for BE with upper endoscopy [77]. The mechanism(s) by which obesity and/or central adiposity lead to the development of BE and EAC are not well-understood. One common hypothesis is that elevated BMI increases the intragastric pressure, which promotes gastroesophageal reflux, although the relative importance of this mechanism is debated [24, 25, 55]. Other related mechanisms of BE/EAC development associated with obesity and GERD include the presence of an increased gastroesophageal pressure gradient and anatomic disruption of the normal gastroesophageal junction [63].

Yet the association between obesity and BE/EAC has been shown to persist even in the absence of GERD. A recent meta-analysis that found a positive association between obesity and BE/EAC noted that obesity remained a risk factor for BE even after controlling for GERD (adjusted odds ratio (OR) = 2.04, 95 % confidence interval (CI) = 1.44-2.90) [73].

It is worth noting that BE and EAC are much more common among males: although GERD occurs with similar frequency among women and men, BE and EAC are roughly twice and seven times more common in men than in women, suggesting gender-related factors play a role in the formation of both BE and EAC [80]. A biologic explanation to explain this paradox is that the adipocytes themselves, which are metabolically active, might promote the development of BE and EAC in men preferentially [24]. Adipocytes located within the visceral compartment (mesentery and omentum) are more metabolically active than subcutaneous fat cells. This might account for the finding that central obesity, more than overall body weight, is a major risk factor for BE and EAC [12]. Thus, the fact that BE is more common in men than women might also be explained in part by the finding that male-pattern obesity is associated with excess abdominal adipose, i.e., an increased waist-to-hip ratio (WHR) [16]. For example, a case-control study by Edelstein et al. compared measures of central adiposity in a group of patients with newly diagnosed BE compared to matched controls and found high WHR was associated with a BE risk (OR = 2.4, 95 % CI = 1.4–3.9) [24].

Metabolically active intra-abdominal fat may lead to alterations in the expression of hormones, cytokines, and adipokines, which in turn may lead to the development of clinical metabolic disorders including insulin resistance and type 2 diabetes [35, 95]. Altered levels of these metabolically active substances have also been associated with the promotion of cancer, possibly related to their pro-inflammatory effects and their effects on angiogenesis, insulin signaling, apoptosis, and metastasis [31]. Leptin and proinflammatory cytokines, which are produced by visceral adipocytes, have been linked to inflammation seen in BE samples [61], and these cytokines have been shown to inhibit apoptosis and increase proliferation in BE and EAC cell lines [62].

Other adipokines, including free fatty acids, produced by adipocytes can lead to the development of insulin resistance which is associated with high blood insulin levels and the promotion of tumor formation [8, 86, 94]. In another case-control study of 284 newly diagnosed BE patients compared to 294 GERD control subjects and 285 population control subjects, adiponectin levels were positively associated with the risk of BE among patients with GERD [2]. However, another recent study evaluated 135 BE patients, 133 refractory GERD patients, and 1157 control subjects using multivariate logistic regression models for waist-to-hip ratio and found an inverse relationship between serum adiponectin levels and BE (tertile 1 vs. tertile 3 OR = 0.42, 95 % CI = 0.22–0.80) [30]. A meta-analysis, which included both of these studies, found that total serum adiponectin was not associated with the risk for BE compared with GERD control subjects (OR = 1.20, 95 % CI = 0.69–2.10) or population control subjects (OR = 0.79, 95 % CI = 0.46–1.34) [10]. However, this meta-analysis did find a relationship between BE and serum leptin levels (OR = 2.23, 95 % CI = 1.31–3.78) and serum insulin levels (OR = 1.74, 95 % CI = 1.14–2.65) when men and women were considered together or separately [51]. Thus, there is compelling but inconsistent evidence for adiponectin, insulin, and leptin affecting the formation and progression of BE/EAC; additional investigation will be important to produce a clearer understanding of their role(s).

## Inflammation and Epigenetic Alterations in BE and EAC

Chronic inflammation is a predisposing factor for malignant transformation, and it has been estimated that roughly 25 % of all cancers are associated with chronic infection and/or inflammation [38]. Chronic inflammation in the esophagus might promote Barrett's metaplasia via induction of transcription factors such as *CDX1* and *CDX2*, which play critical roles in intestinal development [32]. Increased expression of these genes has been shown in BE and EAC tissues, but they are not expressed in the normal squamous esophagus nor in the gastric epithelium [32]. Exposure of esophageal cells to bile acid and hydrochloric acid, which is present in gastroesophageal refluxate, has been shown to activate the *CDX* promoters in esophageal cell lines, and CDX expression can be found in the chronically inflamed squamous esophagus as well as in metaplastic BE [75]. Therefore, it appears that chronic acid/bile reflux into the esophagus stimulates CDX expression which might mediate the development of BE.

Inflammation is known to promote direct DNA damage and genetic alterations, but has also been shown to affect DNA expression via epigenetic mechanisms such as DNA methylation [54]. For example, hypermethylation of *CDH1*, the gene for E-cadherin, and *CDKN2A/p16*, which has been demonstrated in metaplastic gastric mucosa of individuals infected with *Helicobacter pylori*, is thought to be a driver of gastric carcinogenesis [9, 57]. The mechanism mediating aberrant DNA methylation at sites of chronic inflammation is thought to be related to the release of HOCl and HOBr by neutrophils and eosinophils, which in turn leads to the production of 5-methylcytosine and 5-bromocystosine [82]. Because neither DNA methyl transferase-1 (DNMT-1) nor methyl-binding proteins are easily able to distinguish these 5-halocystosines from 5-methylcytosine, inappropriate de novo methylation may occur during DNA replication in the setting of inflammation [54, 82].

The inflammatory milieu seen in the esophagus of individuals with BE and EAC likely promotes both genetic and epigenetic alterations. As noted previously, numerous genes have been shown to be aberrantly methylated in BE and/or EAC, and altered DNA methylation appears to be any early event in the BE to dysplastic BE to EAC sequence [50]. A putative mechanism connected with inflammation-associated processes in the formation of BE involves the glutathione S-transferase (GST) and peroxidase (GPX) family of genes, which normally function to protect cells from the damage caused by reactive oxygen species (ROS), which have been shown to accumulate in the setting of chronic gastric reflux [56]. Peng et al. examined the promoter regions of 23 *GST* and *GPX* genes in normal esophagus, BE, dysplastic BE, and EAC cases in conjunction with gene sequencing and gene expression assays and found aberrant DNA methylation of *GPX3* (62 %), *GPX7* (67 %), *GSTM2* (69 %), and *GSTM3* (15 %) in EAC cases. DNA methylation and mRNA expression were inversely correlated for GPX3, GPX7, and GSTM2, and immunohistochemical (IHC) analysis using a tissue microarray confirmed weak/absent staining in EAC for these genes and moderate/strong staining in normal samples [56]. Thus, it is possible that persistent esophageal inflammation related to GERD leads to epigenetic inactivation of genes involved in antioxidant pathways, which could be an important mechanism in the development of EAC.

Another study examined levels of *MLH1* promoter methylation in esophageal tissues, including cancer, BE, reflux esophagitis, as well as normal tissue [84]. This group found significant hypermethylation of *MLH1* in cancer (63.5 %) and precancer (53.8 %), with the highest level of *MLH1* methylation seen in patients with GERD (88.8 %), suggesting a relationship between inflammation triggered by reflux and aberrant DNA methylation. Of note, altered DNA methylation has been described in other cancer-related inflammatory conditions as well, including ulcerative colitis, cholangiocarcinoma, and chronic pancreatitis [40, 64, 89].

## Obesity, Epigenetics, and BE/EAC

Although the risk of developing BE and/or EAC is associated with obesity [72, 77], and altered DNA methylation is commonly seen in BE and EAC, studies on the effects of demographic factors, such as obesity, on the epigenome in the esophagus are very limited at the time this chapter was written. There is evidence that certain environmental, behavioral, and demographic factors can influence the epigenetic state, which suggests that the behavioral factors associated with BE and EAC may act by inducing alterations in the methylation status of DNA [1]. For instance, alterations in the promoter CpG island methylation status of genes associated with obesity, appetite control, and metabolism have been shown to occur in DNA isolated from blood and breast tissue of obese compared to lean individuals [18, 20, 33, 83]. Hoyo et al. examined *IGF2* methylation in differentially methylated regions (DMR; regions of DNA where multiple adjacent CpG dinucleotides show concordant changes in methylation, which are discussed in further detail below) in umbilical cord blood of newborns and correlated it to newborn birth weight, given previous findings of altered *IGF2* methylation and obesity [37]. This group found that reduced *IGF2* methylation was associated with elevated plasma IGF2 protein levels, with the strongest association seen in infants born to obese women (pre-pregnancy BMI > 30 kg/m$^2$, $p < 0.0001$). Elevated levels of IGF2 were associated with higher infant birth weight even after adjusting for several factors including pre-pregnancy BMI, gestational diabetes, and infant gender. This group concluded that circulating levels of IGF2, a risk factor for esophageal and other cancers, might be affected by altered *IGF2* methylation, which in turn might be affected by pre-pregnancy obesity.

Another group recently evaluated 44 patients with squamous cell cancer of the esophagus (ESCC) in order to determine whether the tumor suppressor gene *CDKN2A/p16* was aberrantly methylated in ESCC tumors and matched normal tissues, and whether this epigenetic alteration was associated with obesity or other risk factors [60]. The authors found aberrant *CDKN2A/p16* methylation in 12/44 (27 %) of ESCC samples and no normal samples. Additionally, they noted that obesity status was positively correlated with *CDKN2A/p16* methylation ($p = 0.001$), with logistic regression analysis demonstrating the risk of methylation for BMI ≥ 25 was 12 times higher than for individuals with BMI < 25 (OR = 12, $p = 0.004$). Although this is a small study, the authors suggest it provides evidence that obesity increases the risk of developing ESCC, possibly by promoting *CDKN2A/p16* methylation.

While it is likely that both somatic genetic and epigenetic alterations play a role in the pathogenesis of BE and EAC, there is currently very little information about the relationship between Barrett's esophagus and esophageal adenocarcinoma, obesity, and aberrant DNA methylation. In an attempt to provide insight into this important question, our research group has used methylation microarrays to examine epigenome-wide methylation patterns in a sizable collection of esophageal samples for which demographic information, including BMI, was available. We analyzed methylation patterns of 46 DNA samples isolated from individuals with BE ($N = 15$),

BE with low-grade dysplasia (BE+LGD; $N=14$), BE with high-grade dysplasia (BE+HGD; $N=9$), and EAC ($N=8$) cases using HumanMethylation 450 BeadChips (HM450, Illumina). We stratified these samples into those obtained from individuals with either high BMI (BMI > 30) or low BMI (BMI ≤ 30). We used the data from the HM450 array to compare the methylation levels of more than 485,000 individuals CpG dinucleotides between the high and low BMI cases. We considered a locus to be "differentially methylated" if the $p$ value was <0.0001 and the methylation level (also known as the beta value) differed by at least 10 % between the low and high BMI cases. Using these criteria, we found a total of 974 differentially methylated loci (DML) in BE, dysplastic BE, and EAC samples when comparing the high and low BMI groups. In general, the high BMI cases showed increased methylation at the DML in the esophageal tissues, with 872 out of 974 DML (89.5 %) demonstrating increased methylation in high vs. low BMI cases.

The DML were found in various functional regions of the genome: 226 were located in gene promoters, 471 in gene bodies (intragenic), and 277 in between genes (intergenic). We also evaluated the location of DML with respect to CpG islands, including CpG dinucleotides located in promoter CpG islands, non-promoter CpG islands, and CpGs outside of islands. Analysis of the regions outside of promoter-related CpG islands is notable because an understanding of methylation alterations in areas with relatively low CpG density is becoming increasingly recognized to be important in diseases such as cancer. It has been shown that CpG-rich regions (i.e. CpG islands) demonstrate more stable DNA methylation across tissues and cell populations, whereas methylation is more dynamic in CpG shores (within 2 kb of a CpG islands) and CpG shelves (within 4 kb of a CpG island). Furthermore, the methylation status of CpG shores and shelves appears to regulate gene expression, which would provide a mechanism through which epigenetic alterations in these regions could affect BE and EAC formation [39, 97]. We found 182 DML were located in CpG islands and 376 were located in CpG island shores (within 2 kb of a transcription start site).

As part of our studies, we also assessed whether differentially methylated loci in the high vs. low BMI groups might be associated with esophageal adenocarcinoma (EAC). We defined "cancer associated loci" as loci whose methylation status differed between a group of 12 normal squamous esophagus and 24 EAC cases which we assayed using HM450 methylation arrays. Using this definition, we found 352 DML (36.1 % of the total 974 DML) that were cancer-associated. This is more than 2× as many cancer-related DML than we would expect by chance alone since just 16 % of the total probes on the array are "cancer related" by our criteria. The top 20 most significant annotated DML associated with BMI are shown in Table 8.1.

There are also differences in methylation patterns when comparing high to low BMI cases when examining the various esophageal tissue types separately. We compared methylation in the high BMI ($N=4$) vs. low BMI ($N=11$) BE cases, the high BMI ($N=7$) vs. low BMI ($N=7$) LGD cases, and the high BMI ($N=9$) vs. low BMI ($N=8$) HGD/EAC cases. Table 8.2 summarizes the DMLs found when comparing these groups. The methylation status of the high compared to low BMI BE cases with respect to genomic regions and CpG island location is shown in Fig. 8.1. In

**Table 8.1** Top 20 differentially methylated loci: high vs. low BMI cases (BE, LGD, HGD/EAC combined)

| Probe ID | Gene | Avg. β low BMI | Avg. β high BMI | *p* value | Probe location | Relation to CpG Island | Relative Expression BE vs. normal^ | Relative Expression EAC vs. normal^ | Cancer associated? |
|---|---|---|---|---|---|---|---|---|---|
| cg11839020 | *LRRC8D* | 0.29 | 0.41 | 4.06E-09 | 5'UTR | Shore | 1.59–1.78* | 1.44–1.86* | N |
| cg11027822 | *ITGA6* | 0.62 | 0.72 | 1.97E-07 | Body | Open Sea | 1.57–3.96* | 1.48–2.66* | Y |
| cg25872281 | *TMUB1* | 0.66 | 0.81 | 3.56E-07 | TSS1500; Body | Island | 1.32* | NS | N |
| cg22984132 | *TMUB1* | 0.51 | 0.62 | 3.64E-07 | TSS1500; Body | Island | 1.32* | NS | N |
| cg26314478 | *ESPNP* | 0.49 | 0.60 | 3.86E-07 | Body | Shelf | NS | NS | N |
| cg06393286 | *FAM43B* | 0.48 | 0.36 | 4.64E-07 | 1stExon | Island | NS | NS | Y |
| cg09058554 | *SLC25A33* | 0.39 | 0.51 | 6.40E-07 | Body | Shore | NS | 1.23* | Y |
| cg14950321 | *PLIN5* | 0.31 | 0.42 | 7.21E-07 | Body | Shore | 4.17* | NS | N |
| cg25302888 | *TMUB1* | 0.65 | 0.77 | 1.23E-06 | TSS1500; Body | Island | 1.32* | NS | N |
| cg05137975 | *FAXC* | 0.53 | 0.70 | 1.52E-06 | Body | Open Sea | – | – | N |
| cg00831127 | *EPHB2* | 0.26 | 0.49 | 2.33E-06 | Body | Shore | 1.62–6.30* | 1.40–7.83* | Y |
| cg19513232 | *CAMK2A* | 0.39 | 0.52 | 2.43E-06 | Body | Open Sea | NS | NS | N |
| cg10976975 | *BMP10* | 0.72 | 0.83 | 2.53E-06 | 5'UTR;1stExon | Open Sea | NS | NS | N |
| cg04025965 | *TMUB1* | 0.58 | 0.72 | 2.66E-06 | TSS1500;Body | Island | 1.32* | NS | N |
| cg02233614 | *PFKFB2* | 0.29 | 0.39 | 2.82E-06 | 5'UTR | Shore | 1.32* | 1.12* | N |
| cg08526705 | *MYC* | 0.61 | 0.74 | 2.99E-06 | Body | Shore | NS | 3.89* | N |
| cg08943714 | *HECA* | 0.28 | 0.42 | 3.66E-06 | Body | Open Sea | NS | NS | N |
| cg17161520 | *PPP1CA* | 0.46 | 0.57 | 3.92E-06 | Body | Shelf | NS | NS | N |
| cg26780915 | *CDHR3* | 0.42 | 0.56 | 3.96E-06 | | Shore | 1.44–1.52* | 1.38* | Y |
| cg10583322 | *MEGF11* | 0.49 | 0.65 | 4.15E-06 | Body | Open Sea | NS | 1.69* | Y |

*5′UTR* 5′ untranslated region, *TSS1500* 1500 bp's upstream from transcription start site, *Shore* DNA sequence up to 2 kb from CpG island, *Shelf* DNA sequence 2–4 kb from CpG island, *Open Sea* DNA sequence >4 kb from CpG island, *NS* not significant; ^ = relative gene expression data obtained from www.oncomine.org; *$p \leq 0.05$

**Table 8.2** Differentially methylated loci: high-BMI vs. low-BMI cases separated by tissue type

| Tissue | Total No. DML | Promoter DML (%) | Intragenic DML (%) | Intergenic DML (%) | CpG island DML (%) | CpG shore DML (%) | Cancer-associated DML (%) |
|---|---|---|---|---|---|---|---|
| BE | 288 | 85 (29) | 129 (45) | 74 (26) | 113 (39) | 108 (37) | 60 (21) |
| LGD | 372 | 120 (32) | 166 (45) | 86 (23) | 226 (61) | 166 (45) | 252 (68) |
| HGD/ EAC | 270 | 73 (27) | 156 (58) | 41 (15) | 53 (20) | 111 (41) | 40 (15) |

DML defined by $p$ value <0.001 and Δβ value (high-BMI vs. low-BMI) > 0.10 while controlling for age

general, in the BE and EAC cases, methylation levels were increased in gene promoters, bodies, and intergenic regions of high BMI patients compared to the low BMI patients.

Ultimately, alterations in methylation levels of particular genes in obese patients might promote metaplasia or neoplasia in the esophagus by affecting certain signaling pathways based on the expectation that a subset of the gene loci that show abnormal DNA methylation will have alterations in their expression levels. In order to investigate this, our group assessed the methylation status of CpGs located in genes associated with signaling pathways and biological mediators implicated in obesity-associated cancers [35, 36, 96] in the esophageal tissues from the subjects with low vs. high BMI. We were interested in determining whether alterations in methylation of obesity-related pathways might correlate with BMI status, which would provide a plausible mechanism for obesity-related gene expression changes. As described above, the insulin/IGF-1 pathways are frequently perturbed in obese individuals, and these alterations can be associated with an elevated cancer risk. With regard to these pathways, we observed increased methylation of *IGFBP1* (average beta = 0.11 in low BMI cases and 0.27 in high BMI cases) and *IRS2* (average beta = 0.11 in low BMI cases and 0.36 in high BMI cases) in the high BMI compared to low BMI BE cases. We also examined molecular pathways associated with adipose inflammation, which has been shown to mediate obesity-related cancer [36], and found the proinflammatory gene IL-1β (*IL1B*) to be hypermethylated in high vs. low BMI cases when we assessed the combined esophageal tissue sets. We also found hypermethylation of *IL1B* in the HGD/EAC cases from high BMI subjects. For the combined cases, the average beta was 0.25 in low BMI cases and 0.35 in high BMI cases, and for the HGD/EAC cases, average beta was 0.20 in low BMI cases and 0.38 in high BMI cases. We also evaluated the adiponectin and leptin pathways, which as discussed above have been implicated in obesity-associated cancer [42, 59]. In our studies, we did not observe any differences in the DNA methylation status of genes involved in leptin or adiponectin pathways in any of the esophageal tissue sets in the high vs. low BMI subjects.

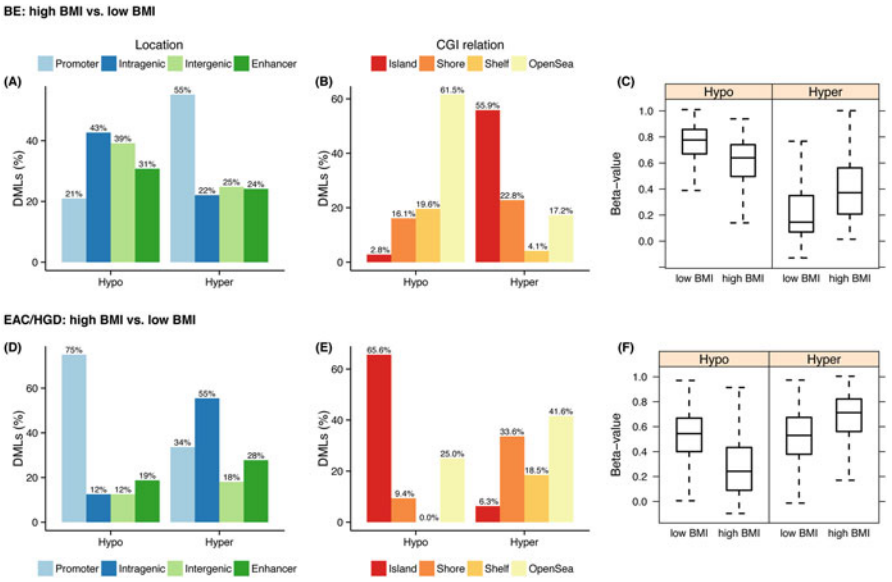


**Fig. 8.1** Genomic location, relationship to CpG islands, and methylation status of DML when comparing high vs. low BMI esophageal samples. In each panel, "Hypo" refers to percentage of DML that are hypomethylated in high BMI vs. low BMI samples; "Hyper" refers to percentage of DML that are hypermethylated in high BMI vs. low BMI samples. On the *Y* axis, DMLs (%) refers to the percentage of the total DML that are associated with a particular genomic location (*Panels A* and *D*) or CGI relationship (*Panels B* and *E*). Percentages may be up to more than 100 % because some probes have been classified with more than one designation. Beta values are equivalent to percent methylation. *Panel A*: DML when comparing high BMI to low BMI BE cases by genomic region. *Panel B*: Location of DML when comparing high BMI to low BMI BE cases with respect to CpG island location. *Panel C*: Box and whiskers plot showing distribution of DML that are hypomethylated in the high vs. low BMI BE cases (*left*) and hypermethylated in the high vs. low BMI BE cases (*right*). *Panel D*: DML when comparing high BMI to low BMI HGD/EAC cases by genomic region. *Panel E*: Location of DML when comparing high BMI to low BMI HGD/EAC cases with respect to CpG island location. Panel F: Box and whiskers plot showing distribution of DML that are hypomethylated in the high vs. low BMI HGD/EAC cases (*left*) and hypermethylated in the high vs. low BMI HGD/EAC cases (*right*)

Our group also utilized several databases to search for cancer-related or other molecular pathways that might be altered by differential methylation patterns in individuals with high vs. low BMI. We used the NCI Pathway Interaction Database (NCI-PID), Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the list of Gene Ontology (GO) terms to identify biological processes or pathways that were over- or underrepresented based on genes containing DML between the esophageal tissue sets in the subjects with either high- or low-BMI status. We restricted our NCI-PID analysis to only cancer-associated DML (DML when comparing EAC cases to squamous cases on the microarray) in order to improve the likelihood that altered molecular pathways would be biologically relevant.

Among the BE cases, we found one NCI-PID pathway, "direct p53 effectors," which includes the differentially methylated gene *RDX* from our dataset, associated

with methylation differences between high and low BMI groups. There were 13 KEGG pathways (including "cell adhesion molecules") and 77 GO terms (including "response to growth hormone" and "biological adhesion") that were represented in the differentially methylated genes in the BE samples from the high vs. low BMI subjects. With respect to the EAC cases, there were no NCI-PID pathways that were significantly associated with methylation differences between high and low BMI status after restricting our analysis to only cancer-related genes. There was one KEGG pathway ("Wnt signaling") and 87 GO terms (such as "tissue morphogenesis" and "response to TGF-beta") differentially methylated between HGD/EAC cases from subjects with high BMI vs. low BMI ($p$ value <0.05).

*TP53*, the gene for p53, is a well-known tumor suppressor gene that is frequently lost early in BE through mutation or loss of heterozygosity (LOH) [69]. *TP53* LOH has been shown to identify a subset of BE patients who are at risk for progression to EAC [19, 68]. The finding of differential methylation involving the p53 pathway in BE from subjects with high vs. low BMI suggests a relationship between obesity and DNA methylation of cancer-related genes in the esophagus. Similar results have been found in other studies comparing methylation in obese to lean individuals. In a recent study of 345 breast cancer cases, the majority (87 %) of CpG sites analyzed showed elevated methylation in obese patients, particularly in estrogen receptor-positive tumors. Obesity was associated with the aberrant methylation of cancer-related genes involved with the immune response, cell growth, and DNA repair [33]. Several prior studies have compared DNA methylation in whole blood or peripheral blood leukocytes among obese and non-obese individuals [15, 17, 26]. In two of these studies, the gene *HIF3A* was found to be hypermethylated in the blood cells and adipose tissue of obese adults, suggesting perturbation of the hypoxia-inducible transcription factor pathway in those with elevated BMI.

## Conclusions

Barrett's esophagus, a metaplastic condition involving the esophagus which develops in the setting of chronic gastro-esophageal reflux and esophageal inflammation, is the precursor lesion for esophageal adenocarcinoma. Thus, GERD is a well-known risk factor for the development of BE and EAC. Obesity, in particular central adiposity, is another important risk factor for the development of these conditions. Traditionally, it has been assumed that obesity augmented the risk of BE/EAC by inducing mechanical or physical changes such as increasing intra-abdominal pressure and/or altering the integrity of the gastro-esophageal junction, leading to increased GERD and reflux esophagitis. However, it has become increasingly clear that the adipose tissue itself, in particular the metabolically active visceral fat more commonly seen in males, may directly promote inflammation and cancer development.

Epigenetic alterations, which are commonly seen in BE and EAC, have been associated with chronic inflammation in the esophagus and also with obesity in other tissues. Data regarding the relationship between aberrant methylation in the esophagus

and obesity are limited, although there is preliminary evidence that elevated BMI is associated with altered methylation in BE, dysplastic BE, and EAC tissues. There is also some evidence that genes involved in cancer-related pathways (p53) and pathways implicated in obesity-related cancers and adipose inflammation (insulin , IGF-1) demonstrate altered methylation in individuals with elevated BMI compared to those with low BMI. Future studies, ideally combining gene methylation, gene expression, and demographic data, will be useful to clarify the mechanism by which obesity elicits alterations in DNA methylation that associate with the risk of BE or EAC.

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# Chapter 9
# Epigenetics, Obesity, and Colon Cancer

**Ruifang Li and Paul A. Wade**

**Abstract** Obesity is an important risk factor in incidence of colorectal cancer, particularly in males. The molecular underpinnings of increased risk are unclear. Recent work has suggested that altered epigenetic regulation resulting from complex factors related to obesity may play a role in the increased incidence of colon cancer. Here we review the relationship of colon cancer and alterations in epigenetic regulation in obesity. The colon epithelial cell is impacted by multiple signaling inputs subject to alteration in obese individuals including adipokines, alterations in metabolism, changes in intestinal microbiota, and chronic inflammation. Obesity-related changes in these pathways likely result in alterations in the epigenome of colonic epithelial cells, with the potential to influence cancer development and/or progression.

**Keywords** Epigenetics • Enhancer • DNA methylation • Cancer • Histone • Obesity

## Introduction

Colorectal cancer is the third most common cancer worldwide, with nearly 1.4 million new cases diagnosed and 694, 000 deaths in 2012 (World Cancer Research Fund International, http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/colorectal-cancer-statistics; WHO, http://www.who.int/mediacentre/factsheets/fs297/en/). It is predicted that 2.4 million colorectal cancer cases will be diagnosed annually worldwide by 2035. The high incidence of colon cancer is largely attributed to the rapid renewal of colonic epithelium every 3–5 days [1–3]. Risk factors for colon cancer include genetic predisposition and lifestyle and environmental factors. It is estimated that approximately 35 % of the risk is due to genetic factors, while the remaining 65 % is due to lifestyle and environmental factors [4]. Among nongenetic risk factors, obesity has gained increasing attention due to the worldwide rapid rise of its prevalence rate in the recent decades [5–7]. Even

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though the close link between obesity and colon cancer is well established, the underlying molecular mechanisms are still not completely understood. The development of colon cancer is thought to be a multifaceted process involving gradual accumulation of genetic and epigenetic aberrations during the rapid renewal process [8, 9]. The recognition of epigenetic changes as a driving force in colorectal neoplasia triggers great interest in studying the epigenetic effects of obesity on colon cancer. However, our current knowledge on this is poor, so rather than providing a comprehensive coverage of this topic, we intend to highlight outstanding research questions in this field.

## Epigenetics and Colon Cancer

Colon cancer has been classically defined as a genetic disease with mutations progressively accumulated during cancer development. A stepwise progression model has been proposed to explain the etiology of colon cancer from benign neoplasia to adenocarcinoma [10]. However, it is becoming more appreciated that epigenetic alterations play a major role in the initiation and progression of colorectal cancer [8, 9]. Colon cancer has been currently viewed as the result of progressive accumulation of both genetic and epigenetic aberrations which act synergistically in the transformation of normal colonic epithelium to colon adenocarcinoma. The epigenetic mechanisms playing roles in cancer development include DNA methylation, histone modifications, noncoding RNAs, and nucleosome positioning [11]. Aberrant DNA methylation is the most extensively studied epigenetic alteration in colon cancer.

Altered DNA methylation patterns have long been associated with tumor formation and represent one of the earliest molecular markers of human cancer. It is well accepted that the genome simultaneously undergoes widespread loss of DNA methylation and locus-specific gain of DNA methylation during tumorigenesis. Compared with normal colon, extensive global hypomethylation was found in colon cancer predominantly at repetitive DNA sequences such as LINE-1 and satellite repeats [12]. These hypomethylated domains coincided with late-replicating, lamina-associated nuclear regions [13]. Local hypermethylated regions in colon cancer located primarily at CpG islands [13]. As a common molecular mechanism of gene inactivation, de novo DNA methylation at CpG islands within promoters of tumor suppressor genes is an early event during tumor progression [14, 15]. Tumor suppressor genes that are hypermethylated in colon cancer include MLH1, MGMT, RB, p16, RARB, DCC, SFRP, and UNC5C [16–21].

A subtype of colon cancer was identified based on DNA methylation profiling at 33 regions, termed CIMP (CpG island methylator phenotype) cancer, which is characterized by a particularly high frequency of methylated loci [22]. In an extension of the CIMP concept, genome-wide studies of DNA methylation in colon cancer have identified three groups of colon cancer: CIMP1, CIMP2, and CIMP negative [23]. The three groups have a unique association with genetic mutations and the

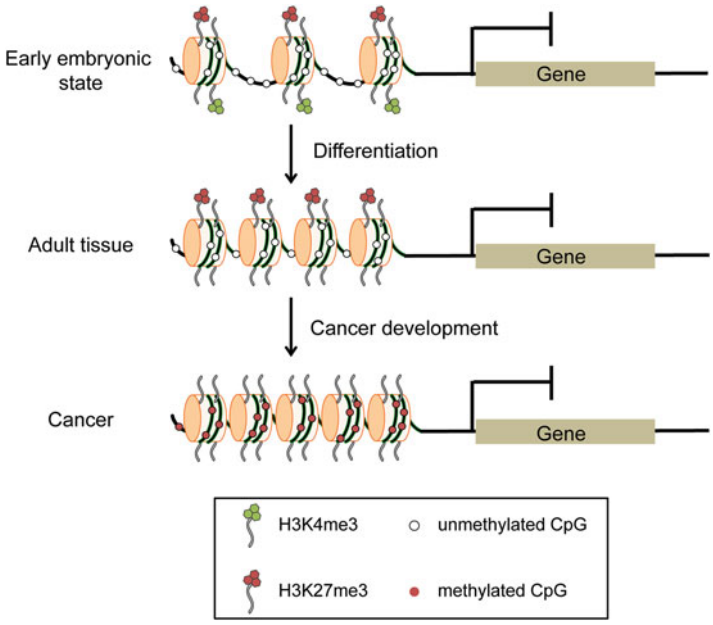


**Fig. 9.1** Aberrant DNA methylation in cancer. Genes that become aberrantly methylated in cancer tend to have characteristic patterns of histone modifications during development and differentiation. In early development, this class of genes is characterized by so-called bivalent chromatin—meaning the simultaneous presence of repressive (i.e., trimethylation of lysine 27 on histone H3; H3K27me3) and active (i.e., trimethylation of lysine 4 on histone H3; H3K4me3) marks. Genes that have bivalent chromatin at their promoters are typically not transcribed during very early development but frequently have critical tissue-specific roles during tissue- and cell type specification. During differentiation of adult tissue types, bivalent genes are maintained in a repressed state (except in cell/tissue types in which their action is required for normal differentiation) characterized by H3K27me3. In cancer development/progression, this class of genes is frequently the target of aberrant methylation of promoter DNA, resulting in stable silencing of their expression

genome instability status of the cancer. CIMP1 was characterized by microsatellite instability (MSI) and BRAF mutation but rare KRAS and p53 mutations; CIMP2 was associated with high KRAS mutation, but rare MSI, BRAF, or p53 mutation; CIMP negative had high p53 mutation and low for MSI, BRAF, and KRAS mutations. It is now well accepted that epigenetic alterations cooperate with genetic mutations to drive the transformation of normal cells to cancer cells through activation of oncogenes and inactivation of tumor suppressor genes which regulate signaling pathways controlling hallmark behaviors of cancer [24].

A close association exists between aberrant DNA methylation and histone modifications. Polycomb-mediated methylation on H3K27 has been implicated in the predisposition of promoters for de novo methylation in cancer [25]. Promoters aberrantly methylated in cancer are enriched with bivalent genes in ESC [25–28], which are important for regulating lineage determination [29–31] (Fig. 9.1). Aberrant DNA methylation in colon cancer was also found in sequences up to 2 kb away from

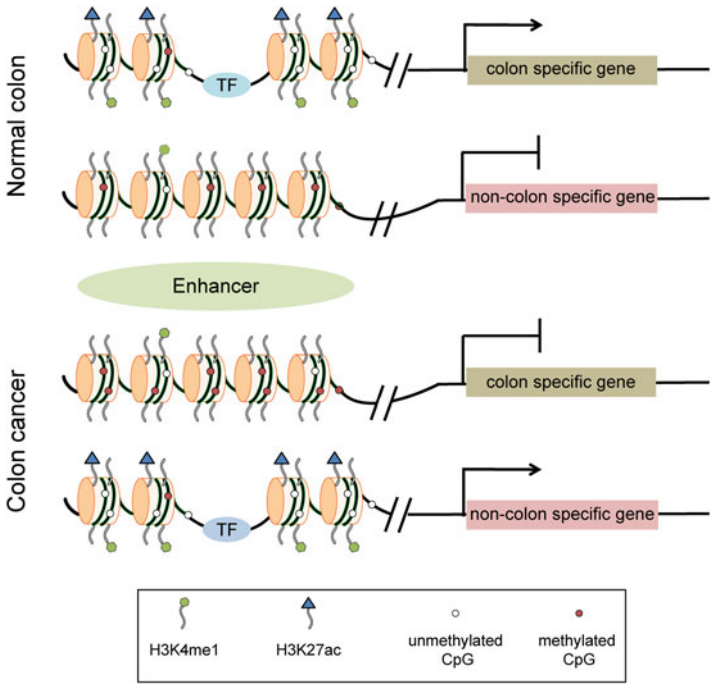


**Fig. 9.2** Enhancer landscape alterations in colon cancer. A defining difference between normal colon and colon cancer is enhancer utilization. Enhancers, *cis*-acting gene regulatory elements, are characterized by prototypical epigenetic marks including monomethylation of lysine 4 of histone H3 (H3K4me1) and acetylation of lysine 27 of histone H3 (H3K27Ac). During cancer development/progression, enhancers driving expression of genes integral to colon epithelial cell identity are decommissioned, leading to decreased expression of linked genes. In contrast, genes with little tissue/cell type specificity characteristically have de novo acquisition of active enhancer marks at regulatory DNA associated with increases in expression

CpG islands termed "CpG island shores." CpG island shore methylation was strongly related to transcriptional repression, and it tends to be tissue specific with the ability of distinguishing normal tissues [32]. Differential methylation of cancer-specific CpG island shores can also distinguish induced pluripotent stem cells and embryonic stem cells from differentiated cells [33]. Thus, it is proposed that malignant reprogramming probably employs the same mechanisms of differentiation and reprogramming in normal development. In support of this, Peter Scacheri and his colleagues found that the enhancer landscape in colon cancer is different from that in normal colon, characterized by gain of non-colon-specific enhancers and loss of colon-specific enhancers which reflects stem cell-like features. These "variant enhancer loci" were enriched with genetic risk variants of colon cancer and predictive of gene expression in colon cancer [34] (Fig. 9.2). In summary, cancer epigenome has been linked to the epigenetic dynamics in normal development. Epigenetic alterations affecting tissue-specific differentiation may be the predominant mechanism which leads to cancer.

Epigenetic changes could be the earliest events during tumorigenesis even before cellular transformation [35, 36]. Aberrant epigenetic alterations were frequently found in normal tissues adjacent to colon cancers [37–39]. It was shown that 50 % of tumor-adjacent histologically normal tissue carried detectable methylated MGMT when the primary tumor had methylated MGMT, whereas only 12 % of histologically normal tissue from cancer-free control patients had MGMT promoter methylation [40]. Aberrant DNA methylation at metabolic genes was found in normal colon mucosa from patients with colon cancer [41]. The early occurrence of epigenetic alterations may prime the colon mucosa to undergo transformation by allowing subsequent accumulation of both genetic and epigenetic alterations that promote cancer development and progression.

## Obesity and Colon Cancer

The prevalence of obesity is increasing at an alarming rate in both developed and developing countries. The number of overweight and obese individuals worldwide has increased from 857 million in 1980 to 2.1 billion in 2013. Obesity has become one of the world's greatest public health burdens. In 2002, the International Agency for Research on Cancer (IARC) claimed that obesity is an avoidable cause of excess cancers in several tissues [42]. It has been estimated that approximately 20 % of all cancer cases and 15–20 % of all cancer deaths in the United States were due to obesity [43]. As the global prevalence of obesity continues on an upward trajectory, the cancer burden related to obesity will rise. It is estimated that continuation of existing trends in obesity will lead to about 500,000 additional cases of cancer in the United States by 2030 (http://www.cancer.gov/about-cancer/causes-prevention/risk/obesity/obesity-fact-sheet).

Among obesity-associated cancers, colorectal cancer is closely related to lifestyle factors. Obesity has been recognized for decades to be a risk factor for colon cancer [44, 45]. Numerous epidemiological studies reported that obesity is associated with increased risk of colon cancer [46–49]. *Rodent* animal models also support this notion [50–53]. Any 1 kg/m$^2$ increase in BMI causes additional risk (HR = 1.03) [54]. Consistently, weight loss may be associated with decreased CRC incidence [5]. A 39–60 % decrease in cancer-related mortality was observed in patients with weight loss after bariatric surgery [55, 56]. Gender difference has been observed consistently across studies and populations. Obese men are more likely to develop colorectal cancer than obese women [5]. The relative risks of colon cancer in obese men is ~1.5–2.0, while in obese women is ~1.2–1.5 [7].

### *Molecular Mechanisms Linking Obesity to Colon Cancer*

Even though sufficient evidence exists for the link of obesity and increased risk of colon cancer [57], the underlying mechanisms of how obesity promotes carcinogenesis in the colon remain poorly understood. It is almost certainly a multifactorial

process. Several possible mechanisms have been proposed to explain the molecular association between obesity and colon cancer, including the direct and indirect effects of obesity. Obesity is typified by an excess of adipose tissue, as an endocrine organ, which secretes a combination of factors referred to as adipokines. Increased leptin and decreased adiponectin were observed in obese individuals [58, 59]. Secondary effects of obesity include chronic inflammation and metabolic and endocrine effects such as abnormal levels of steroid hormones, insulin, and insulin-like growth factor-1 (IGF-1) [5–7, 60]. Obesity can be considered to be a state of chronic low-grade inflammation as demonstrated by increased systemic levels of inflammatory cytokines [61]. A growing body of evidence suggests that pro-inflammatory signaling in the obese state is intimately linked to the development of cancer [62]. Chronic inflammation is associated with 1/5 of all human cancers [63, 64]. Plasma levels of insulin and free IGF-1 are increased, while IGFBP-1 and IGFBP-2 levels are decreased in obese subjects compared with lean controls [65], leading to activation of the insulin-IGF signaling. Chronically, increased insulin levels have been associated with colon cancer [66, 67]. These putative molecular mechanisms linking obesity and colorectal cancer have been previously reviewed extensively [65, 68–70].

## *Gut Microbiota, Obesity, and Colon Cancer*

Gut microbiota represents an emerging field of interest [71, 72]. Normally, the human gut contains approximately 100 trillion bacteria belonging to 500–1000 species [73, 74]. Bacterial cells outnumber cells in the human body by 10 to 1 [75, 76], making them one of the most important environmental factors with enormous impacts on host physiology and metabolism [77]. Gut microbiota is highly plastic and rapidly responds to physiological and pathological changes. Profound alterations in the composition and metabolic functions of gut microbiota were observed in obese individuals compared with lean controls [78–81], although the mechanism behind these changes is unknown. Gut microbiota in obese individuals is characterized by decreased *Bacteroidetes* to *Firmicutes* ratio as well as a reduction in microbial diversity [82–84]. Obesity-associated changes in the relative abundance of *Bacteroidetes* and *Firmicutes* are conserved between mice and human, leading to increased capacity of the host to harvest energy from the diet [85]. Consistently, diet-induced weight loss was associated with increased *Bacteroidetes* and decreased *Firmicutes* [82]. Patients with colon cancer harbored different groups of bacteria compared with individuals with normal colonoscopy. A case-control study found that, similar to changes in obese individuals, people with colorectal adenoma had a significantly lower relative abundance of *Bacteroidetes* than controls [86]. Genomic analysis of the highly conserved 16S rDNA sequences revealed a significant enrichment of *Fusobacterium* in colorectal carcinoma [87, 88]. Animal studies also support the effects of gut microbiota on colon cancer development. Using a spontaneous colorectal cancer mouse model (TCRβ and p53 double-knockout mouse), it was found that the mice did not develop colon cancer under germ-free condition, whereas

70% of mice in the conventionalized group possessed adenocarcinomas [89]. Similarly, germ-free rats developed smaller and fewer intestinal tumors than conventionally colonized littermates when treated with azoxymethane [90]. In summary, gut microbiota could have a direct pro-tumorigenic role during colorectal carcinogenesis [91].

A causal relationship between gut microbiota and disease state was demonstrated by fecal transplant experiments in germ-free mice. Germ-free mice receiving fecal microbiota from twins discordant for obesity adopted phenotypes of the donors; mice receiving microbiota from the obese twin developed increased total body and fat mass and obesity-associated metabolic abnormalities [92]. There is growing appreciation of the role of gut ecology in the promotion of obesity [82, 93, 94]. Changes in the gut microbiota after Roux-en-Y gastric bypass (RYGB) surgery drove weight loss and the reduction of adiposity [95]. Likewise, tumorigenesis is transmissible as demonstrated by transferring the fecal samples from mice with intestinal tumor to genetically predisposed mice [91]. These studies proved the contributions of intestinal microbiota on obesity, metabolic disease, and the development of CRC in susceptible individuals.

The phenotypic changes caused by microbiota alterations are related to the metabolic functions of gut microbiota and their effects on the host metabolism. Disturbed gut microbiota can produce cytotoxic substances which increase cancer risk. The colon microbiota can form structures, termed biofilms, which can alter the metabolome in colon tissue facilitating oncogenic transformation of colonic cells by upregulation of polyamine metabolites [96]. Bile acids which are produced in the liver by the metabolism of cholesterol are converted to secondary bile acids by gut microbiota. These secondary bile acids can promote cancer development [97, 98]. Gut microbial-derived short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate, are produced by fermentation of undigested dietary fiber. Butyrate serves as the main energy source for colonocytes [99]. In addition, SCFAs exert several other beneficial influences on colonic mucosal health. First, SCFAs lower the PH in the colon lumen and thus prevent the growth of PH-sensitive pathogenic bacteria and inhibit generation of carcinogenic secondary bile acids and decrease the solubility of free bile acids to reduce their carcinogenic activity [100]. Second, SCFAs possess anti-inflammatory effects by inhibiting pro-inflammatory cytokine-induced NF-kB activation [101, 102]. Third, butyrate acts as epigenetic regulator by inhibiting histone deacetylase (HDAC) [103], playing important roles in anti-proliferation and cell apoptosis to reduce colorectal tumorigenesis [104–106]. Fourth, SCFAs function as signal transduction molecules via G protein-coupled receptors. In summary, the level of SCFAs may influence the development of bowel disorders and cancer [107, 108].

It is now clear that gut microbiota plays a central role in host physiology, metabolism, and nutrition [109–111]. Characterization of gut microbiota and its metabolism can be expected to provide the key to colonic health and disease. Intestinal carcinogenesis can be a result of dysbiosis in gut microbiota with an increased proportion of deleterious bacteria while a decreased proportion of beneficial bacteria. The role of obesity-related changes in gut microbiota in colon cancer deserves further attention.

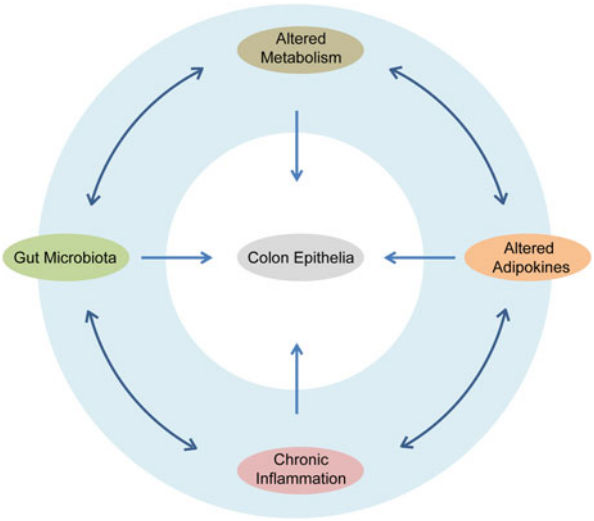


**Fig. 9.3** Crosstalk of direct and indirect effects of obesity and their impact on colonic epithelia. The model depicts several sources of signals to colon epithelial cells that are altered in obese individuals. Each pathway has the potential to impact epigenetic marks in colonocytes. All the pathways depicted have the potential for substantial crosstalk

## The Epigenetic Link Between Obesity and Colon Cancer

Obesity is often accompanied by a series of physiological and/or pathological changes, including inflammation, metabolic and endocrine changes, and altered gut microbiota, which may directly or indirectly promote tumorigenesis in the colon. Most of obesity-associated changes mentioned above are interrelated (Fig. 9.3). For example, obesity and microbiota can induce inflammation and metabolic abnormalities, obesity and metabolic syndrome can alter gut microbiota; conversely, gut microbiota can promote obesity, and secondary bile acids which were produced by gut microbiota can alter the composition of gut microbiota and favor the development of tumor. Because obesity modifies whole-organism physiology with changes mentioned above happening concurrently and intertwining with each other, it is difficult to disentangle the complex changes in obesity and dissect the precise role of each change in colon cancer. The molecular underpinnings that integrate these events to prime intestinal epithelial cell fate and contribute to the acquisition of a tumorigenic phenotype still remain largely unknown.

Cellular phenotype is determined by the genes expressed in the cell which are predominantly regulated by epigenetic mechanisms. Epigenetic mechanisms allow cells to respond to both internal and external environments through changes in gene expression thus contributing to the development of abnormal phenotypes. Cellular epigenome is dynamic during normal cell differentiation, modifiable in response to environmental signals and potentially heritable in daughter cells. It serves as a plastic platform for incorporating various internal and external perturbations to the cell

[112]. Aberrant epigenetic modifications are well established as one of the underlying causes in cancer development. Our previous work has demonstrated that obesity altered the enhancer landscape in mouse colonic epithelium. In this study, obesity was associated with alterations in enhancer usage at loci enriched for binding sites of transcription factors integral to colon development and specification of colonic epithelial cell identity. In addition, loci enriched for binding sites of transcription factors downstream of signaling pathways integral to the initiation and progression of colon cancer were also altered in obese animals compared to lean controls. Despite these alterations in enhancer utilization that closely resemble the differences observed between normal colon and colon cancer [34], obese animals did not develop overt cancer. These data were interpreted to suggest that obesity primes the colon epithelium for cancer by epigenetic alterations at enhancer loci [113]. Thus, obesity-associated epigenetic alterations may predispose obese individuals for colon cancer providing the molecular mechanistic explanations for the well-defined link of obesity to colon cancer. Current understanding of the epigenetic effects of obesity on colon cancer is rudimentary despite the potential importance of this knowledge for detection, prevention, and treatment of the disease.

## *Epigenetic Alterations in Obesity*

Extraordinary interests in the role of epigenetic dysregulation in obesity were exhibited due to the high plasticity of epigenetic process to environmental stimuli [114]. Obesity and obesity-associated secondary effects have been reported to be associated with epigenetic changes in various cell types. DNA methylation is the most extensively studied epigenetic mark in obesity. Body weight was associated with DNA methylation patterns in peripheral blood mononuclear cell and adipose tissue [115, 116]. The composition of gut microbiota was also correlated with DNA methylation patterns in blood cells [117]. Epigenetic alterations, in particular DNA methylation changes, have been observed during inflammation and in inflammation-associated carcinogenesis [118]. Key mediators of inflammation-induced DNA methylation changes appear to be oxidative stress and increased pro-inflammatory cytokines, including IL-6, IL-1B, TNFα, and interferon-γ [119–122]. Obesity-induced pro-inflammatory cytokines increase DNMT1 expression and its enzymatic activity [123]. It has been shown that inflammatory signals induce hypermethylation of DNA methylation valleys (DMVs) contributing to inflammation-induced cellular transformation in enterocytes [124]. A variety of studies demonstrated that obesity is associated with altered epigenetic modifications at a number of metabolically important genes. In skeletal muscle, obesity was associated with aberrant DNA methylation levels at promoter regions of PDK4 and PGC-1a, which were restored to nonobese levels after RYGB-induced weight loss [125]. Prospective cohort intervention study showed that DNA methylation level at SCD1 promoter was associated with weight change in peripheral blood cells [126]. Adiponectin promoter region was hypermethylated in the adipocytes of obese compared with

lean subjects [123]. A twin-based study reported that serotonin transporter (SLC6A4) gene promoter was hypermethylated with increased BMI and waist circumference levels in peripheral blood leucocytes [127]. Obesity was associated with altered DNA methylation at circadian rhythm genes such as CLOCK and BMAL1 in white blood cells [128]. Diet-induced obesity led to genome-wide chromatin remodeling at regions bound by HNF4A, CEBP-α and FOXA1 in liver [129]. Association of obesity with DNA methylation was also found at inflammatory genes in periphery blood cells, such as IL1B, IL6, and TNFα [130, 131]. Increased methylation of proopiomelanocortin (POMC) [132] in periphery blood cells were found in obese compared with lean individuals. Increased methylation at the HIF3A locus is associated with increased BMI in adults of European origin in blood cells and adipose tissue [133]. A genome-wide methylation study in peripheral leukocyte observed 23,305 differentially methylated CpG sites comparing obese with lean individuals, which were enriched at genes associated with obesity and related diseases [134]. Another two genome-wide studies found relatively rare differentially methylated sites in the obese compared with lean controls. One study analyzed ~4 million CpG sites in 74 individuals using comprehensive array-based relative methylation (CHARM) analysis and found four regions showing covariation with body mass index, which located near genes related to body weight regulation [135]. The other study observed one CpG site in UBASH3A gene with higher methylation level and one CpG site in TRIM3 gene with lower methylation level in the obese [136]. The vast majority of epigenetic studies in obesity published to date focused on only DNA methylation. However, obesity is associated with not only DNA methylation changes but also histone modification changes, such as histone acetylation alterations at TNFα and Ccl2 genes in mouse liver [137] and histone methylation (H3K4 and H3K9) changes in human adipocytes [138].

Collectively, these studies have identified multiple obesity-associated differentially methylated sites mostly in blood cells. Peripheral blood cells are attractive for epigenetic studies in obesity, because they can be easily obtained. However, environmentally induced epigenetic changes are likely to be tissue specific as epigenetic regulation is cell type specific. Studies in easily accessible tissues such as peripheral blood may not represent what happens in the colon. In order to understand the epigenetic effects of obesity on the etiology of colon cancer, it is essential to explore the epigenomic changes in the colon comparing obese with lean individuals. Colon tissue contains multiple different cell types; each cell type has a unique epigenome. An epigenetic change observed in analysis of a complex tissue may not be due to the reprogramming of the locus but rather reflect the change in the relative proportion of cell types that have different epigenetic states. Thus, epigenomic profiles in the cellular origin of colon cancer, that is, the colonic epithelium, are required. With the relative inaccessibility of the colon tissue for analysis, to advance our understanding of obesity-associated epigenetic dysregulation in colonic epithelium, animal studies will remain essential to provide mechanistic insights and answer underlying biological questions that are challenging to address in humans. The diet-induced obesity mouse model closely mirrors human obesity and thus serves as an appropriate animal model.

Multiple levels of epigenetic control account for appropriate orchestration of gene expression in healthy and dysregulated gene expression in disease. DNA methylation, histone modifications, small RNAs, and nucleosome positioning participate to maintain the chromatin conformation defining the cellular transcriptome, identity, and function. Research studying the genome-wide epigenetic effects of obesity on colon cancer risk is in its infancy. To successfully address the substantial knowledge gap, comprehensive exploration of obesity-associated epigenomic changes in colonic epithelium is required. This will significantly expand our knowledge on the mechanistic basis of obesity in promoting colon cancer and may provide novel avenues of therapeutic intervention in colon cancer.

Integration of epigenome and transcriptome data will also be crucial to obtain a more complete picture of how obesity affects the regulation of gene expression through epigenetic modifications. A major current challenge to the chromatin field is that the vast majority of epigenomic changes occur at noncoding regions in the genome including intragenic and intergenic regions, which are not readily connected to genes. In order to discover which genes are regulated directly by loci with epigenetic alterations, information on chromosome conformation and enhancer-promoter contacts is required. The three-dimensional organization of the chromatin within the nucleus of colonic epithelium has been poorly explored so far. Defining the topographic structure of the mammalian epigenome and how it functions in regulation of chromatin regions in normal and obese states are of significant interest to the field. The elucidation of the topography of the epigenome will address how enhancer structures are regulated and how boundaries are maintained or abnormally lost in colonic epithelium from obese individuals. In summary, extensive genome-wide profiling of the epigenome in colonic epithelium will advance our understanding of how obesity impacts the epigenome to predispose an individual to colon cancer. But deeper mechanistic insights are required to establish a clear link between obesity and epigenomic changes in colonic epithelium.

## *Linking Cell Signaling Pathways and the Epigenome in Obesity*

Epigenetic regulation of chromatin is dynamic and enables control of gene expression for the cell to respond to various external and internal signals. The direct and indirect effects of obesity lead to aberrant cellular signaling. Obesity is associated with increased leptin and decreased adiponectin [139], hyperinsulinemia, and insulin resistance typified by the activation of insulin-IGF axis [65, 140], low-grade chronic inflammation defined by increases of a variety of pro-inflammatory cytokines, and altered metabolites of gut microbiota, such as SCFAs and secondary bile acids, which serve as ligands of specific cellular signaling pathways. Signal transduction pathways are responsible for the integration and interpretation of such signals into specific transcriptional states by modulating chromatin structure to activate or repress transcription at particular loci. Our previous work showed that obesity-induced changes in gene expression and enhancer utilization in mouse colonic

epithelium reflected the alterations of cellular signaling in obesity [113]. Further investigation is needed to decipher the detailed molecular links between signal transduction and its consequent changes in chromatin structure and gene expression in obesity [141, 142].

On the other hand, aberrant epigenetic landscape can lead to misinterpretation of signals received by the cell, resulting in dysregulation of gene expression and selected growth advantage of transformed cells. The DNA methylation level at GPR41 (SCFA receptor) promoter region was significantly lower in the obese compared with lean individuals, which could potentially influence signaling via SCFAs [143]. Aberrant DNA methylation of polycomb target genes in cancer leads to tight transcriptional repression of those genes preventing their subsequent activation by signal transduction events [144, 145]. Obesity-induced aberrant cellular signaling and abnormal epigenetic states may collaborate with each other to create multiple "hits" needed for colon tumorigenesis. Further understanding of the interrelationship between signaling pathways and epigenomic changes in obesity may provide an avenue for preventive and therapeutic strategies to reduce cancer risk and mortality in an increasingly obese population.

## *Linking the Metabolome and Epigenome in Obesity*

The epigenetic effects of obesity on colon cancer can also be viewed through the lens of metabolism. Most epigenome-modifying enzymes require intermediate metabolites as substrates or cofactors. Altering the availability of substrates necessary for the reactions or cofactors modulating the activity of the enzymes will affect chromatin modifications widely. *S*-adenosylmethionine is a universal methyl donor for methyltransferases [146], synthesized in the methionine cycle from precursors in one's diet. Lack of methyl donors from diet can result in DNA hypomethylation in rodent liver [147] and brain [148]. Metabolic reprogramming during cellular differentiation is also accompanied with epigenetic changes. During the transition from quiescence to proliferation, skeletal muscle stem cells experience a metabolic switch from fatty acid oxidation to glycolysis. This metabolic change decreased the intracellular NAD+ level leading to reduction in the activity of the histone deacetylase SIRT1 and increases in H4K16 acetylation [149]. Therefore, appropriate levels of substrates/cofactors, such as phosphate, acetyl, and methyl groups, are required to elicit the modifications in response to environmental factors. The link between metabolism and epigenetics has reached a previously unappreciated level [150, 151].

Obesity is associated with extensive metabolic changes [152], which may lead to changes in the levels of intermediate metabolites and influence epigenetic modifications such as DNA methylation and histone acetylation. It was reported that diet-induced metabolic syndrome was associated with global changes in DNA methylation level in blood cells, 20 % decrease in males and 15 % increase in females [153]. It is rational to postulate that obesity-related metabolic changes in

colon epithelium, if they exist, will also drive epigenetic alterations in colon epithelial cells. Clear metabolic changes in the colon were observed in obese compared with lean state [154]. The limitation of this study is that instead of colonic epithelium, the experiment was performed in colon tissue. So far, we are unaware of any metabolome profiling in colon epithelium comparing obese with lean state, which is urgently needed to study the effects of obesity-associated metabolic changes on the epigenome which may promote colon cancer. Although we anticipate that obesity-associated changes in cellular metabolism will have an impact on the epigenome in colon epithelial cells, further experiments are required to confirm this hypothesis.

Numerous connections between products of intermediary metabolism and chromatin-modifying proteins have been identified. The regulation of epigenetic programs by metabolites is emerging as an exciting new area of research [155, 156]. On the other hand, obesity-associated aberrant DNA methylation occurred at metabolic genes, suggesting that epigenetic changes associated with obesity reciprocally influence cellular metabolism. Mechanistic insights into the crosstalk between cellular metabolism and epigenetic regulations are required to discover how localized fluctuations in levels of metabolites control chromatin modifiers in space and time, translating a dynamic metabolic state into the "epigenetic code." Understanding the impact of obesity on cellular metabolism and its influence on the epigenome of colon epithelium will likely pave the way for new therapeutic strategies of obesity-related colon disease.

Transient metabolic states could be potentially translated into more stable transcriptional states via stable epigenetic alterations to lock the cellular status leading to long-term effects on the phenotype. Stable epigenetic alterations enable the persistence of altered cellular metabolism even in the absence of the original stress/stimulus which initiates them. "Metabolic programming" serves as a good example of this. In addition, transient metabolic changes in adulthood can also affect phenotypes over extended periods of time. Diet-induced obesity (DIO) mouse model exhibit metabolic dysfunctions mimicking the dysfunctions observed in obese humans [157, 158]. DIO mice transitioning from high-fat to low-fat diet did not completely revert to the same state as mice maintained only on low-fat diet [159], and they developed significantly more colon tumors if challenged with azoxymethane (AOM), suggesting that the transient obese state is recorded in colon epithelium of DIO mice even after dietary switch restored normal body weight. Interestingly, no difference in the number of aberrant crypt foci were observed in these diet-switching mice compared with control mice, suggesting that the effects of obesity appear to be on subsequent stages of tumor development when early preneoplastic lesions transition into adenomas [51]. Consistently, we found that obesity-induced gene expression changes and enhancer alterations were related to colon cancer progression in a DIO model [113]. We propose that obesity-related disorders may be perpetuated via stable epigenetic alterations predisposing obese individuals to colon cancer. Thus, the epigenome may learn from its experience of a prior obese state.

## *Obesity-Associated Epigenetic Alterations in Colon Epithelium Are Reversible (Or Not?)*

Epigenetic modifications are potentially reversible making it possible to respond reversibly to environmental cues. This raises the question whether obesity-associated epigenomic changes are reversible or not. Individuals with obesity are widely recognized to be at increased risk of developing age-related disease such as cancer. Tissue age can be evaluated using an epigenetic biomarker of aging referred to as "epigenetic clock" (based on DNA methylation level of 353 CpG sites). It was reported that the "epigenetic clock" was irreversibly accelerated in the liver of patients with obesity, which possibly explains why these individuals are at high risk of developing liver cancer [160]. In contrast, obesity-associated aberrant DNA methylation in skeletal muscle was reversed to nonobese levels after RYGB-induced weight loss [125]. The controversy suggests that the reversibility of obesity-associated epigenetic alterations may be tissue-dependent. In addition, the timing of epigenetic alterations is also important. Epigenetic shifts during prenatal stages appear likely to be maintained and less tissue specific, while epigenetic alterations occurring in adulthood appear to be more flexible and tissue specific [161]. It remains unclear to what extent obesity-associated epigenomic alterations in colon epithelium are reversible, and the underlying factors which regulate the reversibility of those epigenetic changes have yet to be determined. Deeper mechanistic insights into the reversibility of obesity-associated epigenetic alterations in the colon will raise the hope of developing preventive and therapeutic approaches to decrease the incidence of obesity-related colon cancer.

## *Integration of "Omics" Datasets*

Obesity alters the physiology in the whole organism with direct and indirect effects of obesity playing roles in colon cancer. To better understand the biological link between obesity and colon cancer, we need to consider the various changes in obesity as a whole. The high-throughput profiles of epigenome, transcriptome, and metabolome in obesity will yield an unprecedented view of changes in the cellular regulatory networks in colonic epithelium from obese subjects. An important challenge is how to understand the incorporation of multiple changes associated with obesity from a more systemic perspective. The field is transitioning from high-throughput discovery to detailed mechanistic studies. To get a more comprehensive understanding of the mechanistic link between obesity and colon cancer, we need to integrate high-throughput profiling information from multiple "omes," such as epigenome, transcriptome, proteome, and metabolome. Data integration is probably the most promising strategy for depicting all the regulatory mechanisms involved and how they coordinate with each other to promote the development of colon cancer in obese individuals.

## Summary

Because the epidemic of obesity continues on a worldwide scale, mechanistic insights into how obesity increases the risk of colon cancer are urgently needed. Obesity and obesity-induced secondary changes may directly or indirectly contribute to a number of neoplastic conditions. To comprehensively understand the mechanistic link between obesity and colon cancer, it seems promising to focus on a stage at which they converge, that is, the epigenome. The epigenome integrates environmental inputs to regulate the transcriptional machinery, so that cells can respond to and incorporate adaptations to the current environment. Aberrant epigenetic modifications are likely a driving force in colorectal neoplasia. Therefore, epigenetic changes induced by obesity may provide a molecular link between obesity and increased risk of colon cancer. Obesity-induced epigenomic alterations once established may prime colon epithelium for further tumorigenic events later in life [113].

Obesity-associated aberrant cellular signaling and metabolic changes may be the cause of epigenomic alterations. Reciprocally, aberrant epigenomic landscape in obesity can interfere with signaling pathways and cellular metabolism. Unraveling the precise mechanisms of obesity-related epigenetic regulation in colon epithelium is essential and will provide the basis for therapeutic intervention.

Epigenetic modifications are dynamic and potentially reversible. This exciting opportunity opens new areas of research in the discovery of chromatin-modifying enzymes as new drug targets; clinical trials of drugs targeting histone modification machinery are underway for various diseases [162, 163]. However, it is unclear to what extent obesity-related epigenomic alterations in colon epithelium can be reversed or maintained. Furthermore, relatively little is known about the mechanism of long-term effects of obesity on disease risk. Once these knowledge gaps are filled, in the near-term future, the use of epigenetic drugs could become a realistic possibility for preventing obesity-related colon disease through modifiable epigenetic mechanisms.

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# Chapter 10
# Energy Balance, Epigenetics, and Prostate Cancer

**David Heber, Susanne M. Henning, and Zhaoping Li**

**Abstract** While many genetic alterations have been demonstrated in advanced and metastatic prostate cancer, epigenetic modifications resulting from positive energy balance may play an important role in mediating gene-nutrient interactions that promote the initial development and later progression of this very common form of cancer. Latent prostate cancer incidence increases with aging and is found in 80 % of men aged 80. Among middle-aged men in industrialized nations eating a Western diet, aging increases the incidence of sarcopenia and abdominal/visceral obesity and is commonly associated with increases in insulin-like growth factor 1, inflammatory cytokines, and increased estrogen/androgen ratios. Therefore, the prostate gland is exposed to environmental and endogenous stresses with aging, related to a state of positive energy balance, increasing adiposity, and inflammation. DNA methylation, histone modifications, and microRNA expression in prostate cancer, secondary to positive energy balance and epigenetic modifications, can mediate gene–nutrient interactions in the prostate. The loss of expression of Glutathione-S-Transferase-π 1 (GSTP1) occurs in 90 % of prostate tumors via methylation of CpG islands in its promoter. Soy and green tea polyphenols have been shown to modify prostate tumor epigenetics. Balanced nutritional interventions combined with antioxidant-rich fruits and vegetables together with aerobic and resistance exercise should be examined with regard to their effects on the epigenetics of prostate cancer.

**Keywords** Prostate cancer • Energy balance • Epigenetics • Polyphenols • Soy • Green tea • Sarcopenic obesity

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## Introduction

Positive energy balance as the result of excess energy intake and/or low energy expenditure results in excess body fat and its associated metabolic alterations, such as increased levels of insulin and changes in the bioavailability of insulin-like-growth factor (IGF)-I, cytokines, and steroid hormones often in the absence of obvious obesity. Due to sedentary lifestyle, age-related losses in lean body mass result in reductions in resting energy expenditure, although calorie intake is maintained and commonly leads to the accumulation of abdominal adipose tissue in middle-aged men. Abdominal adipose tissue is a metabolically active endocrine and immune organ secreting adipocyte-derived hormones and cytokines that can impact inflammation and oxidant stress in the prostate gland. These metabolic alterations have been implicated as key contributors to the effects of obesity on common forms of cancer [10, 35].

There is emerging evidence that epigenetic mechanisms involved in typical sporadic prostatic carcinogenesis may be the key to understanding gene–nutrient interactions in prostate cancer including the impacts of obesity and positive energy balance [6]. While epigenetic alterations of the genome are thought of as heritable permanent modifications that impact the expression of genes, there is evidence that environmental and nutritional influences can impact epigenetic changes in the prostate gland.

In one of the most striking demonstrations of the effects of nutrition on gene expression through epigenetic effects, the expression of the Agouti gene in obese heterozygous yellow mice with an increased risk of cancer can be altered in vivo through administration of excess folate and other methyl donors to the mother during gestation. This nutritional supplementation results in the birth from the yellow obese mother of lean brown offspring with a reduced risk of cancer [91]. The Agouti gene, which is lethal in the homozygous condition, programs both yellow skin color and obesity through effects on the melanocortin receptors affecting satiety in the hypothalamus. Methylation of the Agouti gene prevents the expression of altered skin color and obesity resulting in the birth of normal offspring from a maternal phenotype programmed by a mutated gene.

Another important example of the impact of nutrition on epigenetics is imprinting. Imprinting occurs in the placenta and determines the allele-specific expression of a gene based on its parent of origin. Imprinted genes appear remarkably sensitive to environmental changes including diet and oxidative stress. For example, high oxygen environments disrupt imprinting in blastocysts [39].

Loss of imprinting (LOI) of insulin-like growth factor 2 (IGF2) has been shown to occur during aging and is important in tumorigenesis. Oxidative stress, measured by increased NF-kB activity, induces LOI in both cancerous and noncancerous human prostate cells [6]. Decreased expression of the enhancer-blocking element CCCTC-binding factor (CTCF) results in reduced binding of CTCF to the H19-ICR (imprint control region) as a result of increased methylation of DNA, a major factor in the allelic silencing of IGF2. These observations connect inflammation found in aging prostate tissues with an altered epigenetic landscape.

The importance of epigenetic alterations in prostate cancer and their potential modulation by energy balance and antioxidant phytonutrients is the subject of this chapter.

## DNA Methylation in Prostate Cancer

Prostate cancer is a disease of genetic susceptibility as well as epigenetic abnormalities [5]. Epigenetics generally refer to changes in gene expression and chromatin organization that are independent of alterations in the DNA sequence [21]. Epigenetic phenomena are modifiable by dietary and environmental factors. Changes in DNA methylation can be passed on to the next generation [22]. Epigenetic changes (Fig. 10.1) have been identified as promising targets for the prevention and treatment of prostate cancer [65].

DNA methylation is the most widely studied epigenetic modification in mammals. DNA methylation results in the addition of a methyl group to the carbon-5 position in the pyrimidine ring of cytosine in the CpG dinucleotide of genomic DNA. The distribution of CpG dinucleotides throughout the human genome is not uniform and is frequently enriched in the promoter regions of genes, especially in regions of large repetitive sequences such as centromeric repeats, LINE-1, and ALU retrotransposon elements [8]. Short CpG-rich regions are also called "CpG islands" and are present in more than 50 % of human gene promoters [88]. Hypermethylation of CpG islands within gene promoters has been shown to lead to gene silencing, while promoters of transcriptionally active genes are typically hypomethylated [77]. In addition to DNA methylation, other epigenetic changes, such as histone modification and miRNAs, can affect gene expression.

## Histone Modification

Histone modifications typically occur as post-translational alterations at the N-terminal of histones. These histone alterations include acetylation, methylation, phosphorylation, biotinylation, and ubiquitination and play a fundamental role in protein regulation throughout life [11, 14, 53]. miRNAs appear to have a fundamental role in the biology of the cell. They constitute a class of non-coding RNA molecules, which have now emerged as key players in regulating the activity of mRNA. miRNAs are small RNA molecules approximately 22 nucleotides in length, which affect the activity of specific mRNA by influencing their half-life through interference with the normal mRNA degradation process or mRNA translation into proteins [37].

Studies have suggested that alterations in histone modifications are important in PCa [38]. Immunohistochemical analysis of primary prostatectomy tissue samples revealed an association of H3K18Ac, H3K4Me2, H4K12Ac, and H3K9Ac with increasing tumor grade [20]. Remarkably, by analyzing the percentile staining of

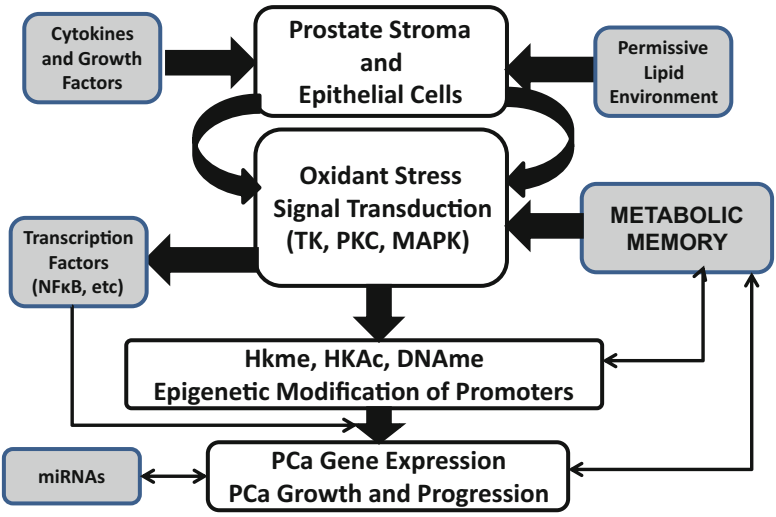


**Fig. 10.1** Epigenetic mechanisms in prostate cancer. Schematic diagram of the role of epigenetic mechanisms in PCa and metabolic memory of prostatic stroma and epithelial cells implicated in prostate carcinogenesis and progression to advanced disease. Inflammation via systemic circulation and local influx of inflammatory cells and a permissive lipid environment can be secondary to sedentary lifestyle and a Western Diet lead to a permissive lipid environment with pro-inflammatory lipids and lipid-derived eicosanoids as well as production of pro-inflammatory mediators such as cytokines and growth factors. Together, these stimuli activate multiple signal transduction pathways including oxidant stress, tyrosine kinases (TK), PKC, and MAPKs leading to activation of transcription factors (TFs) such as NF-kB, and dysregulation of epigenetic mechanisms including HKme, histone lysine acetylation (HKAc), and DNA methylation (DNAme) via the action of corresponding methyltransferases, demethylases, acetylases, and deacetylases. In addition, miRNAs can further fine-tune the expression of key players involved in these pathways. The net outcome of these events is the loss of repressive chromatin marks and gain of activation marks, leading to the formation of open chromatin state at the promoters of pathological genes allowing increased access to transcription factors. Persistence of this altered state of the epigenome through unknown mechanisms can lead to "metabolic memory" represented by enzymatic metabolic pathways of substrate metabolism linked with chronic inflammation, and a shift in metabolic pathways to promote cell growth with continuing field effects in the prostate (*HMTs* histone methyltransferases, *HDMs* histone demethylases, *HATs* histone acetyltransferases, *HDACs* histone deacetylases, *DNMTs* DNA methyltransferases, *DeMet* DNA demethylases)

just two modifications, H3K4Me2 and H3K18Ac, patients could be grouped into lower and higher risk recurrence risk groups. In normal prostate cells, active gene promoters are associated with H3 lysine 4 methylation (H3K4me2 and H3K4me3) and H3 lysine 9 acetylation (H3K9acetyl). These are replaced by repressive marks (H3K9me2, H3K9me3, and H3K27me3) in transcriptionally silenced genes [38]. H4K20me1 specifically identifies CRPC, while H4K20me2 distinguishes different stages of PCa [38].

## Diet, MicroRNA, and Prostate Cancer

MicroRNAs (miRNAs) constitute an evolutionarily conserved class of small non-coding RNAs that are endogenously expressed with crucial functions in fundamental cellular processes such as cell cycle, apoptosis, and differentiation [68]. Disturbance of miRNA expression and function leads to deregulation of basic cellular processes leading to tumorigenesis. A growing body of experimental evidence suggests that human tumors have deregulated expression of microRNAs, which have been proposed as novel oncogenes or tumor suppressors. Recent studies have shown that microRNA expression patterns serve as phenotypic signatures of different cancers and could be used as diagnostic, prognostic, and therapeutic tools [68].

A few studies have analyzed global microRNA expression profiles or the functional role of microRNAs in prostate cancer. Here we have reviewed the role of microRNAs in prostate carcinogenesis by summarizing the findings from such studies. In addition, recent evidence indicates that dietary factors play an important role in the process of carcinogenesis through modulation of miRNA expression, though such studies are lacking in regard to prostate cancer. It has been proposed that dietary modulation of miRNA expression may contribute to the cancer-protective effects of dietary components.

## Nutrition and Epigenetics

Nutrition can potentially affect epigenetic phenomena. For example, nutrition can act at multiple points in DNA methylation [46]. First, nutrients are the main source of methyl groups or act as coenzymes for the one-carbon metabolism that regulates methyl transfer and DNA synthesis. For example, B vitamins, such as folic acid, vitamin B2, B6, and B12, are involved as coenzymes with methionine, choline, betaine, and serine as universal methyl donors [2]. Second, a number of phytochemicals found in plant foods and in dietary supplements alter the epigenetic processes by influencing the activation of enzymes such as 5-cytosine DNA methyltransferase (DNMT).

Phytochemicals including polyphenols (green tea catechins, quercetin, myricetin), soy isoflavones (genistein), parthenolide, curcumin, resveratrol, isothiocyantes, and butyrate, an intestinal product from fiber, affect the activities of methylation enzymes [25, 30, 46, 53, 78]. Third, dietary components, such as retinoic acid and vitamin D, bind to their receptors and modulate gene expression leading to competitive downregulation of methylating enzymes [64, 76]. In addition, other phytochemicals, such as garlic diallyl disulfide, sulfurophane, and indol-3-carbinol, impact the epigenome through histone modulation and regulation of miRNAs [76].

DNA methylation is catalyzed by the enzyme 5-cytosine DNMT with *S*-adenosylmethionine (SAM) as the methyl donor. There are three main DNMT enzymes: DNMT1, DNMT3a, and DNMT3b [26, 70]. DNMT1 is a maintenance methyltransferase maintaining DNA methylation patterns in DNA replication during cell division [78], whereas both DNMT3a and DNMT3b are involved in de novo methyltransferase processes, providing an important function during development (differentiation) [36, 41]. DNA methylation has evolved as an attractive target in cancer therapeutics.

## Alterations in Gene Expression of DNA Methyltransferase Enzymes

Altered DNMT gene expression and enzyme activity is seen in numerous chronic age-related diseases influenced by nutritional status and energy balance including cardiovascular diseases [4, 90], Type 2 diabetes [9], obesity [71], neurodegenerative diseases [3], and several common forms of cancer including prostate cancer [13, 21, 53].

In cancer, both DNA hypo- and hypermethylation have been demonstrated to be associated with disease progression. Methylation during cancer development includes hypermethylation of specific gene promoters, in addition to generalized hypomethylation. DNA hypermethylation in cancer often causes the silencing of tumor suppressors and other genes important for cellular growth, regulation, and differentiation [1]. DNA hypomethylation has been shown to result in chromosomal instability and increased mutation events in several forms of cancer [32, 89].

Yang et al. demonstrated a decrease in global cytosine hypomethylation comparing low-grade prostate epithelial neoplasia, high-grade prostate epithelial neoplasia, and prostate cancer tissue, using immunohistochemistry [93]. However, hypomethylation is not as commonly observed in prostate cancer, with only a handful of specific genes being hypomethylated. The majority of genes in prostate cancer are characterized by site-specific hypermethylation [47]. The evaluation of a panel of methylation markers, such as APC, RARβ2, TIG1, and GSTP1, demonstrated that utilizing the information derived from the methylation status of the gene panel, in combination with histological tissue evaluation, increased the percentage of detection of carcinoma from 64 to 97 % compared with using histological tissue evaluation alone [80]. Analysis of the methylation status of 219 prostatectomy tissue samples using a panel of three genes (*APC*, *HOXD3,* and *TGF*β) demonstrated that an increase in methylation was associated with prostate cancer progression [80]. Evaluation of DNA methylation of these three genes was superior for the prediction of biochemical recurrence compared with individual genes [80]. Importantly, many of these methylation events were also found in early high-grade prostatic intraepithelial neoplasia lesions [93], suggesting that aberrant DNA methylation changes occur early during carcinogenesis [34].

## Aging and Epigenetic Changes in the Prostate

While most cancer incidence is reported as its age-related incidence recognizing that age is a risk factor for most cancers, sporadic prostate cancer is particularly a disease of aging with histologic prostate cancer being found in 60 % of men by the age of 70 years and 80 % of men by age 80 [69]. Epigenetic changes in the prostate may mediate the age-related increase in cancer observed due to the effects of environmental and internal biological factors including oxidant stress. Studies in monozygotic twins have demonstrated epigenetic changes resulting from environmental exposures [23]. A field effect due to these changes has been proposed as an explanation for the observation of multifocal prostate cancers with different genetic signatures in prostatectomy specimens [17]. A number of genetic and epigenetic changes throughout the histologically normal aging prostate have recently been identified.

A shift in the prooxidant-antioxidant balance occurs with aging in many organs including the prostate. As the balance of oxidant stress shifts to produce more reactive oxygen species, inflammation and histologic lesions such as proliferative-inflammatory atrophy (PIA) occur with increased frequency in the aging prostate [15]. When compared to other aging organs, the prostate expresses remarkably high levels of lipofuscein, a breakdown product of oxidatively damaged proteins [57]. Oxidative damage to DNA can be measured by the accumulation of nuclear 8-hydroxy-deoxyguanosine (OHdG) and an accumulation of this adduct occurs in aging prostate tissues [49]. Associated with this increased oxidative DNA damage are alterations in DNA methylation including genome-wide hypomethylation [82], as well as altered methylation at specific CpG islands [29].

## Energy Balance, Diet, and Prostate Cancer

Positive energy balance most commonly is the result of the combined effects of poor dietary habits, physical activity, and common genetic tendency to store excess energy as abdominal fat [50]. The metabolic complications of obesity are due to excess body fat rather than simply excess body weight, and in sarcopenic obesity in elderly men, there is a decrease in lean body mass and an increase in body fat in the presence of normal or increased body weight.

Androgens which tend to fall with aging are important determinants of body composition in men. In healthy men, reductions in serum testosterone levels correlate with reduced lean body mass and positively with fat mass [83]. Androgen deprivation therapy (ADT) is frequently used in advanced prostate cancer and significantly decreases lean body mass and increases fat mass in men with PCa [7, 73, 74, 75, 79]. In two prospective studies of men with non-metastatic PCa, ADT decreased lean body mass by 2.7–3.8 % and increased fat mass by 9.4–11.0 % from baseline to 1 year ($p < 0.001$ for each comparison) [73, 75].

Inconsistent results regarding the association between obesity and prostate cancer have been found. However, these studies need to be interpreted in light of the fact that BMI is an imperfect assessment of excess body fat as excess weight can be due to increased lean or fat in a given individual. Nonetheless, as some studies of adult BMI and the incidences of all prostate cancers have yielded null results, other studies have reported that adult BMI is associated with a decrease in prostate cancer risk among men who are diagnosed before 60 years of age and those with family histories of prostate cancer [67]. However, when the association between obesity and prostate cancer is evaluated while accounting for the stage or grade at diagnosis, adult BMI has been positively associated with the risk of more aggressive tumors (RR BMI.30 kg/m$^2$ 1.23 [95 % CI, 1.00–1.55]) and inversely associated with the risk of less-aggressive tumors (RR BMI. 30 kg/m$_2$ 0.86 [95 % CI, 0.77–1.06]) [67].

Abdominal obesity promotes chronic low-grade inflammation that results in oxidative stress due to the dysfunction of adipose tissue and the alterations of adipocyte-derived hormone secretion and cytokine synthesis. Adipose tissue dysfunction results in increased systemic levels of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-alpha), interleukin 6 (IL-6), C-reactive protein, and matrix metalloproteinases [16]. Inflammation induced by adipocyte dysfunction induces increases in the release and accumulation of reactive oxygen species (ROS). Additionally, obesity alone induces an excessive generation of ROS due to inefficient energy metabolism [12]. Such obesity-related inflammatory and oxidative stress has been hypothesized to be a link between obesity and its comorbidities [84].

Disturbances in energy balance in obesity [95] can lead to stable epigenetic changes in adult tissues and may affect the health of the organism, as recently reviewed by Gut and Verdin [28].

Oxidative stress induces DNA damage (e.g., base modifications, deletions, strand breakages, and chromosomal rearrangements) that reduces the ability of DNA to be methylated by DNA methyltransferases (DNMTs) and results in global hypomethylation [24, 85]. Additionally, ROS can induce the hypermethylation of certain tumor suppressor genes and thus promote carcinogenesis [27, 45]. Moreover, oxidative damage has also been implicated in the regulation of histone modifications and microRNA expression [51, 66, 72]. Inflammation also induces epigenetic alterations in tissues that are associated with disease manifestations, as revealed by recent therapeutic interventions utilizing histone deacetylase and DNMT inhibitors, the effects of certain anti-inflammatory dietary elements on DNA methylation and chromatin remodeling, and the actions of several inflammatory-related transcription factors such as nuclear factor kappa B (NFkB) [55].

## Green Tea and DNA Methylation in the Prostate

Increasing interest in the potential of changing diet and lifestyle or consuming dietary supplements to alter the epigenome has led to a growing body of research focusing on the potential of dietary components and natural products as epigenetic agents in chemoprevention and cancer treatment.

Green tea polyphenols have been shown to inhibit DNA methylation in vitro, leading to hypomethylation and activation of epigenetically silenced genes [20, 38, 44, 54, 56, 60, 92]. Extensive in vitro experiments have been performed in a variety of cancer cell lines to evaluate the effect of green tea polyphenols on DNA methylation. Original studies on the investigation of the effect of EGCG on DNA methylation in cell culture were performed by the laboratory of C.S. Yang (Rutgers, NJ) and are summarized in a review article by Fang et al. [18].

Treatment of LNCaP human prostate cancer cells with a green tea extract (Polyphenon E) was associated with a time- and dose-dependent activation of GSTP1. The hypermethylation and downregulation of GSTP1 has been associated with the development of several types of cancer including cancer of the prostate [63]. The inhibition of DNMT1 protein expression in LNCaP prostate cancer cells treated with 10 μg/mL of Polyphenon E was strongest at 14 days of treatment and was associated with decreased methylation of the promoter region of GSTP-1 [63].

Despite the above-mentioned studies, the ability of EGCG to inhibit DNA methylation remains controversial. Stresemann et al. argued that in some in vitro cell culture conditions, cellular effects induced by EGCG could probably be attributed to the oxidative stress induced by this compound. At neutral or alkaline pH, EGCG undergoes autooxidation resulting in dimerization of EGCG and EGC to form homo- and heterodimers in an alkaline environment with concurrent formation of hydrogen peroxide [61, 62]. This process is ubiquitous in in vitro experiments and during the intestinal digestion, but the degree of autooxidation depends on the cell culture medium. In cell culture medium, the indirect contribution of $H_2O_2$ formation can be avoided by the addition of superoxide dismutase or catalase prior to adding the tea polyphenols [94]. But the majority of cell culture experiments did not address the hydrogen peroxide formation. To summarize, in vitro cell culture studies provide clear evidence that green tea polyphenol treatment can alter DNA methylation, leading to re-expression of silenced genes. To achieve changes in DNA methylation, concentrations of 20–50 μmol/L of EGCG for 3-6 days are needed. These concentrations are much higher than physiologically achievable in mouse or human tissue. These effects of green tea on DNA methylation require further studies in human prostate cancer under appropriate conditions for confirmation of the effect.

Whether EGCG can reverse DNA hypermethylation and reactivate methylation-silenced genes in vivo still remains to be determined. Based on the evidence from in vitro cell culture studies, it is of interest to investigate the effect of green tea polyphenols on epigenetic processes in vivo. Potential mechanisms are the inhibition of DNMT1 activity directly or a competitive inhibition by depletion of its substrate SAM or accumulation of the inhibitor SAH [42, 64]. The delay in the development of prostate cancer by administration of the DNA methyltransferase inhibitor 5-aza-dC to TRAMP mice has been demonstrated as a “proof of principle” that cancer prevention may be achieved through epigenetic modifications [52]. Analysis of untreated TRAMP prostate lesions demonstrated elevated DNMT1 mRNA and protein levels in early stages of prostate cancer development (prostatic intraepithelial neoplasia), which continued through advanced prostate cancer and metastasis. In an 5-Aza-dC intervention study, zero of 14 TRAMP

mice receiving I.P. injections twice weekly on consecutive days of 300 μL 5-Aza-dC (0.25 mg/kg) developed prostate cancer at 24 weeks of age, whereas 7 of 13 (54 %) control mice, injected with PBS, developed poorly differentiated prostate cancer [52].

Fang et al. addressed the question whether the administration of EGCG in mice could lead to a decrease in SAM coinciding with the accumulation of SAH, which in turn could induce competitive inhibition of the DNMT activity [18]. They examined this issue in their ongoing experiments on bioavailability, toxicity, and cancer-preventive activities of EGCG. The results showed that only an acute intra-gastric treatment with high doses of EGCG (500–2000 mg/kg) elevated plasma levels of homocysteine significantly and at the same time decreased the levels of plasma methionine and lowered the concentration of hepatic and intestinal SAM as well as the SAM:SAH ratio [40]. The administration of EGCG (or Polyphenon E) through drinking fluid (0.32 % EGCG or 0.5 % Polyphenon E) decreased intestinal SAM concentrations moderately without increasing the level of SAH. No changes of hepatic SAM or SAH levels were observed with the administration of EGCG in the drinking water [18].

In our investigation of the effect of drinking brewed green tea instead of drinking water on tumor growth and DNA methyltransferase activity in male severe combined immunodeficiency (SCID) mice, we determined an inhibition of DNMT1 protein and gene expression in prostate xenograft LAPC4 tumor tissue [33]. The green tea contained a concentration of 0.07 % of GTPs and was administered for 13 weeks. Tumor volume and weight were also decreased significantly in mice drinking the GT compared to the water control [33].

There is an increase in the use of combinations of natural products in order to overcome multidrug resistance, limited bioavailability, or to target multiple mechanisms concurrently. For example, in our laboratory a combination of GT and quercetin increased the bioavailability and decreased EGCG methylation, leading to an increase in the anticarcinogenic activity in a prostate cancer xenograft mouse model [87]. In addition, cell culture studies demonstrated that the combination of green tea polyphenols with sulforaphane increased apoptosis and altered Nrf2 and AP-1-regulated gene expression in prostate cancer cells [58]. Combining natural products that alter the epigenome will enhance the epigenetic effect since some compounds may alter DNA methylation and other natural compounds may affect histone structure and miRNA regulation [48, 81].

Evidence for the role of green tea polyphenols in affecting DNA methylation in cancer development is mainly based on in vitro cell culture experiments. However, since dietary modifications induce relatively low impact changes on DNA methylation with lower toxicity compared to epigenetic therapeutic drugs, dietary strategies may play an important role in the prevention of carcinogenesis. Moreover, dietary exposures are long-term and potentially repeated several times daily in heavy tea drinkers, for instance. There is a critical need for future investigations in animal and human studies to reveal the potential of different bioactive and dietary components in the epigenetic regulation of chronic disease.

## Soy Isoflavones and Prostate Epigenetics

Population studies suggest that soy isoflavones (genistein and daidzein) may be implicated in the change of incidence of prostate cancer based upon foods typically consumed in different cultures. The consumption of soy isoflavones amounts to about 30 mg/day in Asia, while it is practically zero in the rest of the world [59]. Soy isoflavones have an antiproliferative effect on prostate tumor cells in vitro, demonstrated by an arrest of the cell cycle in G2/M phase after genistein treatment and in G0/G1 phase with daidzein [31] on prostate carcinogenesis in animal models [86].

The epigenetic mechanisms underlying the potentiation of the anticancer effects of chemotherapeutic treatment in prostate cancer have been studied [43]. Recent work studying the effect of a soy isoflavone treatment on cells of prostate cancer has shown a re-expression of *p16*, retinoic acid-related receptors β (*RAR β*), and *O*-6-methylguanine-DNA methyltransferase (*MGMT*) genes by demethylation of their promoters under the effect of genistein [19]. The molecular mechanisms explaining these effects are not yet established.

## Conclusion and Future Research Needs

While it is likely that diet and lifestyle have effects on gene expression that predispose to prostate cancer or contribute to its progression, much more research is needed on polyphenols other than those from green tea and soy. In addition, the effects of positive energy balance and excess intra-abdominal fat have not been adequately studied. The data supporting the systemic inflammatory effects of adipocytes is substantial and there is emerging data on the impact of inflammation on prostate cancer through the development of proliferative inflammatory atrophy in the prostate gland. Research on epigenetics and prostate cancer promises to provide new direction for nutritional approaches to prostate cancer prevention and treatment.

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# Chapter 11
# Effects of Physical Activity on DNA Methylation and Associations with Breast Cancer

**Herbert Yu and Melinda L. Irwin**

**Abstract** Physical activity is associated with reduced breast cancer risk and improved disease outcome. Initial research indicates that physical activity can lower circulating levels of sex steroid hormones, improve insulin resistance, and reduce mitogenic growth factors. Recent investigation suggests that physical activity may have more profound impact on epigenetic regulation. As a key component of epigenetic regulation, DNA methylation has been found to be altered by physical activity. Physical activity may reduce DNA methylation in tumor suppressor genes and increase methylation in the genes encoding pro-inflammatory cytokines. Studies also suggest that physical activity may change the landscape of DNA methylation by increasing genome-wide methylation. These characteristic changes in DNA methylation are also observed in experimental studies in which healthy individuals or breast cancer patients with increased physical activity had significantly reduced DNA methylation in tumor suppressor genes and elevated methylation in the entire genome. All these molecular changes are consistent with the observations that physical activity is associated with low cancer risk and better patient survival, which explains the molecular mechanisms that underlie the beneficial effects of physical activity on cancer prevention and management.

**Keywords** Physical activity • Breast cancer • Tumor suppressor • Inflammation • Exercise-dependent DNA methylation • L3MBTL • Tumor suppression • Methylation exercise

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## Background

Epidemiological studies have demonstrated that high levels of physical activity are associated with lower breast cancer risk [1]. It is estimated that the risk is lowered by 20–40 % [2]. Breast cancer survival has also been found to be improved by physical activity [3]. One study involving 4482 breast cancer patients discovered that women who spent 21 or more Metabolic Equivalent of Task (MET) hours/week (about 5 hours/week) after diagnosis had a 50 % reduction in breast cancer death in comparison to those who spent less than 2.8 MET hours/week in physical activity [4]. This reduction in breast cancer death seems to be independent from other risk factors for disease recurrence, such as disease stage, tumor grade, body mass index, family history of breast cancer, and treatment. The Nurses' Health Study, which identified 2987 breast cancer patients during a 15-year follow-up, also found that patients who spent more than 24 MET hours/week in physical activity after diagnosis experienced a 40 % reduction in breast cancer death when comparing to those who had less than 3 MET hours/week [5]. Two additional patient cohorts assessed the effect of physical activity on disease outcome before diagnosis. One with 1231 Canadian women found high physical activity associated with low breast cancer mortality [6], while the other of 1970 American patients showed no effect on breast cancer death, but reduced all-cause mortality [7]. A review by Ballard-Barbash et al. concludes that evidence of recommended amounts of 2.5 hours/week of physical activity and a lower breast cancer mortality is consistent and compelling [8]. A large pooled study with over 13,000 patients indicates that women who meet the guideline for physical activity at 10 MET hours/week experience a 25 % reduction in breast cancer mortality [9].

The beneficial effect of physical activity on breast cancer risk and survival has been attributed to a number of biologic mechanisms including its influence on sex steroid hormones and mitogenic growth factors. A randomized clinical trial showed that vigorous-intensity physical activity could lower circulating levels of estrogens and androgens among overweight postmenopausal women [10, 11]. Physical activity also prolongs patient survival through improving insulin resistance [12, 13]. Several clinical trials including our own have demonstrated that physical activity can reduce circulating levels of insulin and insulin-like growth factors (IGFs) that are known to be risk factors for breast cancer development and progression [14–18]. Besides its influences on sex hormones and mitogenic growth factors, physical activity's impact on epigenetic regulation has also been speculated and investigated lately.

Aberrant epigenetic regulation is involved in many chronic diseases including cancer. Changes in epigenetic regulation are intimately influenced by various environmental exposures and lifestyle factors, such as cigarette smoking, dietary nutrient intake, obesity, and physical activity. As a major component of epigenetic regulation, DNA methylation shares similar features of alterations affected by exogenous factors [19, 20]. Studies have shown substantial changes in DNA methylation during the development and progression of cancer [21, 22]. The characteristic changes include hypomethylation of oncogenes and hypermethylation of tumor

suppressor genes [23], which leads to the activation of oncogenes and suppression of tumor suppressor genes, respectively. A combination of these changes is known to be the mechanism of carcinogenesis. In addition to the fact that many tumor suppressor genes are found to be hypermethylated in cancer, cancerous cells contain thousands of aberrantly methylated genes compared to normal cells and demonstrate genome-wide hypomethylation and regional hypermethylation [24, 25]. Aggressive tumors also have distinct methylation profiles compared to less aggressive ones [26], and methylation changes occur to both intragenic and intergenic regions [27]. All these observations underscore the importance of DNA methylation in cancer development and provide strong rationale for us to speculate that DNA methylation may mediate the beneficial effects of physical activity on the reduction of cancer risk and mortality.

## Observational Studies on Methylation in Specific Genes and Physical Activity

Several human studies have found indications that DNA methylation may be involved in the beneficial impact of physical activity on cancer risk. Coyle et al. collected breast tissue samples from 45 healthy women and analyzed promoter methylation in two tumor suppressor genes, *APC* and *RASSF1A*, to determine if methylation levels in these genes were associated with physical activity after adjusting for other breast cancer risk factors. The study found that higher levels of physical activity were associated with lower methylation in the *APC* gene, although the associations were not statistically significant [28]. Low methylation in the gene suggests active *APC* expression in physically active women. Since APC (adenomatous polyposis coli) suppresses β-catenin and interacts with E-cadherin, regulating cell adhesion and division, and since loss of APC activity is seen often in cancer, an active *APC* gene is speculated beneficial for preventing cancer development or progression. A similar association between physical activity and tumor suppressor gene methylation was also observed in another small observational study of tumor samples in Japan. Yuasa et al. reported that low methylation in a tumor suppressor gene *CACNA2D3* (a calcium channel-related gene) was correlated with higher physical activity in the past in 106 patients with gastric cancer [29].

Inflammation is another important biologic mechanism that is involved in carcinogenesis and tumor progression. Nakajima et al. conducted an observational study to assess the effect of physical exercise on DNA methylation [30]. The investigation included 230 exercisers aged between 40 and 87 years, as well as 153 elder and 34 young controls. The exercise regimen was high-intensity interval walking, average 52 min per day, for 6 months. DNA methylation was measured in the *ASC* gene in peripheral blood using bisulfite pyrosequencing. The results showed that exercise individuals had significantly higher methylation in *ASC* compared to the controls [30]. ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), also known as PYCARD, has been

reported to up-regulate several pro-inflammatory cytokines such as IL-6, IL-8, IL-1β, and TNF-α. Given these pro-inflammatory connections, exercise-associated hypermethylation of *ASC* is considered to be consistent with the knowledge that physical activity can improve the pro-inflammatory condition resulting from obesity or insulin resistance. This finding provides the first human evidence that physical exercise may affect inflammation through epigenetic regulation. In a follow-up study by the same group of investigators, Zhang et al. compared genome-wide methylation profiles between seven exercisers and six controls using the Agilent Human CpG Island Array 244 K assay [31]. The comparison showed that high physical activity was associated with increased methylation in the *NFkB2* gene, and hypermethylation of this gene was believed to down-regulate its activity that is pro-inflammatory. The authors concluded that this finding was consistent with their previous observation on ASC, which further strengthened their assertion that the health benefits of long-term exercise were partially explained by its ability to curb excessive inflammation. Similar associations between exercise and inflammatory cytokines were also observed in animal experiments (discussed below).

Another study by Shaw et al. [32] also found that elderly men and women who had increased physical activity over time had hypermethylated TNF-α and hypomethylated IL-10 compared to those who had decreased physical activity. As IL-10 is an anti-inflammatory cytokine, these changes in methylation indicates that the activity of pro-inflammation may be declined in physically active individuals through epigenetic regulation.

## Observational Studies on Genome-wide Methylation and Physical Activity

In addition to its effects on specific genes, such as tumor suppressors and inflammatory cytokines, observational studies also found that physical activity could affect changes of genome-wide methylation and that the impact was consistent with the notion that physical activity has beneficial effects on cancer risk. One study measured *LINE-1* methylation in the blood of 647 Caucasian women who had a family history of breast cancer [33]. The study found that physical activity was positively associated with *LINE-1* methylation, i.e., higher levels of activity correlated with hypermethylation, suggesting increased global methylation in physically active women than in physically inactive ones. *LINE-1* (long interspersed element-1) is one of the long repeated elements which appear in high copy numbers in the genome. Methylation in the elements is regarded as an indicator of global hypomethylation, and global hyomethylation is associated with genome instability, resulting in high genetic mutations and increased cancer risk [34]. As cancer cells display global hypomethylation, increasing genome-wide methylation associated with physical activity is consistent with the understanding of physical activity's beneficial effects on cancer. A similar association between *LINE-1* methylation and physical activity

was also observed in a smaller study in which *LINE-1* methylation was measured by quantitative methylation-specific PCR in the peripheral blood of 161 healthy individuals whose physical activities were measured by accelerometer [35]. Using the Illumina HumanMethylation450 BeadChip to measure methylation in nearly half a million CpG sites in pre-diagnostic peripheral blood DNA, Severi et al. found that high global methylation was associated with lower breast cancer risk, a finding that not only supports the notion of global hypomethylation in cancer, but also indicates that the changes in global methylation occur before the diagnosis of cancer, suggesting an etiologic role of the event in cancer [36].

## Experimental Studies on Methylation in Specific Genes and Physical activity

Besides observational investigation, experimental studies also support the speculation that physical activity may enhance the activities of tumor suppressor genes through epigenetic regulation. We conducted a randomized controlled clinical trial to evaluate the effects of physical activity on breast cancer survival. In the trial, we recruited 75 physically inactive postmenopausal women diagnosed with stage 0-IIIA breast cancer [37]. Of them, 37 were assigned to an exercise group and 38 were to a usual care group. Women in the exercise group participated in a six-month exercise intervention consisting of 150 min per week of supervised moderate-intensity aerobic exercise (primarily brisk walking on a graded treadmill). Women in the usual care group were instructed to maintain their regular activities. At baseline and six months of the trial, participants provided blood samples and detailed information on physical activity and diet. Levels of aerobic activity were determined by the MET levels using the established method. To test if DNA methylation was affected by the 6-month exercise intervention, we randomly selected 12 women from the trial participants, six in each group, and analyzed the changes of DNA methylation in 24 blood samples collected before and after the exercise intervention [38]. Genomic DNA was extracted from peripheral blood leukocytes (PBL) and treated with sodium bisulfite. The bisulfite-converted DNA was analyzed for cytosine methylation using the Illumina HumanMethylation27 BeadChip, which interrogates 27,578 CpG sites in 14,495 genes. For each CpG site, a beta (β) value was calculated using the GenomeStudio (Illumina, CA) which estimates the methylation level based on the signal intensity between the methylated and unmethylated alleles. For each subject, a delta beta ($\Delta\beta = \beta_{\text{6-month}} - \beta_{\text{baseline}}$) was computed to determine changes in methylation before and after the trial. Comparing the methylation profiles before and after the exercise intervention, we found 43 genes having significant change in methylation ($p < 5 \times 10^{-5}$). To evaluate the biologic relevance of these methylation changes to breast cancer, we analyzed patient overall survival in association with the expression of these genes in 204 tumor samples. Of the 43 genes, six had significant associations between high expression and better survival. Among

the six genes, three, *IFT172, EPS15* and *PPP2R3A,* had associations which were not consistent with methylation, i.e., high expression and high methylation, and therefore these genes were not considered for further evaluation. However, the other 3 genes, *GLUD1, L3MBTL1,* and *MSX1*, showed associations consistent with methylation, i.e., high expression and low methylation. Based on the deducted relationships, we speculate that exercise may lower the methylation in these genes, that low methylation leads to high expression, and that high expression may improve the overall survival of breast cancer patients. Of the 3 genes, *GLUD1* encodes glutamate dehydrogenase 1, which is a mitochondrial matrix enzyme regulating energy metabolism and has little evidence suggesting its involvement in cancer [39]. *L3MBTL1* is a known tumor suppressor gene, and *MSX1* has a role in mammary gland development [40, 41]. In our microarray-based methylation analysis, *L3MBTL1* methylation decreased 1.48 % in the exercise group, but increased 2.15 % in the control group, resulting in a net difference of 3.63 % ($p = 2.9 \times 10^{-5}$). *MSX1* methylation reduced 2.02 % in the exercisers and elevated 2.75 % in controls, a net difference of 4.76 % ($p = 3.5 \times 10^{-5}$). To confirm the microarray results, we developed two quantitative methylation-specific PCRs (qMSP) analyzing promoter methylation of *L3MBTL1* and *MSX1* in breast tumor samples. Our analysis showed that methylation in *MSX1* was very low in all breast tumors, averaging 1.25 %. Given the low level of methylation, no further analysis was performed to examine *MSX1* methylation and expression. In contrast to *MSX1*, *L3MBTL1* methylation was high in the tumor samples. The average methylation was 65.6 %, ranging from 10.2 to 98.4 %. Methylation in *L3MBTL1* was inversely correlated with gene expression. High levels of *L3MBTL1* methylation were also associated with increased risk of breast cancer death, although the association was not statistically significant. *L3MBTL1* expression was significantly associated with patient overall survival. Patients with high *L3MBTL1* expression had over a 60 % reduction in risk of breast cancer death compared to those with low expression (HR = 0.37, 95%CI: 0.17–0.80, $p = 0.012$). A dose-response relationship between expression and survival was also significant ($p = 0.014$), and the relationship remained significant after adjustment for covariates and confounders, such as disease stage, tumor grade, and hormone receptor status [38]. Using the qMSP, we also measured *L3MBTL1* methylation in the blood samples of 68 women enrolled in our Yale Exercise and Survivorship Study. Of the 35 patients who were randomized to the exercise group, 17 (48.6 %) had declined methylation in *L3MBTL1*, compared to 11 of 33 (33.3 %) in the non-exercise group. After adjusting for age, disease stage, adjuvant treatment, and BMI, the odds of having a decrease in methylation in L3MBTL1 was 2.67 in the exercisers compared to non-exercisers ($p = 0.079$). Although not statistically significant, the analysis indicates that exercise may lower DNA methylation in the tumor suppressor gene *L3MBTL1*.

Based on these results, increasing physical activity in breast cancer patients may lower methylation in the *L3MBTL1* gene and low methylation may increase *L3MBTL1* expression. High expression of *L3MBTL1* in breast tumor samples is found to be associated with better survival outcomes. All these relationships seem to be consistent with the knowledge of L3MBTL1 being a tumor suppressor.

L3MBTL1 (also known as L3MBTL) belongs to the polycomb group (PcG) proteins. Acting as a transcriptional repressor, L3MBTL1 binds to several methylated lysines in H1b, H3, and H4, blocking the access of DNA sequences for transcription [42, 43]. L3MBTL1 is encoded by the *L3MBTL1* gene which is located on chromosome 20q12 in a region that is frequently deleted in patients with myeloid hematopoietic malignancies. Homozygous deletion of this gene causes brain tumors in Drosophila. Other members of the L3MBTL family are also found to be related to cancer. Single or double-strand deletion of *L3MBTL2* and *L3MBTL3* has been reported to occur in medulloblastoma [44]. *L3MBTL4* was found to be frequently mutated or deleted with loss of function in breast cancer [45]. A recent study indicates that *L3MBTL1* suppresses many genes and microRNAs which express in early stages of germ cells and germ line stem cells, and these genes are often reactivated during tumor growth when animals lose the function of L3MBTL1 [46]. Currently, little is known about the role of L3MBTL1 in breast cancer and its relation to lifestyle factors such as physical activity. Given that physical activity has significant physiological impacts on multiple tissues and that L3MBTL1 regulates chromatin activity which can affect many genes, a functional connection between exercise and L3MBTL1 activity seems to be plausible. Furthermore, if a connection between L3MBTL1 and physical activity can be established, it also suggests that exercise-induced methylation changes in certain genes may trigger further reaction in epigenetic regulation which involves histone modifications.

Another experimental study suggests that physical activity may change DNA methylation in genes associated with breast cancer [47]. In a 12-month randomized controlled trial, Bryan et al. measured DNA methylation in the saliva of 64 healthy individuals, 27 exercisers, and 37 controls, using the Illumina HumanMethylation27 BeadChip. Physical activity was measured both subjectively and objectively by self-reported 7-day recall and maximal oxygen consumption, respectively. DNA methylation in saliva samples were compared between the two groups at baseline and 12-month of the trial. A composite variable of DNA methylation was constructed for comparison, and the composite was a linear numerical number consisting of 45 CpG sites in 28 breast cancer-related genes, including *BRCA1* and *BRCA2*. These genes were selected on the basis of prior studies which suggested that these genes were hypermethylated in breast cancer. The investigation showed that physical activity measured by the 7-day recall method was inversely correlated with methylation, i.e., higher activity and lower methylation of these genes. This correlation remained significant after the relationship was adjusted for confounding factors, such as age, body mass index, and baseline physical activity. The observation supports the notion that DNA methylation mediates the beneficial effects of physical activity on breast cancer. Maximal oxygen consumption was not found to be associated with changes in the methylation composite at 12 months of the trial, but at baseline, suggesting that the oxygen consumption may not continue to rise after pulmonary function is improved to adopt the physical need. In addition to methylation in specific genes related to breast cancer, the authors also analyzed the relationship between physical

activity and methylation across all the CpG sites measured by the microarray chip. The analysis showed that higher amounts of physical activity were correlated with increased overall methylation, a phenomenon that is consistent with the findings of observational studies which demonstrated a positive association between physical activity and methylation in *LINE-1* discussed earlier [33, 35]. When comparing the results of this study with others, another interesting finding is that the methylation's connection to physical activity, either based on specific breast cancer-related genes or genome-wide markers, is the same between blood and saliva samples, suggesting that methylation changes affected or induced by physical activity may occur to multiple tissues and organs.

Ronn et al. conducted a 6-month exercise intervention on 23 healthy individuals who were physically inactive at the beginning [48]. The study was designed to evaluate the effect of moderate-intensity exercise on DNA methylation in adipose tissues using the Illumina HumanMethylation450 BeadChip to interrogate nearly half a million of CpG sites. The researchers found that after 6 months of exercise DNA methylation increased globally in these individuals compared to their baseline level. This observation was consistent with the findings of other studies that showed increased global hypermethylation in multiple tissues in association with physical activity. Besides changes in global methylation, this study also found methylation alterations in individual genes after the exercise intervention.

## Concerns on Human Studies of DNA Methylation

Due to practical constraints, collection of tissue samples for epidemiological study of DNA methylation, especially from healthy individuals, is unattainable. Almost all epidemiological and many clinical studies had to use blood samples to evaluate DNA methylation changes in relation to lifestyle factors. The use of blood as a surrogate tissue in methylation studies has raised concerns on the validity of these investigations because DNA methylation is considered tissue-specific and time-dependent. However, as mentioned above, there has been growing evidence suggesting that substantial similarities exist in DNA methylation across multiple tissues and that DNA methylation in PBL can be informative when biologic activities are considered universal or involving multiple tissues and organs. Two studies have shown that DNA methylation in the *IGF-II* and *ER-α* genes is similar between PBL and colon mucosa [49, 50]. Another study analyzed methylation loci in 8 genes in 34 individuals and found half of the genes showed similar methylation patterns between PBL and buccal cells [51]. Shenker et al. found similar DNA methylation changes associated with cigarette smoking in PBL and lung tumor tissues [52]. We found in our study that exercise-related methylation change in a tumor suppressor gene in PBL was indicative of its role in breast tumors and disease prognosis [38].

Studies of monozygotic twins have shown that methylome changes over time from birth to late adulthood and changes are likely to be affected by lifestyles and environmental factors [53]. These temporal changes in methylome also raise

concerns of the validity of one time cross-sectional assessment of DNA methylation in epidemiological studies. However, evidence suggests that the temporal changes do not occur universally across the genome, and some of the changes take a long time to occur. The study by Talens et al. showed that methylation in many genes was stable for decades in PBL and buccal cells [51]. We conducted a similar study to assess short-term stability of methylation in PBL using the HumanMethylation450 BeadChip [54]. In the study, blood samples from 24 women collected twice in a 10-month interval were analyzed, and comparison of the paired samples revealed that methylated loci with large interpersonal variations had very little changes over time, suggesting methylation in those genomic loci can be assessed reliably in cross-sectional studies if substantial person-to-person variation exists. Genomic loci that have little interpersonal variation are those unmethylated CpG sites, and these loci are unlikely to be the focus of methylation research because of low or no methylation. Another concern over the use of blood samples for methylation study is the heterogeneity of blood cells that may mask the differences in methylation across individuals. Some temporal trends in methylation may also be interpreted as changes in cell compositions. Talents et al. investigated this issue and found that cellular compositions of blood samples contributed little to the variation of DNA methylation between individuals [51]. We examined this issue in our study and found cell compositions did not explain methylation differences when high interpersonal and low intrapersonal variations exist [54].

## Animal Experiments

To explore the biologic mechanisms underlying the protective effect of physical activity on colon cancer, Buehlmeyer et al. conducted an animal experiment in which they compared gene expression profiles in colon mucosa between 6-week old male Wistar rats, which completed a stress-free voluntary treadmill exercise for 12 weeks, and matched sedentary controls. The exercising rats showed significant expression changes in many genes, and one of the genes which had a substantial increase in expression encodes betaine-homocysteine methyltransferase 2 (BHMT2), a DNA methylation enzyme [55], suggesting possible subsequent alterations in DNA methylation catalyzed by this enzyme. In another animal experiment, Lira et al. found that long-term exercising rats had significant reductions of inflammatory cytokines in skeleton muscles, including IL-6, IL-1β, TNF-α, and IL-10, compared to the sedentary animals [56]. This finding supports the observations in humans that exercise can improve the condition of inflammation caused by obesity or insulin-resistance, and as discussed earlier, this connection may be mediated through epigenetic regulation [57].

## Future Perspectives

Studying the effect of physical activity on DNA methylation and cancer risk has two significant implications. It will help not only to understand the mechanisms of physical activity in reducing cancer risk and improving patient survival, but also provide potential opportunities to identify molecular markers that may help to monitor the effect of physical activity on cancer prevention and patient management. Given that the intensity, endurance, and personal preference of physical activity vary widely from person to person, the beneficial effects of physical activity on cancer risk and tumor progression need to be assessed and monitored effectively. If epigenetic markers can be used for these purposes, we can significantly improve the compliance and effectiveness of physical activity in health promotion and disease prevention.

In summary, epidemiological studies have shown that physical activity is associated with reduced breast cancer risk and improved patient survival. Emerging evidence suggests that changes in epigenetic regulation may play an important role in mediating the beneficial effects of physical activity on cancer risk and survival. Both observational and experimental studies have indicated that physical activity may heighten genome-wide DNA methylation, reduce promoter methylation in tumor suppressor genes, and increase methylation in pro-inflammatory cytokines (Fig. 11.1). All these epigenetic changes are beneficial to prevention of cancer

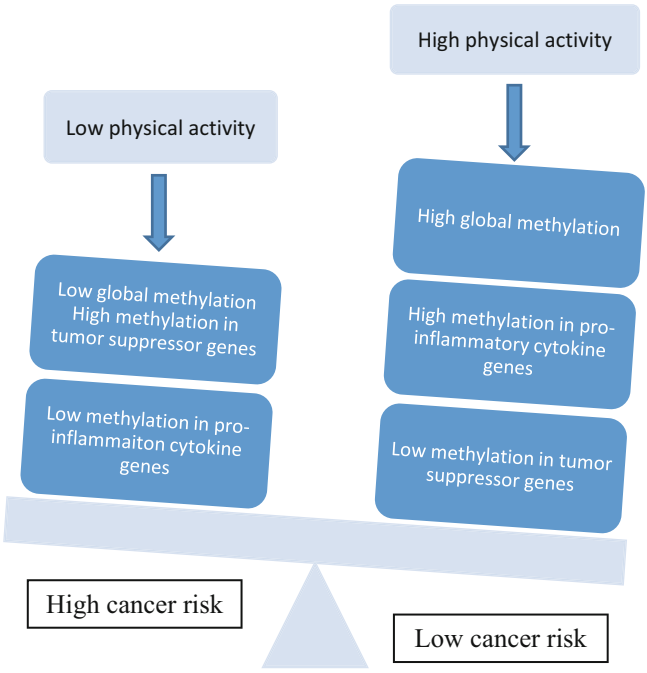


**Fig. 11.1** Possible mechanisms of DNA methylation in connecting physical activity to cancer risk

development and suppression of tumor growth. However, these study findings are still very preliminary and require further validation from large well-designed investigations. If methylation changes can be confirmed to reflect the effects of physical activity, these molecular features may be considered for potential biomarkers to monitor the health benefits of physical activity on cancer prevention.

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