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Metabolic defects provide a spark for the epigenetic switch in cancer

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Abstract

Cancer is a pathology that is associated with aberrant gene expression and an altered metabolism. While changes in gene expression have historically been attributed to mutations, it has become apparent that epigenetic processes also play a critical role in controlling gene expression during carcinogenesis. Global changes in epigenetic processes including DNA methylation and histone modifications have been observed in cancer. These epigenetic alterations can aberrantly silence or activate gene expression during the formation of cancer; however, the process leading to this epigenetic switch in cancer remains unknown. Carcinogenesis is also associated with metabolic defects that increase mitochondrially derived reactive oxygen species, create an atypical redox state, and change the fundamental means by which cells produce energy. Here, we summarize the influence of these metabolic defects on epigenetic processes. Metabolic defects affect epigenetic enzymes by limiting availability of the cofactors like *S*-adenosylmethionine. Increased production of reactive oxygen species alters DNA methylation and histone modifications in tumor cells by oxidizing DNMTs and HMTs, or through direct oxidation of nucleotide bases. Lastly, the Warburg effect and increased glutamine consumption in cancer influences histone acetylation and methylation by affecting the activity of sirtuins and histone demethylases.

Introduction

Carcinogenesis is operationally divided into three discreet steps; *initiation*, an irreversible genetic alteration or mutation that predisposes a clonogenic cell to cancer formation; *promotion*, the clonal expansion of an initiated clonogenic cell that increases the likelihood of additional events occurring on the background of the initiating mutation; and *progression*, the acquisition of additional genetic and epigenetic changes that lead to the generation of diverse phenotypes within a solid tumor during its evolution. During the progression stage in particular, gene expression is globally altered in cancers cells compared to the tissues from which they arise. These changes in gene expression have classically been attributed to the increased rate of mutation and genomic instability seen in cancer. However, over the past decade numerous studies have suggested that epigenetic alterations can be just as effectively alter gene expression in cancer. Epigenetics is managed by two major processes: cytosine methylation, and the post-translational modification of histone tails. Wide-ranging changes are observed for both processes in most types of cancer, and these changes constitute an epigenetic switch. Characterization of this epigenetic switch has clearly established epigenetic dysfunction as an

intrinsic mechanism of carcinogenesis; however, while the effect of the epigenetic switch in cancer is well characterized, its cause remains elusive.

Another hallmark of tumor cells is a metabolic defect which is responsible for altering how tumor cells produce and utilize metabolites. Such metabolic changes lead to increased glycolysis, dysfunctional mitochondrial electron transport, aberrant production of oxidants, and the formation of an atypical redox state. Roles for each of these have been hypothesized to be causal in the initiation, promotion, and progression of the malignant phenotype [1-4]. These hypotheses have centered on the ability of these metabolic changes to elicit genetic alterations during carcinogenesis; however, these alterations are also concomitant with the epigenetic switch in cancer mentioned above. A connection between the epigenetic switch and metabolic defects of cancer was first suggested by Peter Cerutti in 1985. Cerutti aptly proposed that epigenetic processes were disrupted by metabolic defects to causally change gene expression in cancer [5]. However, since Cerutti the depth and breadth of our knowledge regarding the mechanisms of epigenetics and their complexity has grown significantly. We have previously reviewed the ability metabolic changes to elicit epigenetic changes during development [6]. The central theme for this review will be to discuss the novel relationship between metabolic defects and altered epigenetic processes in cancer. We will first discuss how aberrant production of mitochondrial oxidants influences the epigenetic cofactor SAM. Next we will discuss the relationship between the altered redox status of cancer cells and changes in epigenetic processes. Lastly we will introduce the novel concept of how defects in oxidative metabolism might directly influence epigenetics.

The metabolic defect of cancer

As early as the 1920's Otto Warburg and others were measuring fundamental changes in tumor cell metabolism [7,8]. Today, gross metabolic alterations are found in all forms of cancer and center around two major changes: the Warburg effect, and alterations in mitochondrial electron transport. The Warburg effect describes the increased glucose consumption and glycolytic activity of tumor cells (for a recent review see [9]). Increased glucose consumption and glycolytic activity are common in rapidly dividing normal and tumor cells, however the Warburg effect of cancer is accompanied by increased lactate dehydrogenase activity to recycle NADH back into NAD+ and remove pyruvate [10,11]. Lactic acid fermentation, or negative regulation of pyruvate dehydrogenase disrupts the use of pyruvate as a carbon entry source into the Krebs cycle [12,13]. Combined, these defects shift the NAD^+/NADH ratio. It is this aspect of the Warburg effect that we will relate to epigenetic processes in carcinogenesis. Even with limited amounts of pyruvate entering the Krebs cycle, tumor cells continue to produce limited energy via mitochondrial electron transport. Instead of pyruvate, tumor cells use amino acids, like glutamine, as carbon sources for the Krebs cycle. Beginning in the 1970's several groups reported increased glutamine oxidation in cancer [14-16]. Amino acids such as glutamine are great carbon sources for the Krebs cycle because their high concentrations facilitate their passive diffusion in cells, and they can easily be converted into Krebs cycle intermediates [10,17]. Before glutamine can be used as a carbon source for the Krebs cycle it is first converted to glutamate by phosphate-activated glutaminase (PAG) then into α-ketoglutarate by glutamate dehydrogenase (GDH) [18,19]. Modern reports suggest that aberrant glutamine consumption can be attributed to increased expression of both PAG and GDH [18]. Because of lactate fermentation, α-ketoglutarate derived from glutamine, not pyruvate from glucose, becomes the metabolite of entry into the Krebs cycle. This phenomenon has been termed a “truncated Krebs cycle” by Loris Baggetto in which the flux of carbon from α-ketoglutarate to oxaloacetate are much higher than that of citrate to α-ketoglutarate, suggesting carbon is entering the cycle by a means other than pyruvate (i.e. α-ketoglutarate) [17]. The most intriguing aspect of the metabolic defects mentioned above is that their severity increases with cancer progression. The Warburg effect and glutamine consumption are both

higher in poorly differentiated advanced stages of cancer [17-19]. This observation suggests metabolic defects may have a causal role in carcinogenesis.

Free Radicals and Cancer

Tumor cells have increased production of reactive oxygen species (ROS) and an atypical redox balance. The fundamental change in tumor cells that increases their ROS production are defects in mitochondrial electron transport. Tumor cell mitochondrial are rife with structural and functional defects, and may either be the cause or result of increased ROS levels [20]. Confounding this relationship between ROS and mitochondrial defects is that these same cells often exhibit altered expression of antioxidant enzymes. Nevertheless, there is considerable evidence to suggest that the aberrant production of reactive oxygen species in the mitochondria leads to the accumulation of damage that drives carcinogenesis [1,3,4,21]. The expression of antioxidant enzymes like MnSOD, GPx and catalase are often altered in cancer, thus leaving these cells more susceptible to damage from ROS [22,23]. To remedy this, tumor cells decrease their use of mitochondrial electron transport and utilize pyruvate produced during glycolysis as a scavenger of peroxides [24]. But how can aberrant production of ROS drive carcinogenesis? It was first hypothesized by Oberley and Buettner in 1979 that the pro-oxidant state of cancer, created by decreased antioxidant capacity, generates mutations that drive the initiation and progression of cancer [1]. These ideas have since been expanded by others to include metabolic and redox changes in cancer [1,3,21]. The aforementioned hypotheses all centered on the ability of the pro-oxidant state to generate mutations that create the common phenotypic changes associated with cancer. However epigenetic changes are just as capable at inducing phenotypic changes in cancer.

Epigenetics

Epigenetics was a term first coined by Conrad Waddington in 1938. Waddington defined it as “the science concerned with the causal analysis of development” [25]. A more modern understanding of the mechanisms and principles of epigenetics has led to the unified definition of epigenetics proposed by Adrian Bird, where he describes it as “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” [26]. Several epigenetic processes govern the structural adaptation of chromosomes, but paramount among these are cytosine methylation and the post-translational modification of histones. These two mechanisms can work separately, or in concert to control the function of the genome. Epigenetics is most appreciated for its role in regulating transcription; however it has additional roles in DNA repair, DNA replication, and cell division [27,28]. Loss of epigenetic control of transcription, DNA repair, replication, and cell division becomes relevant in many diseases, especially cancer.

DNA methylation

DNA is methylated almost exclusively at cytosines that are part of a CpG di-nucleotides [29]. CpG di-nucleotides are unusual for two reasons. First, they occur at approximately one fifth their expected frequency in the genome compared to the other possible nucleotide doublets, and second they are unevenly distributed towards gene regulatory elements and highly repetitive sequences [30]. CpG dense regions in gene regulatory regions are referred as “CpG islands” by epigeneticists and these have been characterized and operationally defined [31]. As a general rule, methylation of these CpGs is associated with transcriptional repression and condensed chromatin. CpG islands are generally unmethylated in normal cells; however smaller regions, called CpG clusters, can be hypermethylated and silence gene expression [32,33]. Approximately half of all human genes have CpG dense regions located within their regulatory elements, hinting that this mechanism might play a role to regulate gene expression [34]. Methylated CpGs have also been observed in repetitive elements and centromeric repeats

[35]. Loss of DNA methylation in these regions leads to gross changes in chromosome structure and function as observed in ICF syndrome and possibly cancer [36]. Methylated CpG di-nucleotides influence DNA in two ways, by inhibiting protein binding, and by serving as a substrate for new proteins to bind DNA. The addition of the methyl group to cytosine has little effect on the overall structure of DNA, however it can inhibit the binding of several "methylation sensitive" proteins such as CREB, NFkB, and AP2 [37-39]. Conversely, methylated CpGs can serve as specific binding sites that recruit methyl-CpG binding domain proteins (MBPs). With MBP binding comes histone modifying enzymes that influence chromatin structure at the site of CpG methylation. Thus, these proteins can serve as a linker between CpG methylation and histone modifications.

The methylation of cytosine is a post-replicative event, meaning it occurs after cytosine is incorporated into double stranded DNA. Catalyzing the methylation of cytosine at CpG di-nucleotide are several DNA methyltransferases (DNMTs). These enzymes specifically recognize CpGs as targets for methylation. DNMTs catalyze the transmethylation of cytosine by transferring methyl groups from S-adenosylmethionine (SAM) to position 5 of the pyrimidine ring to create 5-methylcytosine (Fig. 1). Humans have three DNA methyltransferases: DNMT1, DNMT3a and DMNT3b. These enzymes are subdivided into two classes based on their functionality *in vivo:* maintenance methylation, and *de novo* methylation [40]. Maintenance methylation is catalyzed by DNMT1 and occurs rapidly following DNA replication [41]. DNMT1 has high affinity for hemimethylated CpGs (CpG doublets where only one DNA strand is methylated), which it quickly converts into fully methylated CpG di-nucleotides [42]. DNMT1 is expressed in all cell types during S phase of the cell cycle, when its activity is highest [43]. It is through DNMT1 that the pattern of DNA methylation is recapitulated in daughter cells [44]. The *de novo* methyltransferases, DNMT3a and DNMT3b, are so named because they equally methylate hemimethylated and unmethylated CpGs [45]. Different genes encode these enzymes, but both exist as multiple splice variants that give these enzymes functional flexibility [45-49]. *De novo* methylation creates new epigenetic events, meaning these enzymes could possibly initiate gene silencing.

Modification of the nucleosome

Genetic information is packaged into higher order structures by nucleosomes. Each nucleosome encompasses ~146 base pairs of DNA wrapped around an octamer of histone proteins. This octamer contains two H2A, H2B, H3, and H4 histone proteins. Another structural characteristic of the nucleosome are the "histone tails" that extend from the core octamer [50]. These tails consist of the N-termini of the histone proteins and are the main site for their post-translational modification. Modifying histones allows the nucleosome to have dynamic roles in transcription, DNA repair, DNA replication, and the cell cycle. The list of potential modifications includes: acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination, and sumoylation (For review of these topics see [51-53]. A majority of these modifications take place at lysines, arginines and serines within histone tails (Fig. 2). Given the breadth of literature on this topic it is impossible to sufficiently cover all modifications in depth here. Therefore, for the sake of brevity, we will focus on lysine acetylation and methylation citing specific examples of how they control nucleosome function. Lysine acetylation is associated with active gene expression and open chromatin. H3K9ac and H4K16ac are two histone modifications often associated with euchromatin [54]. Histone methylation is more complex because the ε-nitrogen on lysine can be modified by up to three methyl groups, thus creating multiple degrees of lysine methylation. For example, lysine 4 of histone H3 can be umethylated, monomethylated (H3K4me1), dimethylated (H3K4me2), or trimethylated (H3K4me3). The complexity of histone methylation is further increased by the possibility that each degree may have a unique function. Histone methylation is associated with both active and silent genes, or as a fundamental mark of all histones. The recent development

of Illumina® chromatin immunoprecipitation sequencing (ChIP Seq) has identified where these modifications reside and hint at their potential roles [55,56]. Active genes have high levels of H3ac, H4ac, and H3K4me3 at their promoters and abundant H3K36me3 and H3K4me1 within coding regions [57]. Having specific modifications at defined tracks along the axis of expressed genes has led to the hypothesis that histone modifications may regulate both transcriptional initiation and elongation [58]. Modifications such as H3K9me3, H3K27me3, and H4K20me3 at promoters are indicative of transcriptionally silent heterochromatin [59-61]. By what means do these modifications influence DNA? Histone modifications actuate DNA/nucleosome interactions in to ways. First they alter the charge of histone tail to influence the contact between negatively charged DNA and the lysine-rich positively charged nucleosome. Second, modified histone tails can serve as binding sites for effector proteins that manipulate DNA. This facet of nucleosome function forms the central tenet of the "histone code" hypothesis, which proposes that the covalent modification of amino acids, and the interaction with one another, generates a language that is read by a series of effector proteins that act upon DNA [62]. Loss of the either the histone modification or the effector proteins would influence DNA function in similar ways. Two common examples of proteins that read the histone code are transcriptional repressors such as heterochromatin protein 1 (HP1) that binds H3K9me3, and lethal 3 malignant brain tumor 1 (L3MBTL1) which interacts with H4K20me1, both of which organize DNA into heterochromatin and silence gene expression [63-67].

Histone Acetylation

Establishment and maintenance of the histone code is accomplished by a multitude of enzymes that target specific amino acids for modification. Histone acetyltranferases (HATs) use Acetyl-CoA to add acetyl groups to lysines within histone tails. Mammalian histone acetyltransferases are divided into five distinct families: GNAT (Gcn5-related N-Acetyltransferase) superfamily, MYST (MOZ, YBF2/Sas3, Sas2, Tip60) family, p300/CBP, TAFII 250, and nuclear receptors [68]. Each of these families have different roles in regulating chromatin structure, however they share a common enzymatic activity. HATs transfer acetyl groups from acetyl CoA to lysine to form ε-Nacetyl lysine in histones and CoA [69,70]. Recruitment of HAT activity to a gene regulatory element is generally associated with active transcription and open chromatin. Histone acetylation occurs at lysine residues in histones H2B, H3 and H4 (Fig. 2). Once acetylated, histone tails become sites for bromodomain containing effector proteins to bind and act upon the DNA or nucleosome [71]. Acetylation of histone tails is not a permanent modification. Histone deacetylases (HDACs) remove acetyl groups from histones, thus providing a certain degree of plasticity to the epigenetic control of gene expression [72,73]. The presence of HDACs within gene regulatory regions is consistent with epigenetic gene silencing and closed chromatin. The HDACs are divided into three groups (Classes I-III) [74]. Combined, there are a total of ten Class I and II HDACs that utilize hydrolysis to remove acetyl groups, while class III HDACs include the sirtuin family of protein deacetylases. Sirtuins are a class of NAD^+ dependent protein deacetylases that are commonly known for their role in increasing lifespan of yeast and *C. elegans* during caloric restriction [75]. The HDAC activity of sirtuins is intimately linked to metabolism by the NAD^+/NADH ratio. With increased NAD^+, sirtuins can more readily deacetylate histones and other proteins [76,77].

Methylation of histone tails is carried out by several histone methyltransferases (HMTs). Methylation requires concerted activity among several protein complexes and HMTs whose full description go beyond the scope of this review (for reviews of these topics see [51,78, 79]). In the context of this review we will focus on the relationship of HMTs with redox and metabolism. Members of HMT family can methylate lysine or arginines in histone tails (Fig. 2). Like DNMTs, HMTs use SAM as a cofactor during transmethylation and produce SAH as a byproduct. HMT substrate specificity and activity is centralized in SET domains and surrounding motifs [80,81]. This specificity is responsible for creating the progressive

methylation of lysines mentioned above. Some HMTs are exclusively monomethyltransferases (*i.e*. create Kme1), while others progressively methylate lysines to di and tri methylated forms. For example, PR-Set7 is the H4K20 monomethyltransferase that creates a substrate for SUV4-20H1/H2 to progressive methylate K20 to higher degrees of methylation [82,83]. Until recently it was believed that histone methylation was a terminal event. This has changed with the discovery of lysine specific demethylase 1 (LSD1) and the jumonji C (JmjC) family of histone demethylases, collectively known as KDMs [84-86]. LSD1 specifically demethylates H3K4me2 and is a member of several transcriptional repression complexes [84,87-89]. Histone demethylation by LSD1 uses oxygen as an electron acceptor to reduce methylated lysine to form lysine, formaldehyde, and hydrogen peroxide [84]. JmjC demethylases have a different mechanism than LSD1 that allows them to demethylate trimethylated lysine [85]. Like HMTs, the KDMs have target lysines. The most intriguing aspect of these proteins is the cofactors they require. JmjC demethylases all need molecular oxygen (O_2), α-ketoglutarate, Fe^{2+}, and ascorbate to demethylate mono, di or tri methyl lysines [85,90].

The epigenetic switch in cancer

Cancer cells have an altered epigenotype compared to the tissues from which they arise. This subject has received much attention and has been the subject of numerous reviews [91-94]. Overall the epigenetic switch is summarized as changes in the level and placement of both DNA methylation and histone modifications. Many cancers acquire or increase the expression of epigenetic enzymes, yet the products of their reactions (i.e. methylated cytosine and modified histones) do not correlate and suggest there are other factors affecting their activity [95]. For over 20 years it has been known that tumor cells are globally hypomethyalted at CpGs compared to the tissues from which they arise, while at the same time cytosine methylation is increased in specific parts of the genome [96,97]. In normal cells, CpGs within repetitive DNA elements and coding regions of genes are methylated. In tumor cells LINE-1 repeats, satellite DNA, and moderately repeated DNA sequences become unmethylated, while genes containing CpG cluster become hypermethylated, rendering them transcriptionally silent (For review see [35]). Prime candidates for this type of repression are tumor suppressor genes such as p16 and 14-3-3 sigma [98-100]. Methylated CpGs also form mutation hot spots within coding regions of tumor suppressor genes like p53 [101,102]. The deamination of methylated cytosine forms thymine, creating a lesion that is difficult to correct because our DNA repair mechanisms cannot easily discriminate which base is correct in the resulting G:T mismatch. Proportional changes in histone modifications are also observed in cancer. Work from Manel Esteller's group has shown that loss of acetylation of lysine 16 and trimethylation of lysine 20 of histone H4 are common events in cancer cells [103]. Others have reported global decreases in H3K4me3, H3K9me3 and H3K27me3 in cancer [104,105]. In many caners loss of these histone modifications is a predictive marker of disease outcome [104,106]. Histone hypoacetylation can also silence tumor suppressor genes, while hyperacetylation can potentially activate oncogenes [107,108]. Epigenetic alterations in cancer could also be affecting the stability of the genome, given the link between the organization of the genome and its repair and replication [27,28]. While much descriptive work has shown the nature of such changes, the cause(s) of the epigenetic switch in cancer remains unknown. At the core of the epigenetic switch is likely the inability of enzymes like DNMTs, HMTs, HDACs and KDMs to maintain the epigenome of the tissue of origin. One means by which these enzymes may be affected in cancer is through loss of their cofactors.

Epigenetic enzymes are reliant upon metabolic cofactors

Creating and maintaining the epigenome requires the enzymes mentioned above and their metabolic cofactors. Transmethylation by DNMTs and HMTs requires Sadenosylmethionine (SAM) as a methyl group donor, a cofactor whose level and availability is linked to metabolism

and redox. Removing histone modifications requires cofactors linked to glycolysis and oxidative phosphorylation. Histone deacetylation by class III HDACs use nicotinamide adenine dinucleotide (NAD^+) to accept acetyl groups from lysines [109]. Likewise, KDMs remove methyl groups by using α-ketoglutarate as an electron donor [110]. Redox status may also influence the function of epigenetic enzymes and the effect proteins that bind their products. From this brief description, it is apparent that the epigenome relies heavily upon metabolic and dietary cofactors. Below we will discuss how the metabolic defects of cancer metabolism influences each of these cofactors to flip the epigenetic switch in cancer.

Once carbon metabolism and epigenetics

In the past few years a push towards understanding the connection between diet and gene expression has revealed that one carbon metabolism can influence epigenetics. These studies have focused on two metabolites, folate and SAM. Both cofactors are central to methylation reactions in cells. Studies by Randy Jirtle's group have shown that dietary folate can impact the methylation of specific genes, while a growing number of studies have begun to investigate the role of SAM in controlling gene expression during liver injury and carcinogenesis [111-113]. SAM's level is governed by folate and the needs of cells at any given time. In mammalian cells, dietary folate serves as a cofactor to assimilate carbon groups from glycine into one carbon metabolism. Prior to folate's use in cells it is converted to tetrahydrofolate. Inhibition of folate metabolism by methotrexate blocks the conversion of deoxyuridylic acid to thymidylic acid [114,115]. Other than nucleotide biosynthesis, folate is used to produce methionine from homocysteine, and eventually to SAM [116]. SAM is used as the methyl donor in biochemical reactions because the methyl group bound to sulfur can be removed with relative biological ease, an essential requirement for such a donor. SAM is an essential cofactor that is required by several biochemical processes (for review see [117]) (Fig. 3). If SAM levels become too low, methylation reactions participating in epigenetic processes may no longer function properly. The overlying concept is that if folate status is interrupted, cells will no longer be able to maintain epigenetic control and have altered gene expression. Mutations in genes that metabolize folate prior to the synthesis of SAM disrupt genomic DNA methylation [118]. Limiting folate intake decreases the SAM/SAH ratio. Decreasing SAM/SAH ratio also inhibits DNA methylation by affecting the activity of DNMTs [119]. DNA hypomethylation induced by folate deficiency is another risk factor for increased cancer susceptibility [116]. One carbon metabolism appears to have the same impact on histone modifications. Feeding rats a methyl deficient diet induces a global decrease of H3K9me3, H3K9ac, H4K16ac, and H4K20me3 histones and promotes carcinogenesis [105].

S-adenosylmethionine synthesis

Mammalian cells produce SAM by the addition of ATP to methionine by SAM synthetases. Humans have three forms of SAM sythetases: MATI, MATII and MATIII. Both MATI and MATIII are encoded by the *mat1α* gene and are primarily expressed in the liver [120]. While the primary amino acid sequence is identical between MATI and MATIII, the two enzymes exist as tetramers and dimers respectively [121]. All other tissues use MATII, which is a heterodimer of MAT2α and MAT2β subunits [122]. Regardless of the tissue, these enzymes are critical in maintaining cellular SAM levels and the activity of the methionine cycle. Here we will discuss the function of SAM and its role in carcinogenesis. We will then introduce how redox status may influence SAM and SAM levels in cancer.

SAM inhibits tumor formation

SAM levels in cells directly influence carcinogenesis by affecting methylation reactions. Tumor promotion studies using rat liver models have shown that SAM content and the SAM/SAH ratio are decreased in preneoplastic lesions [123]. Consequently, treating initiated animals

with SAM decreases the size and frequency of these preneoplastic lesions after initiation [124]. SAM treatment also blocks the progression of these preneoplastic lesions into hepatocellular carcinoma [112,124]. This has led to the conclusion that SAM may have chemopreventative properties [112]. The effects mentioned above can be attributed to SAM's influence on DNA methylation. Studies by Francesco Feo's group in the early 1990's showed that SAM inhibits liver preneoplastic lesion growth by influencing the expression of proto-oncogenes. Administering SAM to animals after initiation decreased the expression of c-myc, c-Haras and c-K-ras in proliferating liver and nodules [123,125]. They determined that the decreased expression of these three proto-oncogenes was caused by increased DNA methylation at their promoters [123]. Similar observations have been observed when human model tumor cell lines are treated with SAM. For example, treating prostate cancer cell lines with SAM decreases their expression of two known tumor promoting genes, urokinase-type plasminogen activator and matrix metalloproteinase-2. The silencing of these two genes is casually linked to hypermethylation of CpGs within their promoters [126]. Furthermore, pretreatment of prostate cancer cells with SAM also decreases tumor growth rate in mouse xenografts [126]. Given these studies, it seems of great importance to understand what fundamental changes in SAM biochemistry occur during carcinogenesis.

Glutathione production is linked to SAM

Mitochondrial defects lead to the aberrant production of reactive oxygen species like superoxide and hydrogen peroxide. To counter this, cancer cells increase their production of small molecular weight antioxidants like glutathione [127]. Increasing the production of glutathione requires cells to tap their sulfur pools. Cells meet this need by increasing the flux of homocysteine into the transsulfuration pathway and away from the methionine cycle [128]. Homocysteine can enter the transsulfuration pathway or be recycled back into methionine by either methionine synthase or betaine homocysteine methyltransferase. This choice is dictated in part by the current needs of the cell at that time. During a pro-oxidant state, homocysteine is diverted away from the methionine cycle and into the transsulfuration pathway. A pro-oxidant sate accomplishes this by increasing the activity of cystathionine β-synthase [129]. The transsulfuration pathways can account for up to 50 percent of the glutathione produced in some tissues [129]. The synthesis of glutathione directly affects epigenetic processes. When glutathione is depleted by chemical means methyl donors become deficient, leading to genome wide DNA hypomethylation [128,130,131]. This change occurs because the level of SAM required by DNMTs and HMTs can no longer be met. A similar case would arise in tumor cells. The pro-oxidant state of tumor promotion would rob the methionine cycle of tumor cells to feed their need to produce glutathione. Countering mitochondrial oxidants in this manner would lead to a depletion of SAM in tumor cells. The result of which is the global loss of DNA and histone methylation observed in cancers.

Many cancers also increase production of unusual metabolites such as sarcosine that appear to be causally involved in malignant behavior [132]. The first step in the synthesis of this metabolite is catalyzed by glycine N-methyltransferase (GNMT), which transfers a methyl group from SAM to glycine to produce sarcosine and SAH. Unlike DNA and histone methyltransferases, GNMT is only weekly inhibited by SAH, which allows it to freely control the SAM/SAH ratio. It has also been proposed GNMT controls transmethylation reactions by siphoning carbon units from the methionine cycle and back into the folate cycle [120]. In normal cells it is likely this mechanism works efficiently as a way to regulate global transmethylation reactions, since the Km of GNMT for SAM is high and would only redirects carbon groups when folate levels are high [133]. This observation also explains the large SAM/SAH ratio of normal cells. In cancer cells GNMT activity could also create an environment that inhibits transmethylation. Tumor cells often over express GNMT, and could be one factor that creating their decreased SAM/SAH ratios [132,134]. If this is the case, then the increased

GNMT activity of tumor cells could potentially influence epigenetic processes in two ways. First, GNMT would decrease the SAM/SAH ratio, and thus inhibit the activities of DNA and histone methyltransferases, which are highly susceptible to inhibition by SAH. Secondly, GNMT would decrease transmethylation reactions by directly removing SAM from the methionine cycle. There is empirical evidence to support this connection between GNMT and epigenetic processes. Activating GNMT activity with glucocorticoids or retinoids results in global loss of DNA methylation [135]. Combined with our knowledge about GNMT levels in cancer it appears that disregulation of this enzyme could be causally involved in the epigenetic switch of cancer.

Other Metabolic intermediates influence epigenetics

The metabolic defect of cancer alters the levels and fluxes of metabolites through glycolysis and the electron transport chain. These fundamental metabolic changes could both influence the function of epigenetic processes in cancer. It was exactly this relationship between metabolism and redox that led Peter Cerutti to hypothesize that altered nicotinamide adenine dinucleotide (NAD^+) was affecting epigenetic regulation of gene expression of [5]. Cerutti attributed these changes to the poly ADP-ribosylation of “chromosomal proteins”, which were one of the best-characterized histone modifications at that time. Here, with the benefit of our current understanding, we extend upon Cerutti's original ideas and speculate that the metabolic changes effect histone acetylation and methylation as well.

Histone acetylation and NAD^+

Sirtuins utilize NAD^+ to deacetylate histones and other acetylated proteins through the hydrolysis of NAD^+, the results of which are O-acetyl-ADP-ribose, nicotinamide, and lysine [75]. An intimate association among the NAD^+/NADH, sirtuins, and histone acetylation has also been well established by studies investigating lifespan and caloric restriction [75]. Caloric and/or glucose restriction effectively increases the NAD^+/NADH ratio, and in turn dictates the HDAC activity of sirtuins [109]. Currently seven sirtuin family members have been identified in humans: SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 [109,136]. Currently the role of sirtuins in cancer is only emerging. We speculate that the increased glycolytic activity of the Warburg effect may partially influence sirtuin activity by altering the NAD^+/NADH ratio in tumor cells. In the early 1970's it was reported that immortalization decreases the NAD^+/NADH ratio in fibroblasts, however current studies on this topic are lacking [137]. If NAD^+ levels decrease during carcinogenesis a concomitant change in sirtuin activity and histone acetylation must also be occurring. During development, changing NAD^+/NADH ratio of muscle cells has already been established as a means to alter sirtuin activity and affect chromatin structure [138-140]. With cancer, the Warburg effect would drive down the NAD^+/NADH ratio, decrease sirtuin activity, and lead to aberrant gene expression through histone hypoacetylation. Connecting the Warburg effect with gene expression via sirtuins is potentially astounding. Because increased glucose consumption is a common trait in cancer, it has been seen as an avenue for creating novel therapies in cancer. The addition of an epigenetic component to the Warburg effect lends further credence to the development of such therapies for cancer.

Kreb's cycle intermediates also have an intimate link with enzymes that regulate epigenetic processes. As we discussed above, some KDMs require oxygen, Fe^{2+}, α-ketoglutarate and ascorbate to demethylate histones [85,90]. Increased glutamine consumption by tumor cells would affect their concentration of α-ketoglutarate, KDM activity, and histone methylation. The impact of increased glutamine consumption on KDMs would be further affected by dysfunctional electron transport. Defects in electron transport chain complexes are common events and coincide with ROS production, however they can also change the level of Krebs

cycle intermediates [141]. For example, Complex II defects increase cellular levels of succinate, and stabilize HIF-1α through substrate inhibition of α-ketoglutarate requiring prolyl hydroxylases [142,143]. This serves as an elegant example of how metabolism in the mitochondria can directly influence transcriptional activity in the nucleus. We speculate metabolic defects also influence nuclear transcription through KDMs that manipulate the epigenome. Aberrant glutamine consumption by tumor cells is compounded by their electron transport chain defects and creates a sizable pool of α-ketoglutarate to accelerate histone demethylation. This is consistent with the observed decreases of H3K9me3, H3K4me3 and H3K27me3 in cancer, all of which are targeted by KDMs [61,103-106,144,145]. Likewise, mitochondrial defects, such as SDH mutations, could also increase succinate levels and potentially inhibit KDM activity. Regardless if metabolism is activating or inhibiting KDMs, from our discussion above there appears to be strong evidence to support a causal link between Krebs cycle imbalance and altered histone methylation in cancer.

Redox regulation of epigenetics

Cancer cells have an atypical redox status that is dictated by ratio of glutathione to glutathione disulfide (GSH/GSSG). Normal cells have an almost infinite GSH/GSSG ratio because their concentration of GSSG is nearly zero. This high GSH/GSSG ratio makes a good redox buffer that favors healthy biological activity in the reduced state [146]. Tumor cells on the other hand have appreciable amounts of GSSG, which effectively decreases their GSH/GSSG ratio. The result is a change in the redox buffering capacity of tumor cells that alters biological activity by affecting enzyme function. Not all enzymes would be subjected to redox regulation in this manner. Candidates for redox regulation are enzymes or factors that utilize oxidizable amino acids such cysteine in their enzymatic mechanisms or functional motifs. The atypical redox state of tumor cells influences epigenetic processes by affecting the production of SAM, epigenetic enzymes, and effector proteins that bind modified histones.

The synthesis of SAM by SAM synthetases is a redox regulated process. Controlling the tertiary structure of SAM synthetase is its oxidation state and the GSH/GSSG ratio [147]. Blocking glutathione synthesis with BSO reduces the GSH/GGSH ratio and MAT activity. However, treatment with SAM reverses BSO's inhibition of MATs by restoring the redox status of cells. [113,148]. These observations reveal an interesting link between SAM levels, MAT activity, and redox state. Redox buffering also influences MAT activity. High GSH/GSSG ratios have a positive influence on MAT activity by keeping the enzyme in a reduced state, however when the GSH/GSSG ratio falls below 10:1, its activity becomes decreased [149]. These enzymes are amendable to redox regulation because of a conserved critical cysteine residue located within their active sites. Work by Pajares and others have identified cysteine 150 (C150) of rat MATI and III as a critical cysteine residue whose oxidation is believed to be a contributing factor in the development of liver cirrhosis [150,151]. While these studies have focused primarily on liver disease, and not cancer, we can speculate that the influence of redox biology on this process would transition between pathologies. We can draw this conclusion because cysteine 150 is conserved between rat MATI and MATII, meaning redox exerts an influence over SAM levels in tissues other than liver. The GSH/GSSG ratios of cancer cells vary, but are generally lower than 10:1. Based on how redox buffering influences SAM synthetase activity we would expect cancer cells to exhibit lower SAM synthetase activity even if the expression of this enzyme is unaffected. This makes sense because of the metabolic network that exists between SAM and glutathione. Oxidizing conditions would slow down the methionine cycle to feed sulfur into the production of glutathione via the transsulfuration pathway. The chronic redox changes in cancer cells would create a SAM deficit in this manner, leaving insufficient fuel for HMTs and DNMTs. The result would be global epigenetic alterations similar to what is observed when glutathione is depleted.

Enzymes that initiate and perpetuate epigenetic events are also subject to redox regulation by the disruption of their enzymatic mechanisms. When Thomas Jenuwein's group characterized the first mammalian homologue of SUV39H1, they identified a cysteine rich region near its SET domain whose presence was necessary for the enzyme's activity [64]. These cysteine rich “Post-SET domains” are found in several HMTs and often have a role in substrate recognition and enzyme activity [81]. DNA methyltransferases also have highly conserved cysteine residues in their active sites. This active site cysteine is generally deprotonated and serves as a catalytic nucleophile by forming an intermediary covalent bond with cytosine that primes the 5 position of its pyrimidine ring to accept a methyl group form SAM (Fig. 4) [152]. Mutation of this cysteine in DNMT1, DNMT3a and DNMT3b results in a catalytically dead methyltransferase [153,154]. Sulfur nucleophiles like the one in DNMTs are amendable to oxidation, resulting in loss of enzyme activity. The potential for redox to alter the activity of DNMTs via cysteine oxidation is best exemplified by 5-aza-2′-deoxycytidine, a potent inhibitor of DNA methylation [155]. When DNMTs attempt to methylate 5-aza-2′-deoxycytidine that has been incorporated into double stranded DNA, an irreversible covalent bond is formed between enzyme and inhibitor via this catalytic cysteine, resulting in loss of DNMT function and a global decrease in methylated DNA [156]. Oxidation of the catalytic cysteine would stop it from serving as a nucleophile, essentially removing the enzyme from the active pool of DNMTs like 5-aza-2′-deoxycytidine. These examples allow us to speculate that the prooxidant state of cancer cells would render DNMTs and HMTs less active. Both enzyme types often exhibit increased expression in cancer, yet the product of their reactions (i.e. methylated cytosine and lysine) are paradoxically decreased [95]. A mechanism that decreases enzyme activity, but not expression, could reconcile these observations. Oxidation of these cysteine rich domains could be one means by which the pro-oxidant state of cancer impinges on the activity of these enzymes.

Histone modifying enzymes and their effector proteins contain several conserved domains which have cysteine rich regions within them. Plant homeo domains (PHDs) are an ~50-80 amino acid domain that contain a conserved Cys_4-$HisCys_3$ zinc finger motif [157]. PHDs are a common motif in several nuclear proteins, many of which have a profound role in regulating chromatin structure and function. Nucleosome binding of these domains was initially characterized in the HAT p300 and the chromatin remodeling complex subunits ACF1 and NUR301 [158-160]. PHD domains are also present in some DNMTs and may influence the location and specificity of DNA methylation [161]. In the past two years several groups have reported that these domains also bind methylated histone tails, specifically H3K4me2, H3K4me3, and H3R2me2 to regulate transcription and possibly genetic recombination [162-165]. Loss of the PHD domain within RAG2 by deletion or mutation of its cysteine abrogates its function and manifests itself *in vivo* as severe combined immunodeficiency [162]. It is interesting to note that H3K4me2 and H3K4me3 are prevalent histone modifications within the human genome, and many genes are regulated by p300/CBP type HATS. Given this information it seems likely that some signaling mechanism controls local function of proteins to stipulate when their activities are required. We suggest that cysteine rich domains are common at the contact sites between chromatin-associated proteins and the factors that recruit them (i.e. modified histone or transcription factor). One example is the PHD domain bearing protein p300 and its interaction with CREB/ATF transcription factors. From the example of PHDs we surmise that redox status could in part regulate the interactions between different histone modifying and binding proteins. Such a regulatory role for redox status is not without precedence. The REF-1 redox switch controls the function of several transcription factors (for reviews on this topic see [166,167]). For transcription factors, redox switches modulate their binding to cognate sites throughout the genome. Is it possible that the contacts between modified histones and their effector proteins are regulated in a similar manner? The presence of a particular histone modification isn't synonymous with its effector protein binding. For example, phosporylation of 53BP1 is required prior to binding H4K20me2, a histone

modification present on 80% of all nucleosomes [83,168]. Because redox switches and ROS have the ability to activate transcription factors, it seems very likely that affecting histone modifications is another avenue by which they can influence gene expression [3,166,169, 170]. Such an investigation in cancer, or chromatin biology remains unexplored and open for speculation.

Oxidants also influence epigenetic processes by oxidizing nucleotide bases and disrupting higher ordered chromatin structure. The oxidation of nucleotide bases by free radicals creates several unique bases, most common of which is 8-oxoguanine (8-oxoG). This oxidized base can directly inhibit DNMTs and possibly induce DNA demethylation at this site [171,172]. Such a case arises when 8-oxoG is in either strand of a hemimethylated CpG doublet, or the base adjacent to a hemimethylated CpG [171,172] (Fig. 5 A, B). The presence of 8-oxoG within CpG doubles occurs via the direct oxidation of guanine in double stranded DNA, or through incorporation of 8-oxoGTP from the nucleotide pool during S phase [173]. In normal cells 8-oxoG is evicted from double stranded DNA by 8-oxoguanine glycosylase 1 (OGG1) and removed from the nucleotide pool by the 8-oxoGTPase hMTH1 (For review of these topics see [173,174]). However, deleterious mutations have been identified for both OGG1 and hMTH1 in many cancers, thus allowing 8-oxoG to persist as a mutagen, and potentially influence the epigenotype and progression of these diseases [175,176]. Oxidation of 5-meC is another type of base damage that can influence DNA methylation [177]. 5-meC accounts for approximately 3-5% of all cytosine within a cell but is still a target for oxidation by reactive oxygen species [178]. Progressive oxidation of 5-meC can lead to its demethylation to form cytosine (Fig. 6). While this reaction is chemically plausible, its progressive oxidation is complex and has been speculated to lead to the demethylation of CpGs *in vivo* [179,180]. The long-term effects of oxidized 5-meC during carcinogenesis are likely attributable to 5-formylcytosine (5-fC), or 5-hydroxymethylcytosine (5-hmC) both of which are detectable *in vivo* [181-183]. Like 8-oxoG, 5-hmC is removed from DNA by its own specific glycosylase, HMC glycosylase [184]. HMC glycosylases are unique enzymes because they simultaneously correct genetic and epigenetic defects. Work from Lawrence Sower's laboratory has revealed that oxidation of 5meC could potentially hinder the ability of DNMT1 to methylate the nascent strand of DNA following replication [185]. Such changes could lead to demethylation at a specific region or locus and dramatically affect the epigenome. The Sowers group has also shown that oxidation of cytosine or guanine on either strand of a CpG affects the epigenome by inhibiting the activity binding of MBPs such as MBP1, MBP2 and MeCP2 and the chromatin remodeling complexes associated with them [183] (Fig. 5C). Thus, the oxidation of individual methylated CpGs could exert strong effects on local chromatin structure and possibly gene expression. But is this a viable mechanism to alter gene expression? Recent findings suggest that ROS production can lead to demethylation of the E-cadherin promoter [186]. While such studies begin to demonstrate a link between ROS and epigenetic processes, they lack a mechanistic component. Additional studies are required to dissect the fundamental mechanisms behind these observations. Aberrant production of oxidants such as hydrogen peroxide also triggers the degradation of higher ordered chromatin structure through the mobilization of HATS such as CBP/p300 [187,188]. Direct oxidation also influences the interaction between nucleosomes. The addition of reducing agents increases the susceptibility of chromatin to nuclease digestion, however the mechanism by which this occurs remains undefined [189-191]. Base oxidation created by ROS is appreciated as a means to induce mutations during carcinogenesis. From our discussion here it likely affects epigenetic processes as well, and establishes another means by which free radicals can influence carcinogenesis.

Summary

The nuclei of eukaryotes contain two sets of information, genetic and epigenetic, that regulate gene expression. Until recently, the focus of cancer biology has been genetic changes as a

means to drive carcinogenesis. Oberley and Buettner hypothesized 30 years ago that ROS could be causal in carcinogenesis [1]. Today, there is strong empirical evidence to support the ability of ROS, redox state, and metabolic changes to create genetic mutations and the mutator phenotype of cancer [1-4,192]. With our increased understanding of epigenetics we now know that it can accomplish many of the same functions as genetic changes in cancer: loss of gene function by silencing, activation of oncogene expression, and inducing chromosomal aberrations [91-94]. In multi-cellular organisms, epigenetics controls the function of the genome to create various cell types without genetic diversity, while in cancer, genetic and epigenetic alterations most likely collaborate to manifest the malignant phenotype. Is it possible that genetic and epigenetic defects in cancer share a common cause? Peter Cerutti was the first to propose a relationship between epigenetics and the prooxidant state of cancer, and we have described a similar connection in development [5,6]. Our current model for the how each of these metabolic defects influences epigenetic control of gene expression is shown in Fig. 7. In this review we have sought to expand upon current theories regarding the causal role for metabolic defects during carcinogenesis. Given the connections we have outlined above, such theories should be amended to include our new understanding of epigenetic processes in cancer.

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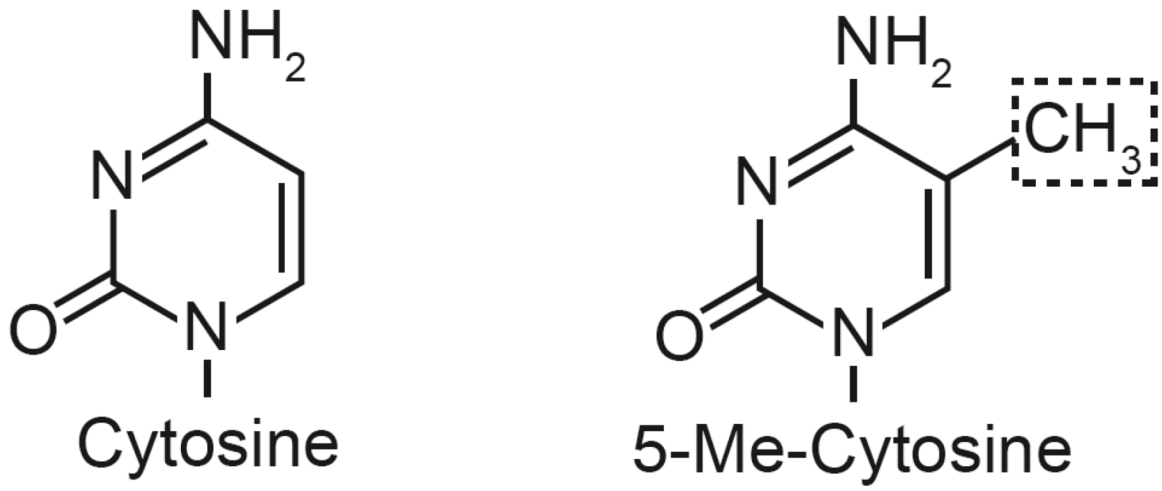
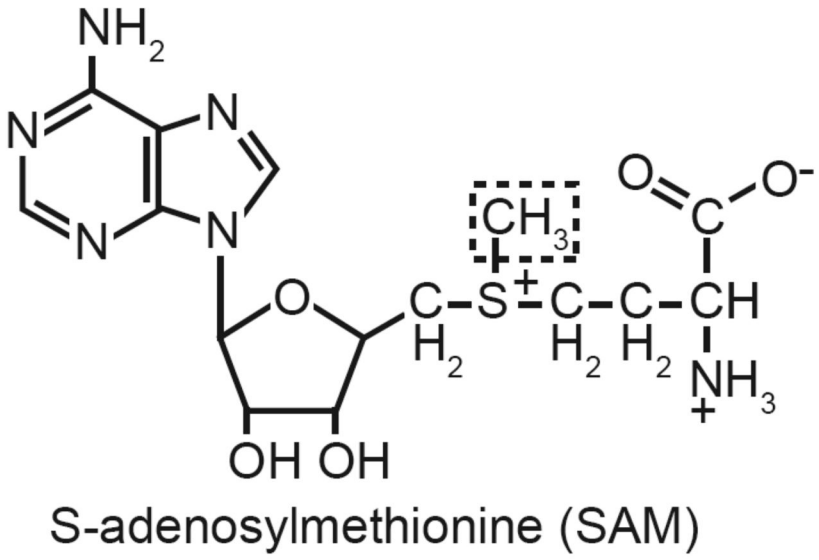


Fig. 1. Pyrimidine ring structures for cytosine and 5-methyl-cytosine (5-Me-C)

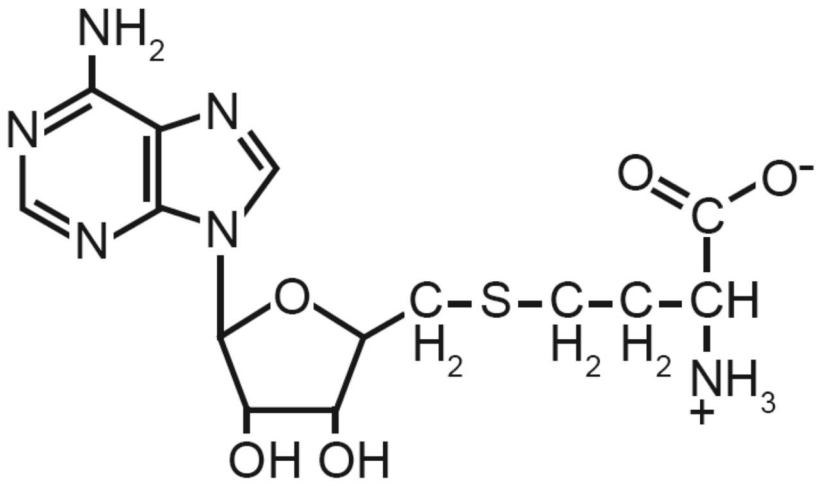
The addition of methyl group to position 5 of the cytosine nucleobase (dashed box) creates 5-Me-C in genomic DNA.

Fig. 2. The sites of lysine acetylation and methylation in histone tails

Histone tails protrude from the central globular domain of histone proteins and can be modified by acetylation and methylation in various ways. (* denotes lysine can be mono, di, or tri methylated)



S-adenosylmethionine (SAM)



S-adenosylhomocysteine (SAH)

Fig. 3. The structures of the epigenetic metabolites *S*-adenosylmethionine and *S*-adenosylhomocysteine

During transmethylation reactions the methyl group of *S*-adenosylmethionine (dashed box) serves as a nucleophile to attack C-5 of cytosine, and the ε-N of lysine. Once the reaction is complete, *S*-adenosylhomocysteine is released and utilized in other biochemical reactions.

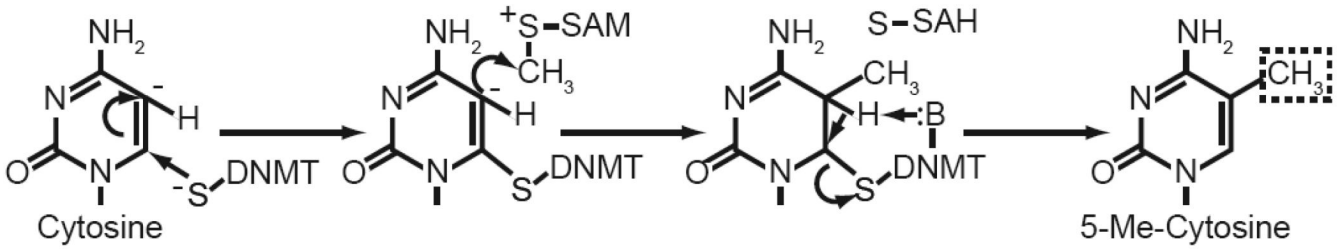


Fig. 4. Enzymatic mechanism of DNA methyltransferases (DNMTs)

Methylation of cytosine begins with the nucleophillic attack of position 6 by a thiolate nucleophile. The resulting electron rich region at position 5 is then at attacked by the methyl group of Sadenosylmethionine (SAM). The reaction then proceeds with the removal of the hydrogen at position 5 by a basic amino acid in the active site of DNMT. In the final step, the double bond is reformed in the pyrimidine ring, resulting in elimination of bond between position 6 and the DNMT.

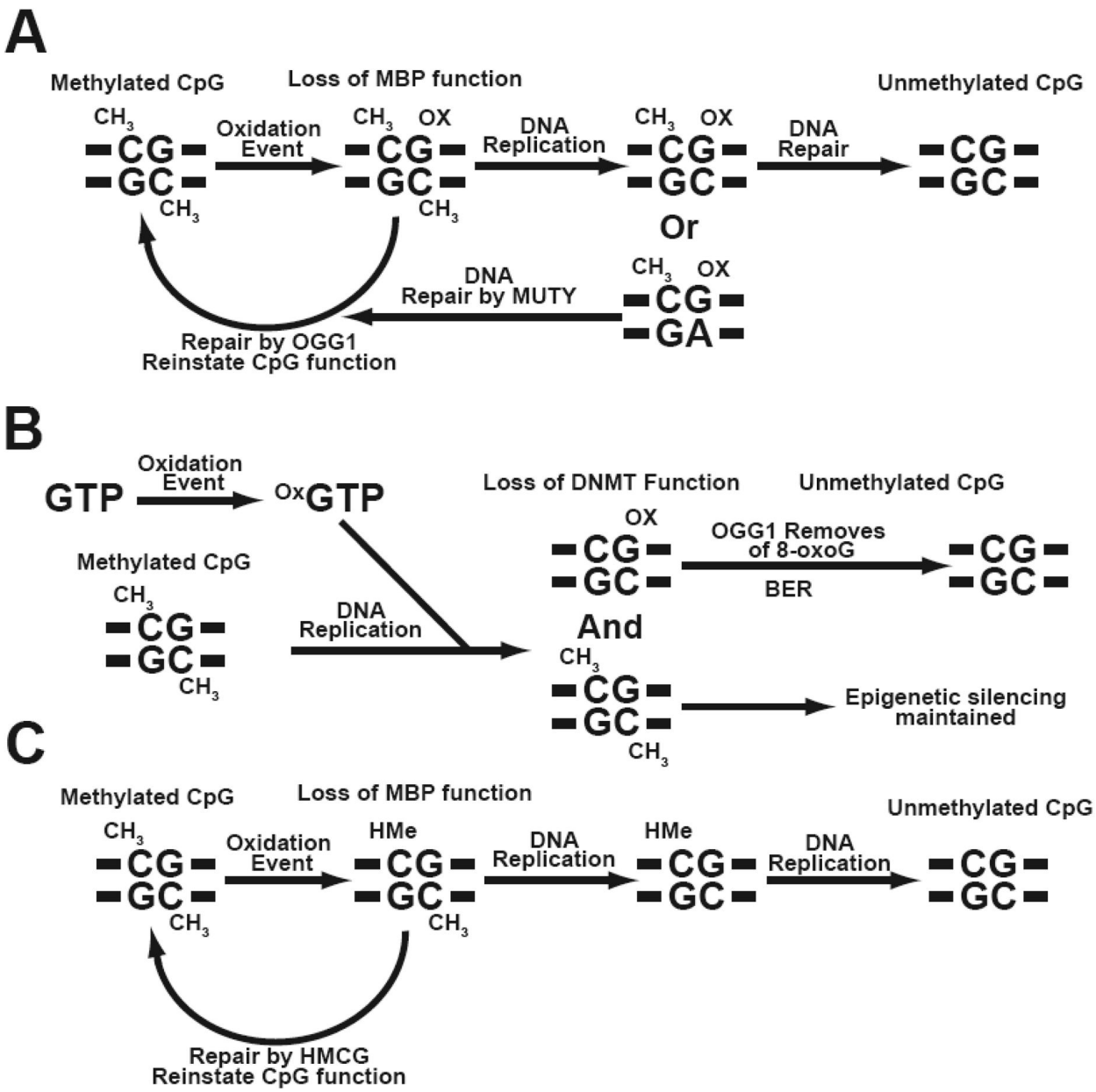


Fig. 5. Oxidation of nucleotide bases within methylated CpGs alters epigenetic processes

(A) Oxidation of guanine within a methylated CpG doublet abrogates MBP function, resulting in an epigenetic change. If these oxidized bases are not removed by 8-oxoguanine glycosylase 1 (OGG1) the epigenetic defect can be passed on during DNA replication and result in an unmethylated CpG. **(B)** Incorporation of oxidized GTP during DNA replication results in a methylated strand, and a hemimethylated CpG that is resistant to DNMTs. Removal of 8-oxoG by OGG1 and repair by base excision repair machinery (BER), creates an unmethylated CpG. **(C)** Oxidation of 5-methylcytosine creates 5-hydroxymethylcytosine (HMe). If this base is not removed by HMC glycosylases (HMCG) MBP function is lost at the site of oxidation and can create a hemimethylated strand following DNA replication. This epigenetic change can then persist as an unmethylated CpG in subsequent cell divisions.

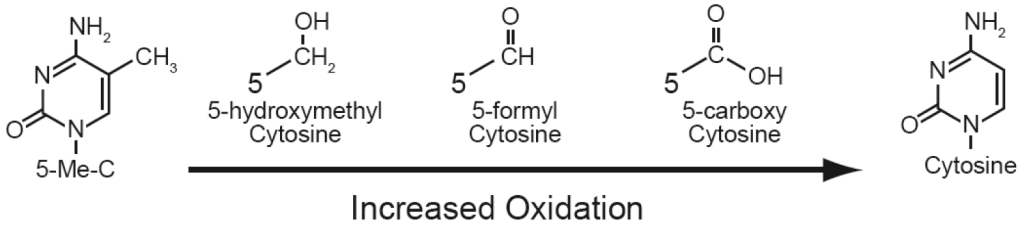


Fig. 6. The oxidative demethylation of 5-Me-cytosine to cytosine

Progressive oxidation of the carbon within the methyl group of 5-methyl cytosine (5-Me-C) results in its demethylation, and formation of cytosine. Each of the intermediates produced during the oxidation process are stable within DNA and affect epigenetic processes in a unique manner.

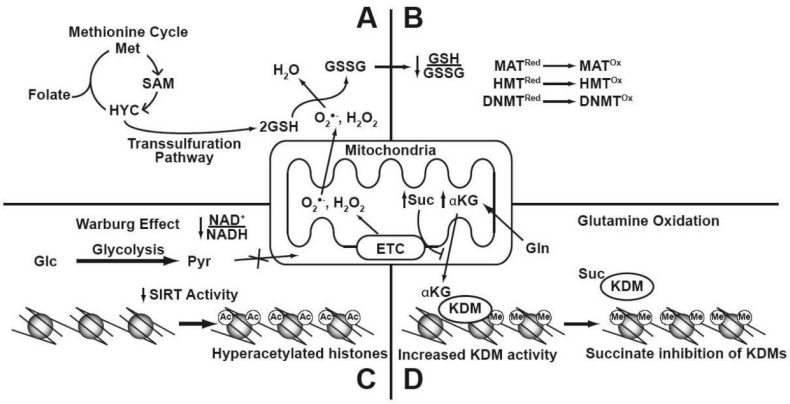


Fig. 7. The current model for the relationship between cancer metabolic defects and epigenetic processes

(A) Tumor cells increase their production of GSH to counter mitochondrially derived oxidants such as $O_2^{\bullet -}$ and H_2O_2. To sustain GSH production, cancer cells divert metabolites away from the methionine cycle into the transsulfuration pathway, resulting in decreased SAM production. **(B)** Aberrant production of oxidants creates an atypical redox state by decreasing the GSH/GSSG ratio which affects the activities of SAM synthetases, DNMTs and HMTs. **(C)** The increased Glc (glucose) consumption of the Warburg effect decreases the NAD^+/NADH ratio and produces Pyr (pyruvate). Decreasing this ratio creates an environment that inhibits the activity of sirtuins, and liberates genes from their negative regulation. **(D)** Oxidation of glutamine (Gln), and dysfunctional electron transport, alters the flow of α-KG (α-ketoglutarate) and Suc (succinate) metabolites within the Krebs cycle. These metabolites can then influence transcription in the nucleus by affecting the activity of KDMs (lysine demethylases).