

## Hypothesis

### Environmental regulation of 5-hydroxymethyl-cytosine by oxidative stress

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**Abbreviations:** 5-hmC, 5-hydroxymethyl-cytosine; 5-mC, 5-methyl-cytosine; IDH, isocitrate dehydrogenase; SDHD, succinate dehydrogenase; FH, fumarate hydratase; TET, ten-eleven-translocation;  $\alpha$ -KG,  $\alpha$  ketoglutarate; 2-HG, 2-hydroxyglutarate; HIF1 $\alpha$ , hypoxia inducible factor 1alpha; DNMT, DNA nucleotide methyltransferase; ESC, embryonic stem cell; AML, acute myeloid leukemia; SAM, S-adenosylmethionine

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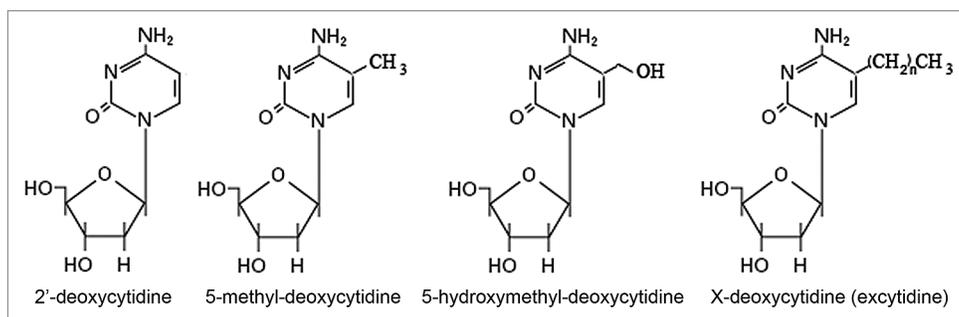
Many environmental toxins, such as heavy metals, air particles and ozone, induce oxidative stress and decrease the levels of NADH and NADPH, cofactors that drive anabolic biochemical reactions and provide reducing capacity to combat oxidative stress. Recently, it was found that the Ten-eleven translocation (TET) protein family members, which oxidize 5-methyl-cytosine (5-mC) to 5-hydroxymethyl-cytosine (5-hmC) in the DNA, were found to be activated under high oxygen conditions by alpha ketoglutarate ( $\alpha$ -KG), a cofactor produced by aerobic metabolism in the citric acid cycle. TET, Jumonji-family histone demethylases and prolylhydroxylase, a repressor of HIF1 $\alpha$  under high oxygen conditions, all require  $\alpha$ -KG as a cofactor for their activation. HIF1 $\alpha$  and TET proteins, which appear to have opposing functions, impact several aspects of human life, including cell growth regulation, embryonic stem cell maintenance, cell differentiation and tumorigenesis. The role of metabolism on the regulation of global DNA methylation and chromatin organization has recently gained greater attention from the biomedical research community. This article will discuss the possible role of TET activation and the regulation of 5-hmC and 5-mC levels in response to environmental stress. We will also discuss how 5-hmC and 5-mC levels at the promoters of specific genes might be a useful biomarker for exposure to environmental toxins.

## Introduction

Environmental factors can alter the way our genes are expressed via epigenetic alterations. These epigenetic alterations dictate heritable patterns of gene expression that arise in the absence of DNA sequence modification and are largely regulated by changes in chromatin structure, mediated primarily by post-replicative methylation of DNA and post-translational modifications of histones.<sup>1,2</sup>

The best-characterized epigenetic mark is a methyl group at the 5-position of cytosine bases known as 5-methyl-cytosine (5-mC) (Fig. 1). 5-mC has been studied extensively, and its role in gene regulation, X-chromosome inactivation, genomic imprinting, long-term silencing of transposons and cancer development is well described.<sup>2</sup> Cytosine exists as a free nucleotide that is incorporated into DNA during replication. The pattern of 5-mC in the genome is accurately preserved by mitotic inheritance through the action of DNA methyltransferases (DNMTs), specifically the maintenance DNA methyltransferase DNMT1, which catalyze the covalent addition of methyl groups to cytosine in newly synthesized DNA.<sup>3</sup> 5-mC is formed by post-replicative addition of a methyl group to cytosine through the action DNMTs, which use S-adenosylmethionine (SAM) as the methyl donor.<sup>3</sup>

5-hydroxymethyl-cytosine (5-hmC) was discovered in mammalian DNA in 1972 (Fig. 1),<sup>4</sup> but it was thought to be an oxidative-damage product of DNA of



**Figure 1.** Cytosine base modifications. The “excytidine” modification refers to yet undiscovered modification(s) to cytosine with unknown functions.

little importance and was therefore largely ignored. 5-hmC was re-discovered in 2009 by the laboratories of Nat Heintz<sup>5</sup> at Rockefeller University and Anjana Rao at Harvard Medical School,<sup>6</sup> and became of interest when it was shown that the TET family of Fe(II) and  $\alpha$ -KG-dependent dioxygenases utilize molecular oxygen to transfer a hydroxyl group to 5-mC in the catalytic conversion of 5-mC to 5-hmC, and that one of these enzymes, TET2, is frequently mutated in myeloid neoplasms<sup>7,8</sup> and both TET1 and TET2 are frequently mutated in glioma.<sup>9-14</sup> The formation of 5-hmC can lead to demethylation of DNA, which may contribute to the dynamics of DNA methylation.<sup>15</sup> Recent evidence linking TET to aberrant DNA methylation has revealed that oxidation of 5-mC occurs in human cells and is catalyzed by TET proteins; the resulting 5-hmC may have opposite or alternative functions as 5-mC. It is possible that other yet undiscovered modifications of cytosine exist with yet-to-be-determined epigenetic effects during development (Fig. 1 and we refer to them as 5-xC or “excytidine”).

Dense methylation found around gene promoters in so-called CpG islands is associated with gene silencing; thus, the distribution of methyl groups in the genome defines regions of varying transcriptional potential.<sup>16</sup> The DNA methylation pattern during embryonic development is tissue specific, which may explain the connection to important aspects of cellular differentiation.<sup>17</sup> The role of 5-hmC in the promoters and gene bodies of genes is less well characterized, but a recent paper from Pastor and colleagues has shown that 5-hmC correlates with “bivalent” chromatin marks in embryonic stem cells (ESCs), with both activating (H3K4me3)

and repressing (H3K27me3) chromatin marks.<sup>18</sup> This suggests that 5-hmC is associated with poised chromatin in ESCs that become activated upon differentiation into specific developmental pathways.<sup>18</sup>

One of the most exciting recent findings in the cancer field is that mutations in metabolic genes are associated with many malignancies.<sup>19</sup> Recent genomic sequencing efforts in acute myeloid leukemia (AML) and in other malignancies have identified new classes of oncogenic disease alleles. One recently identified class of genes mutated in cancer is that of genes coding for enzymes involved in citrate metabolism. The most prevalent of such mutations identified to date affects the genes for cytosolic isocitrate dehydrogenase 1 (IDH1) and its mitochondrial homolog IDH2.<sup>20,21</sup> Other metabolic mutants associated with cancer are succinate dehydrogenase (SDHD),<sup>23</sup> and fumarate hydratase (FH).<sup>24</sup> These findings have highlighted the metabolism, and drugs that affect metabolism, as one of the most important areas of cancer research and drug discovery.<sup>19</sup>

IDH1 and IDH2 are NADP<sup>+</sup>-dependent enzymes that normally catalyze the interconversion of isocitrate and alpha-ketoglutarate ( $\alpha$ -KG; also known as 2-oxoglutarate). A dehydrogenase (also called DHO in the literature) is an enzyme that oxidizes a substrate by transferring one or more hydrides (H) to an acceptor. An oxygenase is any enzyme that oxidizes a substrate by transferring the oxygen from molecular oxygen O<sub>2</sub> (as in air) to it. Dioxygenase is an oxygenase that transfers both oxygen atoms in O<sub>2</sub> to the substrate. Two distinct alterations are caused by the tumor-derived mutations in IDH1 or IDH2: loss of its normal catalytic activity in the production of  $\alpha$ -KG and gain of the

catalytic activity to produce 2-hydroxyglutarate (2-HG) (Fig. 2).<sup>20,21</sup>

IDH1<sup>R132MUT</sup> is a gain of function mutation in isocitrate dehydrogenase that produces the “cancer metabolite” 2-HG, which inactivates TET1 (Fig. 2). 2-HG is a competitive inhibitor of multiple  $\alpha$ -KG-dependent dioxygenases, including histone demethylases and the TET family of 5-mC hydroxylases. The NADP<sup>+</sup>-dependent isocitrate dehydrogenase genes IDH1 and IDH2 are mutated in >75% of low-grade gliomas and secondary glioblastoma multiforme (GBM) and in ~20% of AML leukemia.<sup>20,21</sup> When its normal catalytic activity is lost, mutant IDH1 and IDH2 also gained the function of catalyzing the reduction of  $\alpha$ -KG to produce 2-HG, resulting in an accumulation of 2-HG in IDH1 or IDH2 mutated gliomas and AML. In IDH1 mutated glioma, 2-HG accumulated to extraordinarily high levels of 5–35 mmol/g of GBM,<sup>25</sup> which could be equivalent to 5–35 mM assuming the tissue density of 1 g/ml.

IDH1 and IDH2 mutations were subsequently observed in myeloid malignancies including de novo and secondary AML (15%–30%) and pre-leukemic clonal malignancies including myelodysplasia and myeloproliferative neoplasms (5% of chronic phase and 20% of transformed cases).<sup>26,27</sup> The precise genetic context in which IDH1/2 mutations occur is not known, nor is the mechanism through which they contribute to the malignant phenotype. The most common IDH1/2 mutations in AML and brain tumors, affecting R132 of IDH1 or R140 and R172 of IDH2, have the common feature of acquiring a neomorphic enzymatic activity catalyzing the NADPH-dependent reduction of  $\alpha$ -KG to R(-)-2HG.

The genetic connection has given a major boost to the field of cancer metabolism. Normal cells primarily generate energy aerobically in mitochondria, whereas most tumor cells rely more heavily on glycolysis (the anaerobic conversion of glucose to lactate) to generate energy. Tumors also display other unique metabolic features. But the discovery of a mutated metabolic enzyme was strong evidence that metabolic abnormalities play an important role in oncogenesis (and it set off a frantic effort to understand what the mutation was doing in such common and lethal cancers).

We speculate, based on the insights gleaned from studying the effects of metabolic mutations on cancer epigenetics and the seemingly unrelated field of caloric restriction and longevity, that environmental toxins might also affect global epigenetic patterns by interfering with metabolism (Fig. 3). Developmental exposure to the heavy metal lead (Pb), for instance, alters DNA methylation patterns in human<sup>28</sup> and in mammalian models.<sup>29-32</sup> Furthermore, the studies in the mammalian models have shown that these DNA methylation changes can last several years into the lifespan of the animal (reviewed in ref. 32). The long-lasting effects of Pb on DNA methylation at specific sites might explain the early origins of adult disease observations suggesting that exposure to Pb can have effects on human development decades later.<sup>33</sup>

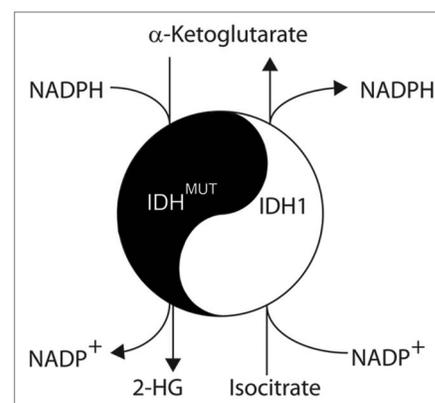
In our model, oxidative stress decreases NADPH and NADH levels, which, in addition to glutathione (GSH), are cofactors that drive anabolic biochemical reactions and provide reducing capacity to combat oxidative stress. Consequently, NAD<sup>+</sup> levels increase in the mitochondria under oxidative stress conditions, and this leads to the activation of the Sirtuin NAD<sup>+</sup>-dependent family of protein deacetylases.<sup>34</sup> Sirt3, a Sirtuin-family member in mammals, was recently found to deacetylate IDH2, the mitochondrial isoform of isocitrate dehydrogenase, and thereby activate it. Activated IDH2, in our model, can then produce increased amounts of  $\alpha$ -KG and lead to changes in 5-hmC and 5-mC patterns in the genome (Fig. 3). Sirt3 was found to

be a key regulator of caloric restriction, which is thought to also increase NAD<sup>+</sup> levels, because Sirt3 is required to prevent age-related hearing loss under caloric restriction.<sup>34</sup>

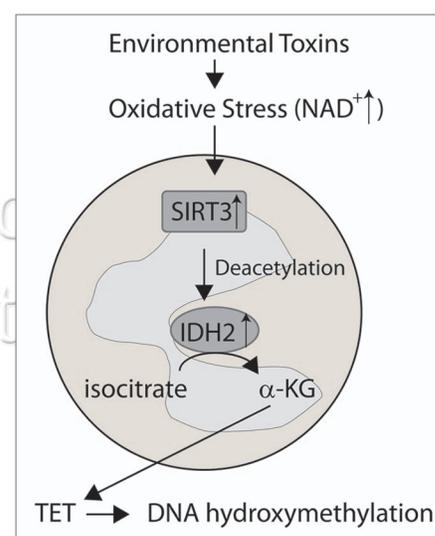
How might 5-hmC and 5-mC levels at the promoters of specific genes be a useful biomarker for exposure to early environmental toxins? We mentioned that developmental exposure to Pb alters DNA methylation patterns in human<sup>28</sup> and in mammalian models.<sup>29-32</sup> We speculate that this altered methylation pattern is mediated in part by oxidative stress-mediated alterations in metabolism, and thereby activated TET and other global chromatin modifying proteins (Fig. 3). We also speculate that each environmental toxin might affect metabolism and chromatin organization in a different manner and, consequently, each toxin has a unique “fingerprint” that reflects both the dose of the toxin and the type of environmental toxin.

If one can determine a genetic “fingerprint” for the dose and type of prenatal toxin exposure, it might then be possible to perform whole genome DNA methylation and hydroxymethylation assays on newborn dried blood spots to determine whether a newborn was exposed to an environmental toxin. These global DNA methylation and hydroxymethylation assays would potentially be much more sensitive than spectroscopic assays for environmental toxins, especially polycyclic aromatic hydrocarbons and other organic environmental toxins, which are difficult to detect. Consequently, global DNA methylation and hydroxymethylation assays are potentially extremely useful future tools for environmental toxicologists to determine the long-term effects of early exposure to environmental toxins on human health.

In summary, we believe that we can combine an understanding of metabolism gleaned from cancer and caloric restriction studies to better understand how early exposures to oxidative-stress inducing environmental toxins might have lifelong effects on a person’s epigenome. We hope that some of the ideas in this review will stimulate further studies on the metabolic effects of environmental toxins and thereby lead to a better understanding of their effects on



**Figure 2.** The Yin-Yang of Isocitrate Dehydrogenase. The wild-type IDH1 produces  $\alpha$ -ketoglutarate, which is used by TET. The IDH1<sup>MUT</sup> produces 2-HG, which inhibits TET and thereby decreases global 5-hmC levels.



**Figure 3.** Regulation of TET by environmental toxins. Environmental toxins produce oxidative stress, which leads to an increase in the ratio of NAD<sup>+</sup> to NADH. Sirt3 is a NAD<sup>+</sup> dependent protein deacetylase, which activates IDH2. IDH2 produces  $\alpha$ -KG from isocitrate and  $\alpha$ -KG activates TET, which leads to global DNA hydroxymethylation.

global chromatin and DNA methylation and hydroxymethylation patterns.

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