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## DNA modifications in models of alcohol use disorders

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### Abstract

Chronic alcohol use and abuse result in widespread changes to gene expression, some of which contribute to the development of alcohol use disorders (AUD). Gene expression is, in part, controlled by a group of regulatory systems often referred to as epigenetic factors, which includes, among other mechanisms, chemical marks made on the histone proteins around which genomic DNA is wound to form chromatin, and on nucleotides of the DNA itself. In particular, alcohol has been shown to perturb the epigenetic machinery, leading to changes in gene expression and cellular functions characteristic of AUD and, ultimately, to altered behavior. DNA modifications in particular are seeing increasing research in the context of alcohol use and abuse. To date, studies of DNA modifications in AUD have primarily looked at global methylation profiles in human brain and blood, gene-specific methylation profiles in animal models, methylation changes associated with prenatal ethanol exposure, and the potential therapeutic abilities of DNA methyltransferase inhibitors. Future studies may be aimed at identifying changes to more recently discovered DNA modifications, utilizing new methods to discriminate methylation profiles between cell types and clarifying how alcohol influences the methylomes of cell type populations and how this may affect downstream processes. These studies and more in-depth probing of DNA methylation will be key to determining whether DNA-level epigenetic regulation plays a causative role in AUD and can thus be targeted for treatment of the disorder.

### Keywords

Alcohol; Epigenetics; DNA methylation; DNMT; DNA hydroxymethylation; TET

## 1. Introduction

The transcription of a gene is controlled, in part, by its availability to the binding of transcription factors, which usually only have access to regulatory regions and promoters if the DNA is in the euchromatin state, unwound from histone proteins, with unmodified, unbound nucleotides. The state of the chromatin (DNA wound around histones and compacted) is controlled by epigenetic modifications - a complex of molecular machinery

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involved in regulation of gene expression at the individual gene and gene network levels. Crucially, epigenetic factors are capable of dynamically regulating gene expression within a cell, which, despite each cell in an organism having the same genotype, results in multiple cell types during development and, at the organism level, allows for expression of varied phenotypes. Epigenetic modifications include chemical residues or “marks” which may be added to or removed from amino acids of histone protein tails or DNA nucleotides. Addition of these marks by enzymatic “writers,” such as addition of methyl groups by methyltransferases, or their removal by “erasers,” such as removal of acetyl groups by deacetylases, enables dynamic regulation of the chromatin state, providing access to the DNA for transcription factors or for “readers,” such as methyl-binding domain (MBD) proteins, which bind certain marks to produce a downstream effect. Expression of these regulatory elements can be influenced by the environment, including exposure to ethanol (ethyl alcohol) and stress, and, through their effects on gene transcription, can lead to behavioral changes in an individual. In this way, epigenetic regulation mediates the complex relationship between an individual’s genotype and environment, resulting in changes in gene expression and downstream phenotypes.

Epigenetic systems have garnered growing attention in the study of disease states, since alterations to chromatin states are capable of creating contexts of widespread, aberrant gene expression that are capable of significantly contributing to development and expression of various complex pathologies. Though understudied in the context of models of alcohol use disorders until the past decade, recent evidence demonstrates that exposure to alcohol is associated with a wide range of epigenetic modifications, which may underlie alcohol-related behaviors. The focus of this review is on DNA-level epigenetic modifications, with emphasis on DNA methylation. We aim to provide relevant background and an overview of DNA modification research in the context of AUD, including alcohol’s effects in brain and peripheral tissues in both human and animal models, the effects of alcohol on the methylome of the developing brain, and the evidence supporting DNA modifications as a potential therapeutic target for treatment of AUD. Challenges and future directions of this research are also discussed.

## 2. DNA modifications

### 2.1. DNMT and 5mC

Epigenetic DNA modifications are made by the addition of chemical groups, such as a methyl group, to DNA bases. The cytosine of 5'-cytosine-phosphate-guanine-3' (CpG) dinucleotides is the most common epigenetically modified nucleotide, with the most frequent chemical mark on these cytosines being the addition of a methyl group to carbon 5 of the cytosine ring by DNA methyltransferase (DNMT) enzymes to form 5-methylcytosine (5mC) (Fig. 1a). CpG dinucleotides are frequently found clustered together to form CpG islands, which could often be found in regulatory regions, such as gene promoters. DNMT proteins catalyze the addition of a methyl group from the cell’s primary methyl group donor, *S*-adenosyl-methionine (SAM; Fig. 1b), which itself is synthesized from adenosine triphosphate (ATP) and methionine by methionine adenosyltransferase as part of the folate and methionine cycles (Hamid, Wani, & Kaur, 2009; Mentch & Locasale, 2016). It was

shown long ago that diets deficient in methyl donors, such as folates, choline, and some B-complex vitamins that work as cofactors in methyl transfer reactions, increased voluntary ethanol consumption in rats, while methyl-enriched diets decreased drinking (Williams, Berry, & Beerstecher, 1949), suggesting that methylation reactions play an important role in regulation of ethanol intake. Correspondingly, chronic ethanol reduces tissue SAM (Hamid et al., 2009) and individuals with alcohol-related liver disease show reduced SAM (Mato & Lu, 2007), supporting the importance of methylation processes in alcohol traits. In addition, alcohol-related reductions in B vitamins and folates lead to buildups of SAM precursor homocysteine (Blasco et al., 2005). Since SAM is the primary methyl donor for DNA methylation, it is possible that dietary changes alter SAM synthesis and affect alcohol use through changes to DNA methylation and downstream gene expression. However, possible actions of SAM are unlikely to be limited to DNA methylation, as SAM is also involved in other processes, including histone methylation and neurotransmitter metabolism (Mentch & Locasale, 2016).

There are two major classes of DNMT proteins involved in genomic DNA methylation, originally termed *de novo* (DNMT3a, DNMT3b; Okano, Bell, Haber, & Li, 1999) and maintenance (DNMT1; Bestor, 1992; Yoder, Soman, Verdine, & Bestor, 1997); however, some studies indicate that the *de novo* methyltransferases also have maintenance activities, and vice versa (Arand et al., 2012; Lorincz, Schübeler, Hutchinson, Dickerson, & Groudine, 2002). The *de novo* activity of DNMTs involves methylation of CpGs on either strand of DNA independent of whether the cytosine of the complimentary CpG dinucleotide of the double-stranded DNA is methylated, while the maintenance methyltransferase activity has high preference for methylating cytosines if one cytosine in a CpG dyad is methylated (a state termed “hemi-methylation”), thus resulting in both of the cytosines of the CpG dyad being methylated (Fig. 1b).

5mC is the most common DNA-level epigenetic modification, constituting ~70% of all CpG cytosines in mammals (though most unmethylated CpGs are found in promoters) (Robertson & Wolffe, 2000), and ~4% of all cytosines in the brain (Münzel et al., 2010). 5mC is most commonly recognized as a repressive mark, especially in the context of methylation of CpG islands in gene promoters. This modification is repressive through the blocking of transcription factor binding (Watt & Molloy, 1988), binding of MBD proteins, including the methyl-CpG binding protein 2 (MeCP2) (Lewis et al., 1992), and recruitment of co-repressor complexes (Klose & Bird, 2006), which results in condensed, transcriptionally repressive chromatin. Conversely, absence or removal of methylation in CpG islands can be derepressive (Fig. 1c). CpG methylation is essential for mammalian embryonic development and is used to preserve a type of molecular “memory” of which genes should be active or inactive - a state afforded through the stability of the 5mC modification. 5mC profiles contribute to cell differentiation by the switching on or off of cell type-specific genes (see Liyanage et al., 2014 for review) and, therefore, vary between different cell types and tissues including cell types and regions in the brain. Furthermore, variations in methylation within a cell type may result in functional and/or morphological differences among cells. Methylation profiles may also be passed down to daughter cells during replication following reprogramming, and can provide parental imprinting and X-chromosome inactivation (Cedar & Bergman, 2012; Finegersh, Rompala, Martin, & Homanics, 2015a; Lane et al., 2003;

Liyanage et al., 2014). Opposite to previously existing viewpoints, CpG methylation and its resulting molecular memory is not permanent, and can be regulated by demethylation pathways. For example, demethylation through the base excision repair (BER) pathway can modify 5mC and further alter gene expression. This typically involves cytidine deamination (e.g. through activation-induced deaminase (AID)/apolipoprotein B mRNA editing enzyme complex) to create 5-hydroxymethyluracil followed by 5hmU:G mismatch repair via DNA glycosylases (e.g. thymine DNA glycosylase (TDG)) and normal BER mechanisms (see Wu & Zhang, 2011 for review). Another demethylation pathways via 5hmC is discussed below.

Though 5mC is most commonly associated with CpG dinucleotides, there has been increasing evidence for methylation of cytosines in dinucleotide pairs with the other bases (A, T, C) -collectively referred to as CpH dinucleotides. CpH dinucleotides also demonstrate high levels of cytosine methylation, which is formed and maintained primarily by DNMT3a in mature neurons. Methylation of CpH dinucleotide cytosines also has a repressive effect on transcription *in vitro* and is able to recruit MeCP2 (Guo et al., 2014a). Further investigation is required into the role of CpH methylation in AUD and broader contexts, as the extent to which CpH methylation affects transcription and/or disease states *in vivo* is presently unknown.

## 2.2. TET and 5hmC

The second most common modification to CpG dinucleotides is the addition of a hydroxyl group to 5mC to form 5-hydroxymethylcytosine (5hmC). 5hmC was only recently described (Kriaucionis & Heintz, 2009; Tahiliani et al., 2009) and research into its profile and potential roles in control of gene expression and downstream processes in the context of substance use disorders (SUD) is still in the nascent stages. The oxidizing reaction of 5mC is catalyzed by a family of proteins called ten-eleven translocator (TET1, TET2, TET3), which operates in an iron-and  $\alpha$ -ketoglutarate-dependent manner and uses  $O_2$  as its oxygen donor (Tahiliani et al., 2009). TET proteins are also capable of further oxidizing 5hmC into 5-formylcytosine (5fC) and 5fC into 5-carboxylcytosine (5caC) (Ito et al., 2011), with both modifications being susceptible to BER through TDG (Fig. 1a) (Guo, Su, Zhong, Ming, & Song, 2011; He et al., 2009; Hashimoto, Hong, Bhagwat, Zhang, & Cheng, 2012a). While 5caC is hypothesized to be primarily an intermediate in the demethylation pathway, there is evidence that 5fC is a stable modification when not further oxidized. Although it is rather lowly abundant *in vivo* in adult brain (Bachman et al., 2015), one study identified more numerous proteins with a strong preference for 5fC as compared to 5mC and 5hmC, implying a role in regulatory functions akin to 5mC and 5hmC (Iurlaro et al., 2013). The functions of these proteins predictably included transcriptional regulation, chromatin modifications, and DNA repair, suggesting that the 5fC mark may play a more important role in transcriptional regulation than previously thought. To date 5fC and 5caC have not been studied in the context of AUD or SUDs.

5hmC is itself a stable nucleotide (Bachman et al., 2014) highly prevalent in brain compared to other tissues, implying a special role for it in central functions (Ito et al., 2011; Kriaucionis & Heintz, 2009; Nestor et al., 2012). Within the brain, 5hmC is most enriched in the cerebral cortex, hypothalamus, and hippocampus. In these regions, 5hmC accounts for

0.7% of all cytosines; other regions see slightly lower levels of 5hmC, ranging from 0.3% (cerebellum) to 0.6% (brainstem, olfactory bulbs) (Münzel et al., 2010). Given the increased abundance of 5hmC in brain tissue, the brain, more than other tissues, may be susceptible to changes in 5mC levels, which can in turn alter gene expression. The exact mechanisms of the effects of 5hmC on gene expression are not fully understood, but it has been proposed to mainly promote transcription via DNA demethylation. 5hmC was proposed to be an intermediate in both active DNA demethylation via AID and BER pathways (Guo et al., 2011) and passive demethylation by blocking of DNMT1 (Hashimoto et al., 2012b). 5hmC produces an activating effect on transcription when expressed in gene bodies (Fig. 1c; Guo et al., 2014b) and also plays a role in control of splice variant expression via demarcation of exon-intron boundaries (Feng et al., 2015; Khare et al., 2012). Interestingly, 5hmC can bind methyl-binding domain proteins traditionally associated with transcriptional repression, but functional consequences of these associations are not well understood. For example, binding of 5hmC to MeCP2 was proposed to facilitate transcription in postmitotic neurons (Mellén, Ayata, Dewell, Kriaucionis, & Heintz, 2012), while binding of 5hmC to the methyl-CpG binding domain protein 3 (MBD3) in bivalent promoters in embryonic stem cells was proposed to contribute to transcriptional repression by the Nucleosome Remodeling and Deacetylase (NuRD) corepressor complex (Yildirim et al., 2011). Furthermore, CpH dinucleotides can also be hydroxymethylated, with subsequent demethylation in the presence of TET1, contributing to transcriptional activation (Guo et al., 2011). Both DNA methylation and hydroxymethylation have been extensively studied in the context of higher brain functions including learning and memory (Kennedy & Sweatt, 2016; Meadows et al., 2015; Rudenko et al., 2013). The current review focuses on their roles in drug addiction.

### 2.3. DNA modifications in SUDs

DNA modifications have been implicated as a factor in a large number of somatic and psychiatric disorders, including cancer (see Esteller, 2008; Varela-Rey, Woodhoo, Martinez-Chantar, Mato, & Lu, 2013 for review), schizophrenia, depression, and bipolar disorders (see Nestler, Peña, Kundakovic, Mitchell, & Akbarian, 2015; Madrid, Papale, & Alisch, 2016 for review). To date, histone modifications have received the bulk of study by SUD research groups. In recent years, however, DNA modifications have garnered increasing attention. For example, in cocaine abuse, DNMT3a is dynamically regulated by acute and chronic cocaine use and withdrawal whereas knockout of *Dnmt3a* enhances cocaine responses in the nucleus accumbens (Anier, Malinovskaja, Aonurm-Helm, Zharkovsky, & Kalda, 2010; LaPlant et al., 2010). *Mecp2* knockout in the nucleus accumbens enhances amphetamine reward (Deng et al., 2010), while knockdown of the MeCP2 protein in dorsal striatum attenuates cocaine responses (Im, Hollander, Bali, & Kenny, 2010). Furthermore, cocaine downregulated *Tet1* but induced increased 5hmC content in some enhancer regions of cells in the nucleus accumbens after repeated cocaine administration in mice, with increases in 5hmC and gene expression at some genomic loci being maintained for up to a month (Feng et al., 2015). Viral knockdown of *Tet1* in the same study resulted in enhanced cocaine place conditioning in a conditioned place preference (CPP) paradigm, whereas overexpression of *Tet1* reduced CPP. Further discussion regarding the epigenetics of SUDs is beyond the scope of this review, though has been covered well in the past (Feng & Nestler, 2013; Nestler, 2014).

### 3. DNA modifications in AUDs

#### 3.1. Human brain

In the context of AUD, most early research has looked at methylation status of individual gene promoters, owing in part to the limitations of whole genome methylation profiling and early-stage sequencing methods. Recent advances in microarray and sequencing technologies have allowed for genome-wide methylation profiling, which has been utilized by several alcohol-related studies that mainly focused on human tissue. In general, differential methylation results from studies of human genes in the brain suggest potential mechanistic roles in central control of alcohol-related behaviors, whereas results from peripheral tissues may be more useful as biomarkers for diagnosis, prognosis, and/or treatment (Andersen, Dogan, Beach, & Philibert, 2015).

At individual gene level, the *PDYN* gene in postmortem brain exhibits differential methylation of CpGs associated with alcohol-risk single-nucleotide polymorphisms (SNPs) in its 3' untranslated region (UTR), underscoring the role differential methylation may play in non-promoter CpGs, and identifying specific AUD risk SNPs (Taqi et al., 2011). *PDYN* encodes prodynorphin, a precursor of several peptides in the endogenous opioid system that is involved in alcohol dependence (Wee & Koob, 2010). Therefore, SNP-related differential methylation at *PDYN* CpGs may play a mechanistic role in susceptibility to AUD. *TET1* expression is also increased in brains of alcoholics that demonstrate comorbid psychotic symptoms as compared to control subjects and psychosis-only subjects (Guidotti et al., 2013).

Several studies measured genome-wide DNA methylation level in postmortem alcoholic brain. For example, a 2012 study by Ponomarev and colleagues used transcriptome microarrays and methylation-sensitive reverse transcription-qPCR and observed reductions in *DNMT1* transcript levels and accompanying reductions in methylation of the repeat element Long Terminal Repeat (LTR) retrotransposon, a type of endogenous retrovirus found throughout the genome, in human alcoholic cortex compared to matched controls (Ponomarev, Wang, Zhang, Harris, & Mayfield, 2012). Since LTR repeats constitute about 8% of the human genome, the researchers concluded that alcoholic brain exhibits global hypomethylation. This reduced methylation at LTRs could have occurred during primordial germ cell or embryonic demethylation stages through epigenetic reprogramming, but it has been demonstrated that LTRs are highly resistant to reprogramming-related demethylation in mice (Lane et al., 2003), suggesting that the results from the Ponomarev group were alcohol-related. Corroborating these conclusions, another group used methylated genomic DNA (gDNA) immunoprecipitation and genome-wide promoter methylation microarray methods to probe the genomic methylation profile of human alcoholic cortex; of the ~3,800 differentially methylated genes, the majority (~57%) exhibited higher methylation in controls, or relative hypomethylation in alcoholics (Manzardo, Henkhaus, & Butler, 2012). Most recently, and somewhat contrary to these results, Wang and colleagues used Illumina HumanMethylation450 BeadChip assays to perform whole genome methylation profiling in the prefrontal cortex of alcoholics and control cases from the same brain bank as the previous two studies, and found 1,812 differentially methylated CpGs (including non-

promoter CpGs found in gene bodies and 3' UTRs) after multiple comparison correction, 66.3% of which were hypermethylated in alcoholic subjects (Wang, Xu, Zhao, Gelernter, & Zhang, 2016). Remarkably, differences in DNA methylation were only observed in male subjects, hinting at gender differences in alcohol-induced DNA methylation in brain.

Some discrepancies in the results of these three studies most likely arise from differences in methodology and targeted genomic locations of CpGs. While Ponomarev and colleagues (2012) examined DNA methylation at the repeat elements mainly located in the intergenic regions, the other two studies targeted promoters (Manzardo et al., 2012; Wang et al., 2016) and gene bodies (Wang et al., 2016) of known genes. In addition, compared to the study by Manzardo and colleagues who used an antibody specific for 5mC, Wang and colleagues used the bisulfite conversion method that does not distinguish between 5mC and 5hmC (Huang et al., 2010), and, therefore, depending on the actual distribution of the two marks, may either overestimate or mask differences in DNA methylation. A direct comparison of the latter two studies may point to genomic locations with likely changes in alcohol-related DNA hydroxymethylation. Ultimately, the results of these studies are complimentary, revealing the diversity and heterogeneity of DNA methylation in the alcoholic brain, and each contributes a part of a grander picture relating DNA methylation to AUD. It is important to note, though, that these studies cannot definitively establish whether the observed methylation differences are caused by alcohol or a preexisting condition, or whether differential methylation played any part in regulating alcohol-related behaviors — a major limitation in using human brain. Still, this does not diminish their importance in providing mechanistic insights into potential consequences of chronic alcohol abuse.

### 3.2. Human blood

Peripheral tissue, particularly blood, provides the advantage of being able to sample DNA and probe methylation at various time points with respect to alcohol use, including those after recent use and after periods of withdrawal. These studies may not provide as much insight into mechanisms that drive central functions associated with AUD as similar data from brain tissue, but may prove useful as biomarkers for diagnosis and treatment (Andersen et al., 2015). At individual gene level, alcohol exposure resulted in more hypermethylation. For example, Bönsch and colleagues found hypermethylation at the alpha-synuclein gene (*SNCA*) promoter (2005), along with decreases in *DNMT3A* and *DNMT3B* mRNAs, the latter of which exhibited a significant negative correlation with blood alcohol concentration (2006) in blood of males with AUD. Two other studies reported an elevated methylation at the dopamine transporter (*DAT*) (Hillemacher et al., 2009a) and vasopressin (*AVP*) genes (Hillemacher et al., 2009b). There was also increased methylation at the *OPRM1* gene, which encodes for the  $\mu$ -opioid receptor (Zhang et al., 2012), a gene heavily involved in alcohol reward pathway that is targeted by opioid receptor antagonist naltrexone, an FDA-approved drug for treatment of AUD. The brain-derived neurotrophic factor (*BDNF*) gene codes for a protein involved in cell health and promotion of synaptic growth and differentiation, and methylation at its promoter was increased in blood of alcohol-dependent patients but returned to baseline after 14-day withdrawal (Heberlein et al., 2015). Blood from alcohol-dependent males demonstrated an increase in methylation of the nerve growth factor (*NGF*) promoter between withdrawal days 7 and 14 and corresponding reduced

transcription of that gene (Heberlein et al., 2013), though there was no change in *NGF* methylation from withdrawal days 1–7 in the same subjects. Methylation changes have also been observed in blood at individual genes related to neurogenesis, inflammation, and alcoholism, with an overall trend of hypermethylation (Weng, Wu, Lee, Hsu, & Cheng, 2015).

Global DNA methylation in blood has been studied using various techniques targeting either total proportion of methylated cytosines or genome-wide methylation profiles. Global hypermethylation has been observed in blood of alcoholics compared to controls using restriction enzyme digestion with fluorometric enzyme-linked immunosorbent assays (Bönsch, Lenz, Reulbach, Kornhuber, & Bleich, 2004; Bönsch et al., 2006). On the other hand, genome-wide methylation analysis of blood from male alcoholic cases and controls by bisulfite conversion and microarrays demonstrated that greater than 99% of differentially methylated CpGs were hypomethylated, with associated hypomethylated genes involved in stress, immune response, signal transduction, and alcohol metabolism (Zhang et al., 2013), though bisulfite sequencing cannot differentiate 5mC and 5hmC (Huang et al., 2010), which could contribute to the discrepancies between this study and those that observed alcohol-related hypermethylation. Conversely, hypermethylation was observed in lymphocyte-derived lymphoblasts of females with a history of heavy alcohol use using the bisulfite and microarray techniques (Philibert, Plume, Gibbons, Brody, & Beach, 2012), again, hinting at potential gender differences in alcohol-associated DNA methylation. DNA methylation may also be related to co-occurrence of addiction phenotypes, as global blood DNA methylation and comorbid alcohol and nicotine use was positively correlated (Semmler et al., 2015). A study from the IMAGEN consortium used methylation-sensitive gDNA digestion and genome-wide methylation microarrays for genome-wide methylation profiling in blood of twins discordant for AUD. This study found 77 differentially methylated regions, with ~68% of them being hypermethylated (Ruggeri et al., 2015). Interestingly, most of the differentially methylated regions identified were not in CpG islands. One of the differentially methylated genes, protein phosphatase *PPM1G*, was hypermethylated, showed decreased mRNA expression, and was found to be associated with AUD, early escalation of alcohol use, and increased impulsiveness in 499 adolescents from the IMAGEN group.

DNA methylation in blood appears to be a dynamic process, as one pilot study demonstrated the reversal of differential methylation at several genomic loci in subjects with heavy alcohol use at two time points, from pre-abstinence to post-abstinence, with many of these methylation changes mapping to apoptosis gene networks (though it is worth noting that many of these subjects exhibited comorbid nicotine and/or THC use) (Philibert et al., 2014). Finally, a recent study showed that DNA methylation may, at least, in part, mediate the associations of genetic variations with AUD phenotypes via regulation of gene expression (Zhang et al., 2014b). DNA modification profiles can vary widely between tissues and cell types (Liyanaage et al., 2014) and, therefore, caution should be taken in extrapolating results from one tissue to another, especially from peripheral tissues to brain. Blood methylation profiles may not provide full mechanistic information for central functions, but can be useful as biomarkers of various conditions associated with AUD, including response to treatment.



### 3.3. Animal models

Animal models of alcohol use are effective for exploring and manipulating the interplay between alcohol and DNA modifications. Advantages include the availability of multiple alcohol models that attempt to replicate different aspects of AUD, such as binge drinking (drinking in the dark model; Rhodes, Best, Belknap, Finn, & Crabbe, 2005), voluntary escalation in consumption (chronic two-bottle choice drinking; Osterndorff-Kahanek, Ponomarev, Blednov, & Harris, 2013), or development of dependence (chronic intermittent vapor model; Becker & Lopez, 2004). By probing DNA modifications in brain tissue of animal models, we can explore specific mechanisms underlying causative relationships between DNA modifications, gene expression, and behaviors.

Analysis of CpG promoter methylation using these models has revealed several genes that are affected by the interplay between alcohol and epigenetic regulation and may play a role in alcohol behaviors. Some of these genes exhibit hypermethylated promoters, while others show the opposite trend, suggesting that alcohol's effects on DNA methylation are diverse and may be affected by numerous factors, including developmental stage, functional state of the cell, and specific gene targets in specific cell types (Basavarajappa & Subbanna, 2016). An advantage of studying methylation in animal models is the ability to determine mechanistic relationships between methylation of specific genes and alcohol behaviors. The NMDA receptor subtype 2b (NR2B; *Grin2b*) gene promoter is demethylated and its expression is increased in mouse adult and embryonic cortical neurons exposed to chronic ethanol, though the same gene's methylation status was unaffected by acute ethanol treatment in adult mouse cortex (Ravindran & Ticku, 2004, 2005). Ethanol inhibits excitatory hippocampal NMDA ion currents (Lovinger, White, & Weight, 1989), and NR2B-containing receptors have been shown to be especially sensitive to ethanol (Chu, Anantharam, & Treisman, 1995). Since *Grin2b* methylation is decreased in response to ethanol, it is possible that the downstream increase in NR2B expression is a maladaptive neuroadaptation that leads to withdrawal phenotypes of hyperexcitability (Wong, Tauck, Fong, & Kendig, 1998). In fact, this hypothesis is corroborated by Wang and colleagues, who demonstrated that inhibition of NR2B subunit-containing NMDA receptors reduced operant self-administration and relapse (2010). It can therefore be hypothesized that alcohol-induced changes to methylation at, and to expression of the *Grin2b* gene underlie maladaptations that drive excessive alcohol consumption and relapse.

In a mouse model of chronic intermittent alcohol exposure, TET1 mRNA expression was increased in the nucleus accumbens, but not in cortex, after 4 days of ethanol vapor (Finegersh et al., 2015b). DNA modifications were not measured in this study, but the change in TET1 implies possible downstream changes in 5mC and 5hmC. Acetaldehyde, an alcohol metabolite, has also been shown to inhibit DNMT activity *in vitro* (Garro, McBeth, Lima, & Lieber, 1991). Though alcohol can inhibit DNMT protein activity through acetaldehyde, multiple ethanol dosages yielded increases in DNMT1 and DNMT3a mRNA in rat bed nucleus of the stria terminalis (Sakharkar et al., 2014), a possible compensatory effect. It is also worth noting that the BER pathway may be altered in the nucleus accumbens of alcohol-injected mice through increased expression of GADD45b, which is capable of

recruiting cytidine deaminases and DNA glycosylases, implying the potential for global reductions in DNA methylation (Gavin, Kusumo, Zhang, Guidotti, & Pandey, 2016).

Peripherally, liver tissues of alcohol-exposed young mice have exhibited altered DNA 5hmC content; this result did not extend to older mice, though older mice exhibited reduced *TET2* and *TET3* expression. Interestingly, dietary changes altered 5hmC in the older mice, but not in the younger animals, indicating interactions between aging and alcohol in the liver (Tammen et al., 2014). Furthermore, chronic alcohol has been shown to induce global hypomethylation in rat colon, which has been hypothesized to contribute to colon cancer (Choi et al., 1999).

A bulk of research on DNA modifications in animal models focused on the effects of ethanol on developing brain, which yields the manifestation of numerous abnormalities collectively termed “fetal alcohol spectrum disorders” (FASD). There is abundant evidence from both *in vitro* and *in vivo* models for the effects of prenatal ethanol exposure on DNA modifications, particularly on methylation. For example, embryonic cell cultures of astrocytes exposed to ethanol exhibited reduced levels of DNMT3a and hypomethylation of the tissue plasminogen activator promoter (Zhang et al., 2014a), which is involved in the degradation of the extracellular matrix components and has been shown to exhibit increased expression in the brain of animal models of AUD and FASD (Noel, Norris, & Strickland, 2011). Such activation of tissue plasminogen activator is also associated with neurodegeneration (Skrzypiec et al., 2009). MeCP2 protein and mRNA expression is increased in mouse neural stem cells after continuous ethanol exposure with correspondingly decreased 5mC and increased 5hmC content at MeCP2 regulatory elements as assessed using DNA immunoprecipitation and quantitative PCR (Liyanage, Zachariah, Davie, & Rastegar, 2015). Interestingly, and illustrating potentially important dynamic regulation of DNA modifications, after removal of ethanol from neural stem cell culture media for six days, the cells exhibited reduced MeCP2 protein and mRNA with correspondingly reversed 5mC and 5hmC enrichment at some of these MeCP2 regulatory elements. Globally, 5mC level in these cells was increased after chronic ethanol exposure, whereas 5hmC was reduced after withdrawal (Liyanage et al., 2015). Using an *in vitro* model of FASD, it has been demonstrated that fetuses of ethanol-treated mice exhibit global hypomethylation in addition to inhibited DNMT activity in nuclei isolated from whole fetuses (Garro et al., 1991). In addition, one recent study identified increased expression of both DNMT1 and TET1 in mouse neural stem cells after four days of withdrawal from acute exposure to alcohol; however, there was no change in 5hmC content and only modest changes to 5 mC at the probed target loci (Veazey, Parnell, Miranda, & Golding, 2015).

Alcohol exposure can cause widespread perturbations to methylation programming (Zhou, Chen, & Love, 2011a) and improper cell differentiation (Zhou et al., 2011b), which can contribute to developmental disorders and neurobehavioral deficits that can persist into adulthood (reviewed in Lunde et al., 2016). Prenatal ethanol exposure increases adult mouse hippocampal expression of *Slc17a6*, which encodes the VGLUT2 protein and is associated with hypomethylation of that gene’s promoter, though it is worth noting that VGLUT2 protein expression was decreased (Zhang, Ho, Vega, Burne, & Chong, 2015). VGLUT2 is a vesicular glutamate transporter primarily expressed in the hippocampus in the developing

brain, and the alcohol-induced changes in this protein are consistent with altered glutamate transport and cognitive and behavioral phenotypes associated with FASD. There may be an additional level of epigenetic control via microRNA (miRNA) that may act to balance methylation-related changes to gene expression, as here, the authors speculated that the VGLUT2 mRNA was degraded by a miRNA, which resulted in decreased protein expression (Zhang et al., 2015). In addition, *Bdnf* gene was hypermethylated and its mRNA expression decreased in the olfactory bulbs of rat pups with fetal exposure to ethanol through their mothers (Maier, Cramer, West, & Sohrabji, 1999), which was consistent with olfactory bulb neuronal loss. Furthermore, prenatal ethanol treatment of rats reduced mRNA expression of the astrocytic protein GFAP through *Gfap* promoter hypermethylation in fetal astrocyte cultures and postnatal brain tissue (Valles, Pitarch, Renau-Piqueras, & Guerri, 1997). These results underscore a central aspect of FASDs, in that early (*in utero*) alcohol exposure produces molecular perturbations that persist after birth and into adulthood.

While prenatal alcohol models typically focus on maternal exposure, there is increasing evidence that paternal ethanol exposure may lead to a modified sperm epigenome, such as reduced DNMT1 expression and possible DNA hypomethylation, and downstream expression of normally imprinted, silent paternal alleles in offspring (Bielawski, Zaher, Svinarich, & Abel, 2002). Paternal drinking may lead to altered DNMT activity and methylation profiles in gametes, which can potentially result in altered methylation reprogramming and epigenetic inheritance (reviewed in Finegersh et al., 2015a). Importantly, it has been established that paternal ethanol exposure contributes to low drinking and elevated ethanol sensitivity in male mouse offspring, along with altered *Bdnf* expression and hypomethylation of the *Bdnf* promoter (Finegersh & Homanics, 2014). Paternal preconception alcohol use has also been associated with offspring risk for psychosocial abnormalities (Finegersh et al., 2015a). Despite an increasing interest in epigenetic inheritance research, the exact mechanisms of these transgenerational phenomena remain unclear (Heard & Martienssen, 2014). The majority of other research into FASD is beyond the scope of this review, but has been reviewed elsewhere (see Liyanage et al., 2014; Lunde et al., 2016; Basavarajappa & Subbanna, 2016).

Together, these studies illustrate that alcohol-induced methylation states can vary both *in vitro* and *in vivo*, and that these effects depend on particular time points during development and during ethanol exposure, which underscores the dynamic nature of DNA modifications. Differential methylation of genes like *Grin2b* and *Bdnf* suggests that neurons may be particularly vulnerable to changes to the DNA methylome, which may play a significant role in alcohol-related neuroadaptations. Neural activity has been shown to alter DNA methylation in memory-related genes (Meadows et al., 2015), and conversely, dynamic regulation of DNA methylation has been demonstrated to be essential to memory formation (Miller & Sweatt, 2007), leading to the hypothesis that DNMTs and TETs are involved in tuning synaptic scaling (Kennedy & Sweatt, 2016). Comparably, while most epigenetic research to date in the context of AUD focused on neuronal processes, other brain cell types (e.g., astrocytes) have started to receive increasing attention, showing changes in DNA methylation that may contribute to alcohol-related processes (Zhang et al., 2014a).

### 3.4. DNA modifications as therapeutic target for treatment of AUD

More applicable from a translational perspective is investigation into the effects of alcohol on DNMT and TET functions, and whether manipulations of these proteins can in turn alter alcohol related behaviors. Small molecule inhibitors of these enzymes have the potential to change gene expression and downstream functions via regulation of chromatin structure. For example, DNMT inhibitors can reduce DNA methylation and change downstream gene expression via a reduction in DNMT functions. Some of these molecules are FDA-approved drugs that have been developed to correct abnormal methylation in cancers. Recently, these drugs have been used to study mechanisms underlying the effects of DNA methylation on alcohol functions. For example, an *in vitro* administration of the DNMT inhibitor azacitidine (5-azacytidine, or 5-aza) has been shown to mimic the effects of ethanol on the methylation status of the *Grin2b* promoter (Ravindran & Ticku, 2004). Some other *in vitro* studies demonstrated that ethanol-induced deficits in methylation reprogramming and neural stem cell migration, growth, and differentiation could also be mimicked by application of 5-aza (Zhou et al., 2011a,b).

Excessive alcohol intake increases DNMT1 expression in mouse nucleus accumbens *in vivo*, while systemic i.p. administration of 5-aza 2 h before a drinking session reduced excessive alcohol intake in mice after previous acquisition of escalated alcohol drinking (Warnault, Darcq, Levine, Barak, & Ron, 2013), though this effect did not persist into subsequent drinking sessions. Recent results from our lab support this finding, as i.p. injections of the potent DNMT inhibitor decitabine (5-aza-2'-deoxycytidine) reduced drinking when injected an hour before alcohol access post-baseline drinking in a chronic intermittent model of voluntary drinking, as well as when injected 2 h before a drinking in the dark (DID) procedure (Ponomarev et al., 2016). These results were corroborated in a study that used a rat model of alcohol dependence using vapor exposure, in which i.c.v infusions of the DNMT inhibitor RG108 abolished escalated operant ethanol self-administration in post-dependent rats compared to non-vapor-exposed animals (Barbier et al., 2015). Furthermore, post-dependent rats exhibited increased neuronal DNMT1 and 5mC immunoreactivity after three weeks of abstinence in the medial prefrontal cortex and nucleus accumbens, with correspondingly decreased expression of genes related to synaptic neurotransmitter release, and these changes were also abolished by i.c.v. RG108 infusion. Probing of the whole transcriptome from this experiment also revealed statistically significantly decreased expression of *Tet1* and *Tet3* transcripts, suggesting alcohol-related changes in DNA hydroxymethylation (Barbier et al., 2015). Taken together, these results suggest a generalized role for DNMT inhibitors in attenuating ethanol drinking.

In contrast to these findings, i.c.v. injections of 5-aza before multiple bouts of ethanol vapor exposure has been shown to facilitate subsequent voluntary 2-bottle choice drinking compared to pre-vapor baseline drinking in a chronic intermittent ethanol vapor mouse model. However, i.c.v. injection of 5-aza did not facilitate drinking without vapor exposure, and when 5-aza was administered via i.p. injections, no increase in drinking was observed even after vapor exposure (Qiang et al., 2014). The authors of this study hypothesized that 5-aza may not efficiently cross the blood brain barrier, and encouraged caution when interpreting results based on i.p. injections of this drug. Even more interestingly, subsequent

injection of SAM in these mice prevented escalated drinking (Qiang et al., 2014), lending credibility to the findings from Williams et al. (1949) that suggested a role for methyl donors in attenuating drinking.

These four studies used experimental designs that differed from each other in significant ways, which may explain the discrepancies in the findings. It appears that DNMT inhibitors may alter drinking depending on the time of application, since application before prolonged vapor exposure facilitated subsequent voluntary drinking (Qiang et al., 2014), whereas application right before voluntary intake (Ponomarev et al., 2016; Warnault et al., 2013) or after a period of withdrawal from chronic ethanol (Barbier et al., 2015) attenuated subsequent ethanol self-administration. There may also be an effect of species, as vapor exposure models produced the opposite results in rats (Barbier et al., 2015) and mice (Qiang et al., 2014) in response to DNMT inhibitors, whereas voluntary drinking models in mice showed consistency (Ponomarev et al., 2016; Warnault et al., 2013). Method of injection may play a role as well. Whereas i.c.v. injection of 5-aza facilitated drinking, i.p. injection of the drug from the same study did not (Qiang et al., 2014), though in another study, i.p. injection of 5-aza attenuated drinking (Warnault et al., 2013). This suggests that 5-aza may not be entirely effective at crossing the blood brain barrier. However, i.p. injection of decitabine proved to be effective at attenuating drinking (Ponomarev et al., 2016), and decitabine has been shown to cross the blood brain barrier (Karahoca & Momparler, 2013). This also hints at the possibility that method of injection (i.e. systemic i.p. injection vs. central i.c.v. injection) of these drugs may affect behavior differently, owing to target (brain vs. systemic) or level of stress involved with injection method (including cannulation for i.c.v. injection). These discrepancies between drug, method of injection, and method of alcohol exposure will require further investigation, both to sort out these differences and to affirm their efficacy in reducing and/or preventing drinking.

To add to the complexity of the interactions between DNA modifications and alcohol functions, alcohol produces opposite effects on DNMT1 expression in humans and animal models, a decrease in alcohol-dependent human subjects (Ponomarev et al., 2012) and an increase in mice (Warnault et al., 2013) and rats (Barbier et al., 2015). There are a number of factors that may contribute to this discrepancy, including the fact that rodent models do not capture the full complexity of human conditions. It may also support previous evidence for the dynamic nature of DNA modifications in the context of AUD, which may change from acute to continuous exposure to lifetime consumption and after withdrawal from alcohol. Despite some discrepancies between postmortem human and animal studies, taken together, these results imply a role for DNA-modifying enzymes as potential therapeutic targets for the treatment of AUD. Both 5-aza and decitabine are FDA-approved for treatment of cancer and these studies may lead to the repurposing of these compounds for drug addiction.

#### 4. Concluding remarks

The evidence presented here supports the hypothesis that alcohol use disorders are associated with epigenetic changes to DNA and that alcohol use/abuse disrupts DNA modifications, which can, in turn, mechanistically contribute to alcohol-induced changes in cellular functions and behavior. These results demonstrate a relationship between alcohol

and DNA modifications in a wide variety of contexts. In humans, multiple studies have observed alcohol-associated changes to global methylation in both brain and blood, with a general tendency of hypomethylation in brain and the opposite trend in blood. These differences between the two tissues may reflect the cell type specificity of DNA modification profiles, including a much higher content of 5hmC in neurons, compared to blood cells. The directionality of these changes within a tissue has also not always agreed, likely owing to differences in methodology and targeted genomic locations. The genomic location appears to be an important variable with regard to alcohol-induced changes in DNA methylation. Usually heavily methylated genomic repeats that mainly reside in intergenic regions are less methylated in the alcoholic brain (Ponomarev et al., 2012), while CpGs in promoters and gene bodies show diverse patterns of methylation (Manzardo et al., 2012; Wang et al., 2016). While the alcohol-associated reduction in methylated cytosines has been proposed to be due to a deficiency in methyl donors (Ponomarev, 2013), mechanisms underlying promoter hypermethylation in alcohol-dependent subjects are not well understood.

Epigenetic profiling in somatic tissues, especially in a renewable resource like blood, offers the advantage of studying the dynamic nature of chromatin modifications, as repeated measurements can be obtained from the same subjects over time. It is becoming increasingly clear that many drug-induced epigenetic modifications are transient and some are more long lasting than others. DNA methylation appears to be more stable than most histone modifications, but even this mark can undergo changes within short time periods, as one study showed a reversal of blood DNA methylation profiles in alcoholics after four weeks of abstinence (Philibert et al., 2014). This may, of course, be because the renewed populations of blood cells do not express alcohol-induced changes after this environmental challenge is removed. Nevertheless, epigenetic time course studies are important in helping understand the relationships between DNA modifications, gene expression, and downstream functions, when transcriptome and functional measurements are taken in parallel with epigenetic profiling. In addition, the blood epigenome can provide important biomarkers that can be used in identifying risk factors and individual susceptibility to AUD as well as predicting functional consequences of alcohol abuse and treatment outcomes in clinical settings.

Because of cell type specificity of epigenetic profiles, findings from peripheral tissues cannot be easily extrapolated to central mechanisms. One way to better predict brain changes based on somatic findings is to identify a subset of epigenetic marks that are consistently correlated across tissues. A good example of such an analysis is the identification of the “epigenetic clock,” a panel of 353 CpG sites, which can reliably predict biological aging based on their methylation profile across a wide variety of human tissues (Horvath, 2013). Identifying such a panel in response to alcohol in humans will be challenging, but different animal models are readily available and the importance of this analysis warrants further investigation.

Compared to human postmortem brain studies, animal models offer the advantage of identifying causative factors in the context of alcohol exposure. This includes determining the epigenetic control of alcohol-induced gene expression, specific roles of epigenetic enzymes in different cell types, and the effects of epigenetic drugs on brain and behavior.

Studies identified multiple CpG sites that may mediate alcohol-induced gene expression in neurons and other brain cells. Time course studies also suggest that DNA modifications are involved in neuroadaptive responses to alcohol. Prenatal tissues are especially susceptible to alcohol's effects on the methylome, as disruption of methylation perturbs cell differentiation and leads to abnormal cell functions in adults. Although the majority of FASD research concerns maternal alcohol exposure, some have recently hypothesized that paternal alcohol exposure may have a potential role in methylation reprogramming (reviewed in Finegersh et al., 2015a). FDA-approved DNMT inhibitors consistently alter drinking behaviors, though the results have not always agreed, likely owing to differences in study designs. Still, the potential of using hypomethylating agents in AUD clinical settings looks promising.

Taken together, the results presented above indicate that the relationships between alcohol traits and DNA modifications are quite complex and depend on a number of factors that include species, gender, tissue and cell type, method of alcohol exposure, time point when measurements are taken, and developmental time point of the model. There are multiple challenges for epigenetic research in the context of AUD, as highlighted in recent reviews (Harlaar & Hutchison, 2013; Ponomarev, 2013). Some of the technical challenges have been addressed by recent advances in epigenetic methodology, as discussed in the next paragraph. Perhaps one of the biggest challenges remaining, and not particularly attributable to epigenetic research, is the translation of results from animal models to clinical settings, as it is not clear how well animal models represent different aspects of alcohol use and abuse in humans. Given some reports showing inconsistencies between animal and human studies, it is important to identify mechanistic similarities and differences between human conditions and animal models. This can be done via carefully designed meta-analytical studies across experiments and species and results of such analyses can build a foundation for future clinical studies. Such comparisons can provide insights into the epigenetic mechanisms of AUD, distinguishing pre-existing epigenetically-driven susceptibility to alcohol abuse and alcohol-induced epigenetic profiles that can drive pathological behaviors (see Fig. 2 for a diagram of hypothetical relationships between methylation states, gene expression and AUD). Future studies should also be aimed at determining individual roles of DNA methylation and hydroxymethylation profiles both at CpG and CpH levels, the mechanistic roles of DNMT- and TET-family proteins in different cell populations, and the relationships between DNA modifications at specific genomic locations and the expression of mRNAs, proteins, and downstream cellular functions.

Fortunately, technology to probe these questions has recently become available. As mentioned above, one of the most widely used methylation profiling platforms, bisulfite sequencing, is insensitive to hydroxymethylcytosine, and is thus not able to differentiate between 5mC and 5hmC (Huang et al., 2010) - a consideration that should be made when consulting literature that has used this technique for methylation profiling and when conducting future bisulfite sequencing experiments. With the development of oxidative-bisulfite sequencing, which converts 5hmC to 5fC before the bisulfite reaction, whole genome methylation profiling can be conducted accurately (Booth et al., 2013). Subsequent bisulfite sequencing without the oxidation reaction can also be performed for subtractive analysis to determine 5hmC profiles in the same samples. Further modification and subtractive analyses using methylation-assisted bisulfite sequencing can differentiate 5-

formylcytosine and 5-carboxylcytosine from the other cytosine modifications, though not the two from each other (Neri et al., 2015).

Determination of cell type - specific epigenetic profiles is critical to our understanding of alcohol-induced changes in gene expression, cellular functions and behavior. A relatively new technique that could be useful for study of cell type-specific epigenetic states is the INTACT (Isolation of Nuclei TAGged in specific Cell Types; Deal & Henikoff, 2010), method, which rapidly and efficiently extracts and sorts nuclei from specific cell types for subsequent analyses, including DNA modifications. Alone, this method could be used to probe chromatin states in specific cell types between alcohol and control groups, and, combined with transcriptome profiling, it may reveal the molecular mechanisms contributing to alcohol-induced changes in cellular functions. Additionally, it can help identify cell type – specific changes that were masked by other, more abundant cell types in previous studies probing whole brain tissues. Furthermore, compared to gene expression profiling, which usually provides a snapshot of molecular activity at a single time point, epigenomic states may uncover long-lasting attributes of cellular identity, including patterns of past gene expression, current gene expression, and/or potential future experience-dependent responses (Mo et al., 2015). This notion may, at least in part, explain how drugs of abuse establish long-lasting changes in brain plasticity underlying compulsive drug use, craving, and relapse, even following years of abstinence.

One ultimate goal of this research is to use these epigenetic findings to develop novel therapeutic strategies for the prevention and treatment of AUD. Efforts to repurpose FDA-approved drugs can play a critical role in this development. Several drugs targeting chromatin modifications, including DNMT inhibitors, have been approved by FDA for treatment of cancer and several studies tested some of these drugs in animal models of AUD with some success of reducing excessive alcohol intake. The primary advantage of the drug repurposing strategy is that the time to clinical trial can be greatly reduced. In addition to this strategy, novel compounds that affect epigenetic states can be targeted for preclinical trials. One candidate includes 2-hydroxyglutarate, which inhibits the TET family proteins. Use of such TET inhibitors could potentially alter alcohol consumption in manner similar to the DNMT inhibitor effects. Unfortunately, this TET inhibitor is rather unselective and acts on other dioxygenases (Xu et al., 2011), though its effects have not been explored in the context of AUD models. It is also possible that other FDA-approved drugs can mimic the effects of some epigenetic compounds and normalize alcohol behaviors, so research on drug repurposing is warranted.

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## Abbreviations

<b>AUD</b>	Alcohol use disorder
<b>SUD</b>	substance use disorder



<b>FASD</b>	fetal alcohol spectrum disorder
<b>CpG</b>	cytosine-phosphate-guanine
<b>CpH</b>	cytosine-phosphate-[non guanine base]
<b>DNMT</b>	DNA methyltransferase
<b>TET</b>	ten-eleven translocator
<b>5mC</b>	5-methylcytosine
<b>5hmC</b>	5-hydroxymethylcytosine
<b>5fC</b>	5-formylcytosine
<b>5caC</b>	5-carboxylcytosine
<b>MBD</b>	methyl-binding domain
<b>SAM</b>	<i>S</i> -adenosyl-methionine
<b>BER</b>	base excision repair
<b>AID</b>	activation-induced deaminase
<b>TDG</b>	thymine DNA glycosylase
<b>SNP</b>	single-nucleotide polymorphism
<b>5-aza</b>	5-azacytidine

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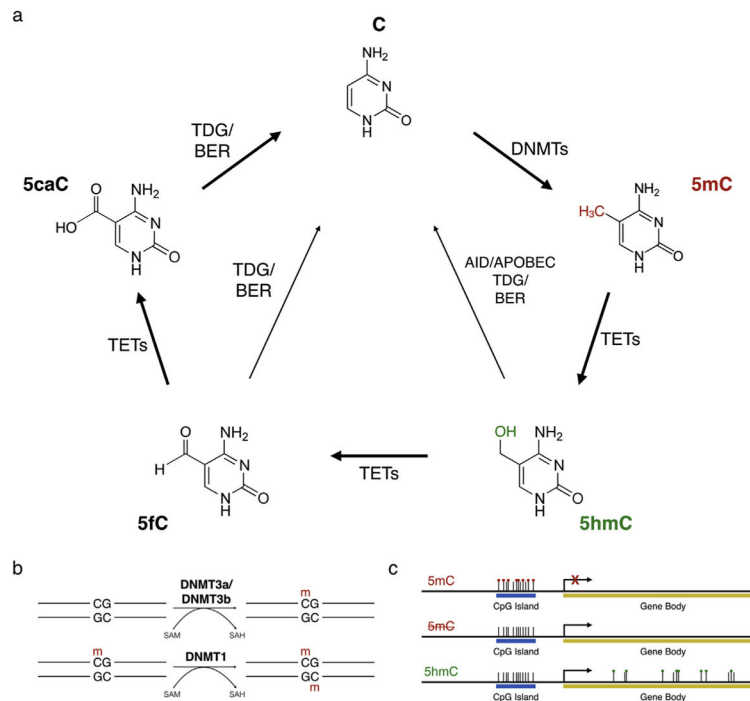
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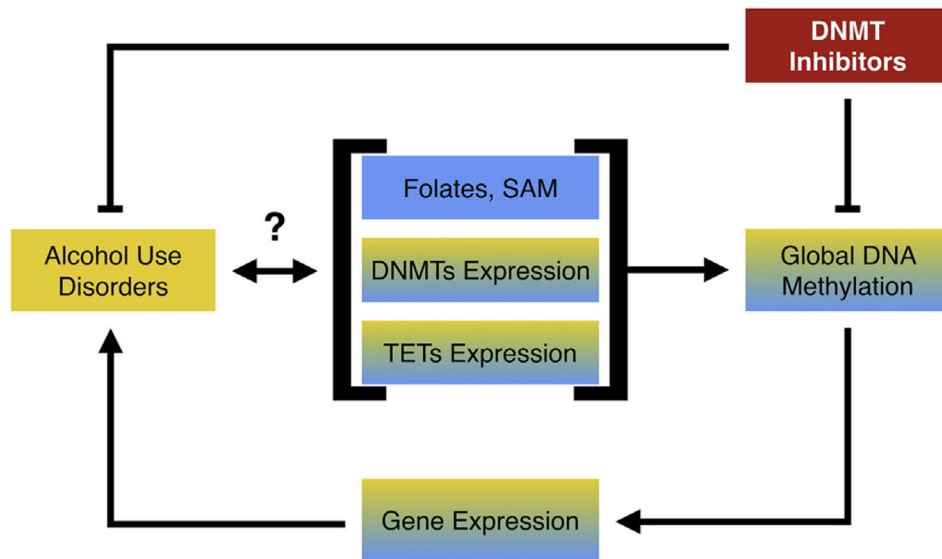
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**Fig. 1.**

DNA modifications and their effects on gene expression. a) Cytosines (C) may be methylated at the 5-carbon by DNA methyltransferase proteins (DNMTs) to produce 5-methylcytosine (5mC, red). 5mC can then be converted to 5-hydroxymethylcytosine (5hmC, green) by Ten-Eleven Translocator proteins (TETs). Further oxidation of 5hmC by TETs yields 5-formylcytosine (5fC), which can be converted to 5-carboxylcytosine (5caC) by TETs. Conversion of 5mC by TETs is the first stage in the active DNA demethylation pathway. 5hmC may be converted to cytosine demethylated through activation-induced deaminase (AID)/apolipoprotein B mRNA editing enzyme complex (APOBEC) to create 5-hydroxymethyluracil followed by creation of an abasic site by thymine DNA glycosylase, which is then repaired to C by base excision repair. 5fC and 5caC can bypass the AID/APOBEC pathway. b) Top: *de novo* CpG methylation. DNMT3a and DNMT3b, the *de novo* methyltransferases, add a methyl group from donor *S*-adenosyl-methionine (SAM) to methylate one cytosine of a complementary pair of unmethylated CpG dinucleotides. Bottom: maintenance CpG methylation. DNMT1, the maintenance methyltransferase, methylates the unmethylated cytosine of a hemi-methylated complementary pair of CpG dinucleotides to produce a complementary pair of CpGs methylated at the cytosines on both strands. c) 5mC is frequently found in clusters of CpG dinucleotides, called CpG islands, which are, in turn, frequently found in gene promoter regions. 5mC in promoter CpG islands (top) is typically a repressive mark and acts by blocking gene transcription as indicated by a red cross over the black arrow. Absence of 5mC in CpG islands (center) and presence of 5hmC in gene bodies (bottom) are often associated with transcriptional activation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Hypothetical interaction between AUD, DNA modification systems, and gene expression. Yellow color indicates generalized increases and blue color indicates general decreases. Alcohol abuse alters epigenetic regulatory mechanisms, such as reductions in abundance of methyl donors folates and SAM, and changes in expression of DNMT and TET genes. Alterations to these factors lead to alterations to global DNA methylation, which correspondingly alters gene expression, which can then drive changes in behavior with respect to alcohol. Evidence points to hypomethylating agents, such as DNMT inhibitors as mainly reducing drinking in animal models, though the mechanism of action remains unclear. It is also unclear (indicated by the question mark) whether the epigenetic regulatory factors are a result of alcohol abuse or are endogenous aberrations that contribute to development of AUD traits, as different studies provide support for both hypotheses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)